NEUROMODULATORY CONTROL OF SPINAL FUNCTION IN HEALTH AND DISEASE

EDITED BY: Brian R. Noga, Shawn Hochman and Hans Hultborn
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NEUROMODULATORY CONTROL OF SPINAL FUNCTION IN HEALTH AND DISEASE

Topic Editors:
Brian R. Noga, University of Miami, United States
Shawn Hochman, Emory University, United States
Hans Hultborn, University of Copenhagen, Denmark

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EDITORIAL
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Editorial: Neuromodulatory Control of Spinal Function in Health and Disease

Brian R. Noga[1]*, *Shawn Hochman*[2] *and Hans Hultborn*[3]

[1] *Miller School of Medicine, University of Miami, Miami, FL, United States,* [2] *Department of Physiology, Emory University School of Medicine, Atlanta, GA, United States,* [3] *Department of Neuroscience, University of Copenhagen, Copenhagen, Denmark*

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Editorial on the Research Topic

Neuromodulatory Control of Spinal Function in Health and Disease

The classical ionotropic transmitters glutamate/ACh (acetylcholine) and glycine/GABA (gamma-amino butyric acid) are, respectively, responsible for the primary excitatory and inhibitory synaptic actions within spinal cord anatomical circuits, be they simple reflexes as the monosynaptic stretch reflex (and its reciprocal inhibition of antagonists), or more distributed and integrated networks along autonomic, sensory, and motor systems. The selection and complex spatiotemporal recruitment of intrinsic spinal circuits (e.g., locomotion) are profoundly sculpted by neuromodulation acting both at pre- and post-synaptic levels.

Neuromodulation denotes to the ability of neurons to alter their electrical and synaptic properties in response to intracellular biochemical changes. Neuromodulation commonly occurs via activation of metabotropic (G protein-coupled) receptors that alter signal transduction pathways. Neuromodulators function to modulate rather than mediate activity and represent a broad class of neuroactive substances. By altering the cellular/synaptic properties of individual neurons embedded in networks, neuromodulators profoundly alter the operation of neural circuits and behavior. They provide flexibility in circuit selection and strength to allow the nervous system to adapt neural output according to the functional requirements and/or demands of the individual to achieve the desired behavioral state. At times, it appears that the neuromodulators have a more primary function in activating and controlling complex networks than the term "modulator" would tend to imply.

Neuromodulatory transmitter systems undoubtedly affect all spinal cord functional systems including locomotion, respiration (via phrenic and other respiratory motoneurons), posture, balance, fine movements, autonomic functions (including control of bowel, bladder, blood pressure, and heart rate), reflexes, and sensory information processing (nociception, etc.). Since many neuromodulatory transmitter systems (e.g., such as those releasing monoamines) originate from neurons outside of the spinal cord, injuries to the spinal cord will necessarily damage their descending projections in addition to other non-neuromodulatory pathways controlling the anatomical network. Such injuries will therefore affect not only the initiation and control of spinal networks, but also the ability to adjust their output according to ongoing functional demands.

The present Research Topic includes review and original research articles that seek to shed light on the neural processes underlying the neuromodulatory control of spinal cord function in health and disease. This Research Topic consists of 24 articles on various aspects of Spinal Cord research contributed by 105 authors. The assembled contributions are summarized below in three thematic categories: (i) locomotion, (ii) descending and segmental pathways, and (iii) disease/injury.

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Edward S. Ruthazer, McGill University, Canada

***Correspondence:**
Brian R. Noga
bnoga@miami.edu

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LOCOMOTION

The mesencephalic locomotor region (MLR) activates spinal locomotor generating neurons via pathways originating in the medial reticular formation and descending through the ventral funiculus. There is evidence that monoaminergic pathways are also activated both during MLR-evoked fictive locomotion and during spontaneous or voluntary locomotion. In the present original research, Noga et al. measured in real time, by fast-cyclic *in vivo* voltammetry, the release of the monoamines serotonin (5-HT) and norepinephrine (NE) in the decerebrate cat's lumbar spinal cord during MLR-evoked fictive locomotion. The time course, the spinal locations and the concentration of the release of these two monoamines were mapped. Monoamine release was observed in dorsal horn, intermediate zone/ventral horn. The results demonstrate that spinal monoamine release is modulated on a timescale of seconds, and that the concentrations are high enough to strongly activate various receptors subtypes and further suggest that monoamine action is, in part, mediated by extrasynaptic neurotransmission in the spinal cord.

The review by Sharples et al. focuses on the role of dopamine in the spinal control of locomotion in vertebrates. It summarizes the biochemistry, pharmacology, anatomy, and function of dopamine (and to a lesser extent other monoamines) in the locomotor networks of various vertebrates including fish (lamprey and zebrafish), amphibians (larval xenopus), and mammalians (rodents). A brief discussion of the effects of dopamine on rhythmicity in invertebrates is also provided. Parallels are drawn with both noradrenergic and serotonergic systems. New experimental approaches (optogenetics, pharmacogenetics) together with more traditional approaches (intrathecal administration/cell transplantation) to the study of dopaminergic function and/or the treatment of gait disorders following SCI are discussed.

Sensorimotor transformations are essential for control of goal-directed behavior and interaction with the outside world. The review by Daghfous et al. examines how the vertebrate CNS integrates sensory signals to generate locomotor behavior by examining the pathways and mechanisms involved in the transformation of cutaneous and olfactory inputs into motor output in the lamprey. The review also explores how serotonin modulates the system through actions on both sensory inputs and motor output. This timely review of mechanisms for sensory control of locomotion is also very thorough and helpful to the uninitiated in this area of lamprey pharmacology.

Wienecke et al. studied the interaction between blood pressure, respiration, and locomotor activities in decerebrate cats. They observed periodic variation (Mayer waves) in blood pressure, which was synchronized with respiratory and locomotor drive potentials recorded in hindlimb motoneurons. The report demonstrates the intricate interrelations between respiratory networks and hindlimb locomotor networks. They conclude that the respiratory drive in hindlimb motoneurons is transmitted via elements of the locomotor central pattern generator. The rapid modulation related to Mayer waves suggests the existence of a more direct and specific descending modulatory control than has previously been demonstrated. The possibility that monoaminergic drive to the spinal cord may mediate the occurrence of Mayer waves is discussed.

In addition to the classical monoamine neuromodulatory transmitters, another group of endogenous monoamines, the trace amines (TAs), may play a role in neuromodulatory actions within the nervous system. They share structural, metabolic, physiologic, and pharmacologic similarities to the classical monoamines and are synthesized from the same precursor amino acids. The paper by Gozal et al. provides evidence for the presence of trace amine synthetic enzymes, trace amine-associated receptors (TAARs), and TAs in the spinal cord. Furthermore, the authors demonstrate effects of TAs on spinal neuronal networks, effects that have a pharmacological profile distinct from that of more classical monoamine neurotransmitters. This data indicates that TAs may function as an intrinsic spinal monoaminergic modulatory system capable of promoting recruitment of locomotor circuits independent of the descending monoamines. These actions support their known sympathomimetic function. This study will likely stimulate further research in this area.

How pattern generators are modulated in the absence of descending control from the brain is highly important for understanding the neural control of movement as well as for developing therapeutic approaches to improve mobility of SCI patients. The review by Cherniak et al. summarizes recent studies of sacral relay neurons with lumbar projections and evaluates their role in linking the sacral and thoracolumbar networks during different motor behaviors. They show that: (1) the activation of the locomotor central pattern generators through sacral sensory input is mediated by a heterogeneous group of dorsal, intermediate and ventral sacral-neurons with ventral and lateral ascending funicular projections, and (2) the rhythmic excitation of lumbar flexor motoneurons, produced by exposing the sacral segments to alpha-1 adrenoceptor agonists, is mediated exclusively by ventral clusters of sacral-neurons with lumbar projections through the ventral funiculus. The mechanisms and physiological implications of their findings are discussed. Given the recent increase in interest in combinatorial approaches to increasing function after spinal injury, this review of neural mechanisms contributing to the sacro-caudally activated lumbar motor rhythm is timely and should be of general interest to spinal cord rehabilitation, plasticity, and regeneration researchers.

The monoamines serotonin (5-HT) noradrenaline (NA) and dopamine (DA) are known to reconfigure spinal circuits and facilitate expression of motor rhythms including locomotion. Beliez et al. explore the common and differential actions of these monoamines in coordinating rhythmic locomotor-related output along the thoracolumbar-sacral axis in the *in vitro* isolated neonatal rat spinal cord. The monoamines generated similar ventral root motor rhythms in terms of period/duration as well as left/right and flexor/extensor phase relations but differed in motor burst amplitude and other temporal characteristics including intersegmental phase relationships. Observed differences likely relate differences in descending behavioral drive linked to separate recruitment of these neuromodulators.

DESCENDING AND SEGMENTAL PATHWAYS

C-boutons are important cholinergic modulatory loci for state-dependent alterations in motoneuron firing rate. Deardorff et al. present an elegant and systematic review of the state of knowledge of one of the major inputs to spinal motoneurons, the C terminals. The authors take the reader on a historical journey from the initial identification of the C-boutons and then introduce the unique molecular organization of the signaling ensemble surrounding the C-bouton synapse and its effect on the firing frequency of the spinal motoneuron via its effect on the post-spike after hyperpolarization. They then describe the C-bouton itself and its cell of origin, and its cholinergic identity and circuitry. Finally, they propose a possible mechanism by which the activity of C-bouton may play a role in enhancing firing rate during periods of increased excitatory drive, whilst also acknowledging alternative explanations.

Bulbospinal systems may influence spinal neurons by classical synaptic and modulatory mechanisms and are involved in motor, sensory, and autonomic functions. Huma et al. report on the brainstem locations of cells of origin of bulbospinal pathways of the rat passing through the medial longitudinal fasciculus (MLF) and the caudal ventrolateral medulla (CVLM). Neurons were identified using anatomical tracing methods, their transmitter phenotypes identified, and their locations mapped onto brainstem diagrams. Cells that form pathways from the brainstem to the lumbar spinal cord passing through the MLF and CVLM for the most part, have overlapping spatial distributions. Although both populations contain crossing and uncrossing axons and similar proportions of excitatory and inhibitory axons, MLF and CVLM reticulospinal neurons have different spinal cord projections. Those in the MLF project more ventrally and are more likely to have direct motor functions than those in the CVLM. In contrast, CVLM projections are predominantly ipsilateral and concentrated within deep dorsal horn and intermediate gray but do not extend into motor nuclei or lamina VIII. CVLM pathway may function to coordinate activity of premotor networks.

Johnson and Heckman provide a comprehensive review of the neuromodulatory control of the electrical properties of spinal motoneurons. Gain control of motoneuron output is important for generating the enormous range of forces required for the wide dynamic range of the normal movement repertoire. For diffuse neuromodulatory systems such as the monoaminergic projection to motoneurons, independent control of the gains of different motor pools is not feasible. In fact, the system is so diffuse that gain for all the motor pools in a limb likely increases in concert. Additionally, if there is a system that increases gain, probably a system to reduce gain is also needed. In this review, they summarize recent studies that show local inhibitory circuits within the spinal cord, especially reciprocal and recurrent inhibition, have the potential to solve both of these problems as well as constitute another source of gain modulation.

DISEASE/INJURY

In the manuscript by Becker and Parker, the lamprey model is used to compare cellular and synaptic properties of neurons above and below the lesion site in spinal cord injured (SCI) animals and in normal spinal cord of uninjured animals. They also examined the effects of lesioning on the modulatory effects of 5-HT. Based on their results, the authors suggest lesion specific changes occur in cellular and synaptic properties and in serotonin modulation. Therefore, pharmacological approaches to facilitate functional recovery should not be based on the effects reported in uninjured spinal cords. Although, the cellular and synaptic properties of motor neurons and spinal interneurons caudal to lesion site has been demonstrated previously by the same group, the strong differences between larval and adult stages justified the analysis in young adult lampreys. This is the first investigation on the effects of a spinal lesion on the modulatory effects of serotonin, and thus the study has the potential to make a strong contribution to the related literature.

The original paper by Kou et al. describes the changes in the neural circuits in streptozotocin (STZ)-induced diabetic rats. Diabetic polyneuropathy (DPN) is one of the most common complications of diabetes mellitus but is not a single entity and encompasses several neuropathic syndromes, including sensory and motor defects. In this paper, the authors discuss both progressive mechanical allodynia and impaired locomotor activity. The alterations in myelinated nerve fibers, unmyelinated non-peptidergic nerve fibers, and peptidergic nerve fibers might be involved in the early stages of the development in DPN. The underlying mechanism of DPN might be addressed by the dysfunction of those subpopulations of afferents from the peripheral nervous system to the CNS. Both the allodynia and locomotor defects could be prevented and reversed by intrathecal insulin injection.

The review by Fields and Mitchell addresses the well-known ability to exhibit plasticity of the neural system controlling breathing. The focus of the review is the less appreciated ability to exhibit metaplasticity, i.e., a change in the capacity to express plasticity ("plastic plasticity"). Key examples of metaplasticity in respiratory motor control, and our current understanding of mechanisms giving rise to spinal plasticity and metaplasticity in phrenic motor output is discussed. The metaplasticity is especially seen after pre-conditioning with intermittent hypoxia. This metaplasticity is not confined to the respiratory network but is also seen in motor networks involved in limb movements.

The broad review by Ghosh and Pearse covers the promotion or facilitation of serotonergic signaling (including specific receptor systems) to enhance motoneuron excitability, stimulate CPG activity, and restore locomotor function following spinal cord lesions. These strategies have included pharmacological modulation of serotonergic receptors, through the administration of specific 5-HT receptor agonists, or by elevating the 5-HT precursor 5-hydroxytryptophan, which produces a global activation of all classes of 5-HT receptors. Another approach has been to employ cell therapeutics to replace the loss of descending serotonergic input to the CPG, either through transplanted fetal brainstem 5-HT neurons at the site

of injury that can supply 5-HT to below the level of the lesion or by other cell types to provide a substrate at the injury site for encouraging serotonergic axon regrowth across the lesion to the caudal spinal cord for restoring locomotion. This approach is one direction at the forefront of research for generating putative interventional approaches for the treatment of SCI.

Serotonergic systems are important for activation and modulation of locomotor circuits. However, locomotor circuits function differently following SCI that damages descending serotonergic (and other) pathways. The study by Strain et al. examines how the spinal cord adapts to sensory perturbations after injury when the serotonergic system is activated. They observed differences in the intralimb and interlimb coordination in SCI animals during serotonergic agonist-induced locomotor movements following sensory perturbation (range-of-motion restriction) in comparison to that seen in intact animals. Differences were observed for hindlimb and forelimb locomotor movements controlled by the distal and proximal (to spinal transection) areas of the spinal cord, respectively. The number of hindlimb steps observed following quipazine treatment was also significantly greater than intact controls, suggesting that the effects of quipazine treatment are related to "supersensitivity" of spinal segments distal to the site of injury. The results have implications for design of rehabilitation strategies to treat paralysis following SCI.

The central molecular changes that might influence neurotrophic signaling pathways and modulate locomotor recovery and pain following SCI are investigated in the study by Strickland et al. Based on their previous work, they assess the expression of select miRNA species that might influence neurotrophic signaling pathways and functional recover following noxious peripheral electrical stimulation. The data show that uncontrollable nociception which activates sensorimotor circuits distal to the injury site, influences SCI-miRNAs and target mRNAs within the lesion site. SCI-sensitive miRNAs may well mediate adverse consequences of uncontrolled sensorimotor activation on functional recovery. However, their sensitivity to distal sensory input also implicates these miRNAs as candidate targets for the management of SCI and neuropathic pain.

The long-term effectiveness of opiates for the treatment of pain is limited by the development of tolerance. This is thought to be the result of dysfunction of the μ-opioid receptor (MOR) and dopamine (D) receptor mediated second messenger pathways in the brain. Brewer et al. examine the role of the spinal cord in the development of tolerance since it plays a prominent role in the processing of nociceptive information and has both dopamine and MOR receptors in the dorsal horn. They reconfirm that D3 receptors are necessary for morphine analgesia *in vivo* and show for the first time that acute block of D3 receptors in the lumbar spinal cord prevents modulation of spinal reflex amplitude by morphine *in vitro*. Their data suggest that the D3 receptor modulates the MOR system in the spinal cord, and that a dysfunction of the D3 receptor can induce a morphine-resistant state. They propose that the D3KO mouse may serve as a model to study the onset of morphine resistance at the spinal cord level, the primary processing site of the nociceptive pathway.

Involuntary movements and spasms may be the result of hyperactive motor networks. Regulation of such networks may be accomplished by intrinsic modulatory systems releasing transmitter such as purines. Such is the case for the ventral horn of the spinal cord. Carlsen and Perrier report on findings in the postnatal mouse that indicate that ventral horn astrocytes produce a tonic and a phasic inhibition of excitatory synaptic transmission in ventral horn neurons by releasing ATP. ATP is rapidly hydrolyzed to adenosine which then acts on presynaptic receptors and thereby decreases the probability of transmitter release. While a role of purinergic processes in synaptic modulation by astrocytes, at least *in vitro*, is established in diverse brain regions, this has been studied in less detail for (ventral) spinal neural circuits.

Maturation of spinal motor circuits are influenced by the development and maintenance of descending serotonergic projections. The review article by Gackière and Vinay describes how 5-HT plays a role in the maturation of locomotor patterning and GABAergic synaptic transmission via actions on 5-HT_2 and 5-HT_7 receptors. They describe how postsynaptic inhibition is reduced after SCI and can be accounted for by a 5-HT_2 receptor-mediated dysregulation of chloride transport in motoneurons. Evidence suggests that 5-HT enables restoration of locomotion after SCI via 5-HT receptors involved in the activation of signal transduction pathways that restore the chloride gradient needed for synaptic inhibition (5-HT_2) and facilitate interneuronal activity (5-HT_7).

Sławinska et al. compare the effects of pharmacologic actions of 5-HT_2 and 5-$HT_{1A/7}$ receptor agonists, applied alone or in combination, on observed recovery of hindlimb treadmill locomotor function after low thoracic spinal transection in adult rats. Assessment of independent drug actions demonstrated that agonists act on complementary circuits: 5-HT_2 receptors by facilitating motor excitability directly, and 5-$HT_{1A/7}$ receptors by promoting locomotor circuit generating interlimb coordination. These findings add to earlier studies that support combined receptor targeting in locomotor recovery strategies while acknowledging limitations of potentially competing pharmacologic actions on afferent feedback and other intraspinal circuits.

The neurotrophins nerve growth factor (NGF), brain derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) play an important role in neural circuit development and plasticity. Neurotrophins and their receptors are normally present in the spinal cord. Boyce and Mendell review how neurotrophic actions modify spinal sensory and motor circuit function including nociception, reflexes and stepping. Experimental interventions have targeted the introduction of exogenous neurotrophins to recapitulate their known trophic actions to improve function in disease states and after injury. This review focuses on more recent findings relevant to these translational issues.

Grau et al. reviews the complex interplay of neuromodulatory factors that alter the capacity for activity-dependent adaptive reflex plasticity (spinal learning) and locomotor recovery after SCI. Adaptive or maladaptive behavioral outcomes are interpreted in relation to prior "priming" events that alter experimental outcome (meta-plasticity). Broadly, adaptive

plasticity and motor recovery are tied to an up-regulation of BDNF signaling while maladaptive plasticity and motor deficits are associated with induction of a pro-inflammatory phenotype including expression of the cytokine tumor necrosis factor (TNF). An understanding of spinal cord neuromodulatory status after injury may provide a useful framework for training interventions in the clinical population.

Previous experiments implicate cholinergic brainstem and spinal systems in the control of locomotion. Jordan et al. undertook a pharmacological exploration on the capacity of the spinal cholinergic system in modulating spinal locomotor networks with emphasis on capacity to facilitate recovery after SCI. Contrary to expectations, cholinergic (muscarinic) receptor activation disrupted locomotor recovery while receptor block (atropine) greatly facilitated expression of locomotion and recruitment of cutaneous reflexes. Their temporal correspondence supports the view that there is a tonic muscarinic inhibition of afferent feedback onto locomotor circuits thereby identifying a new opportunity for restoring locomotion after injury.

Spinal motoneurons can exhibit differences in excitability early in development in amyotrophic lateral sclerosis (ALS). As serotonin, noradrenaline, and dopamine already modulate motor activity at early postnatal ages, Milan et al. used the SOD1G93A (SOD1) ALS mouse model to examine possible differences in their expression and function. In ventral horn, there were no differences in HPLC-detected content of these monoamines and metabolites between WT and SOD1 mice (P1 or P10). Similarly, no differences on the locomotor rhythm were observed in the SOD1 mouse when the monoamines were bath applied in the isolated spinal cord (P1–P3). However, NA generated a larger amplitude motor response in SOD1 mice, suggestive of adrenoceptor-based motor excitability increases at early postnatal ages.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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ORIGINAL RESEARCH ARTICLE
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Alterations in the neural circuits from peripheral afferents to the spinal cord: possible implications for diabetic polyneuropathy in streptozotocin-induced type 1 diabetic rats

Zhen-Zhen Kou, Chun-Yu Li, Jia-Chen Hu, Jun-Bin Yin, Dong-Liang Zhang, Yong-Hui Liao, Zhen-Yu Wu, Tan Ding, Juan Qu, Hui Li* and Yun-Qing Li*

Department of Anatomy, Histology and Embryology, K. K. Leung Brain Research Centre, The Fourth Military Medical University, Xi'an, China

Edited by:
Hans Hultborn, University of Copenhagen, Denmark

Reviewed by:
C. J. Heckman, Northwestern University, USA
Scott Hooper, Ohio University, USA

****Correspondence:***
Hui Li and Yun-Qing Li, Department of Anatomy, Histology and Embryology, K. K. Leung Brain Research Centre, The Fourth Military Medical University, No. 169, West Changle Road, Xi'an 710032, China
e-mail: deptanat@fmmu.edu.cn; li_hui@fmmu.edu.cn

Diabetic polyneuropathy (DPN) presents as a wide variety of sensorimotor symptoms and affects approximately 50% of diabetic patients. Changes in the neural circuits may occur in the early stages in diabetes and are implicated in the development of DPN. Therefore, we aimed to detect changes in the expression of isolectin B4 (IB4, the marker for nonpeptidergic unmyelinated fibers and their cell bodies) and calcitonin gene-related peptide (CGRP, the marker for peptidergic fibers and their cell bodies) in the dorsal root ganglion (DRG) and spinal cord of streptozotocin (STZ)-induced type 1 diabetic rats showing alterations in sensory and motor function. We also used cholera toxin B subunit (CTB) to show the morphological changes of the myelinated fibers and motor neurons. STZ-induced diabetic rats exhibited hyperglycemia, decreased body weight gain, mechanical allodynia and impaired locomotor activity. In the DRG and spinal dorsal horn, IB4-labeled structures decreased, but both CGRP immunostaining and CTB labeling increased from day 14 to day 28 in diabetic rats. In spinal ventral horn, CTB labeling decreased in motor neurons in diabetic rats. Treatment with intrathecal injection of insulin at the early stages of DPN could alleviate mechanical allodynia and impaired locomotor activity in diabetic rats. The results suggest that the alterations of the neural circuits between spinal nerve and spinal cord via the DRG and ventral root might be involved in DPN.

Keywords: diabetic polyneuropathy, neural circuit, spinal cord, dorsal root ganglion, primary afferent, rat

INTRODUCTION

Diabetic polyneuropathy (DPN) is one of the most common complications of diabetes mellitus (DM; Dyck et al., 1993). DPN is not a single entity but encompasses several neuropathic syndromes, including sensory and motor defects (Yasuda et al., 2003; Boulton et al., 2005). Because DPN is rather complex and poorly understood and is most likely affected by multiple factors, diagnosis of DPN is seldom successful in the early to intermediate stages of DM (Tesfaye et al., 2010).

A large proportion of diabetic patients who suffer from DPN describe abnormal sensations such as pain in the early stages. Painful DPN in human subjects is usually characterized by the development of tactile allodynia, a condition in which light touch is perceived as painful (Baron et al., 2009). Tactile allodynia is observed in a large proportion (30–47%) of human subjects with DM (Bastyr et al., 2005). In contrast to sensory disturbance, motor function has rarely been studied in DPN (Andersen, 2012). However, previous reports demonstrate that type 1 diabetic patients exhibit impaired strength of the ankle and knee extensors and flexors, indicating that DPN is often a mixed sensory-motor neuropathy (Andersen, 2012; IJzerman et al., 2012). Despite considerable research on DPN, evidence for pathophysiological changes in the nervous systems underlying the impairment of motor function still lacks neurostructural evidence.

Previous studies have focused on the role of peripheral nerves in DPN. Increasing evidence now indicates that the insult of DPN might be at all levels of the nervous system, including both the PNS and the CNS (Eaton et al., 2001; Fischer and Waxman, 2010). Although the involvement of both PNS and CNS in DPN has previously been recognized, the underlying mechanisms remain poorly understood, particularly in the changes of the neural circuits between spinal nerves and spinal cord via the DRG and ventral root. Thus, investigating the alterations in the neural circuits is essential for understanding the development of the sensory and motor defects in DPN.

The spinal nerves conduct mixed signals, including sensory and motor information from nerve endings in the PNS (Todd, 2010). Spinal nerves have both dorsal and ventral roots. The dorsal root carries sensory axons originating from the sensory neurons in the dorsal root ganglion (DRG), while the ventral root

carries motor axons originating from the motor neurons within the ventral horn of the spinal gray matter. The various types of DRG neurons send their central axonal processes to different parts of the spinal dorsal horn in the CNS (Duce and Keen, 1977; Todd, 2010). Three main types of nerve fibers originate in the DRG neurons and terminate in different laminae of the spinal dorsal horn: (1) unmyelinated non-peptidergic afferents are observed in laminae I and II; (2) peptidergic afferents are found in laminae I, II and V; and (3) myelinated afferents are located in laminae III and IV.

There are useful reagents for identifying and investigating the different nerve afferents. Cholera toxin B subunit (CTB) is selectively taken up by myelinated but not unmyelinated primary afferents (Tong et al., 1999; Shehab et al., 2004). When CTB is injected into sciatic nerves, it is selectively taken up by myelinated afferents and transported transganglionically to label fibers and terminals in laminae III to IV of the spinal dorsal horn and neurons in lamina IX of the ventral horn (Tong et al., 1999). For the unmyelinated afferents, the plant *Bandeiraea simplicifolia* I-isolectin B4 (IB4) binds to a subtype of small DRG neurons, specifically those that lack neuropeptides (Michael and Priestley, 1999). The antibody for calcitonin gene-related peptide (CGRP) recognizes small peptidergic neurons in the DRG and their afferents in spinal cord (Karanth et al., 1991).

It has been confirmed that DPN is irreversible when nerves are destroyed, so early intervention is very important to prevent neuropathic complications in patients with diabetes (Boulton et al., 2005; Tesfaye et al., 2010). Therefore, in the present study, we sought to clarify the changes of neural circuits at the early stages (within 4 weeks) of DPN. Using the model of streptozotocin (STZ)-induced type 1 diabetic rats, we examined the distributions and alterations of CTB-labeled myelinated, IB4-labled nonpeptidergic unmyelinated, and CGRP-immunopositive peptidergic fibers and their cell bodies in both DRG and spinal cord. We also applied insulin through intrathecal injection in diabetic rats to observe the effects of treatment on sensory and motor activities in behavioral tests.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

All animal studies were conducted using approved protocols and carried out in accordance with the Principles of Laboratory Animal Care (NIH Publication no. 85-23, revised 1985). Male Sprague-Dawley rats weighing 220–250 g were obtained from the Laboratory Animal Center of The Fourth Military Medical University (Xi'an, China). In accordance with our previous studies (Zuo et al., 2011; Kou et al., 2013a), rats were injected with a single injection of 60 mg/kg STZ (Sigma, St. Louis, MO, USA), which was freshly dissolved in ice-cold sodium citrate (pH 4.5), while age-matched control rats received injections of a similar citrate buffer. Diabetes was confirmed on the third day by measurements of blood glucose concentrations in samples obtained from the tail vein using a strip-operated reflectance meter (Active; Roche Diagnostics, Mannheim, Germany). Only rats with blood glucose concentration >20 mM were used. All animals were housed in standard conditions (12 h light/dark cycles) with water and food available *ad libitum*.

PAIN BEHAVIORAL TEST

The experiments were performed in accordance with our previously reported protocols (Mei et al., 2011; Kou et al., 2013a). To quantify the mechanical sensitivity of the hind paw, animals were placed in individual plastic boxes and allowed to acclimate for 30 min. A series of calibrated *von* Frey filaments (Stoelting, Kiel, WI, USA) ranging from 0.4 to 60.0 g were applied to the plantar surface of the hind paw, with sufficient force to bend the filaments for 5 s or until paw withdrawal. Applications were separated by 15 s intervals to allow the animal to cease any response and return to a relatively inactive position. In the presence of a response, the filament of the next lower force was applied. In the absence of a response, the filament of the next greater force was applied. A positive response was indicated by a sharp withdrawal of the paw. Each filament was applied 10 times, and the minimal value that caused at least six responses was recorded as the paw withdrawal threshold (PWT). All behavioral studies were performed under blind conditions.

OPEN FIELD TEST

An open field test was used to analyze the rats' locomotor activity, as in our previous report (Quan-Xin et al., 2012). An animal was placed in one corner of the open field (100 × 100 × 48 cm). Movement of the rat in the area during the 15 min testing session was recorded. After 15 min, the rat was removed to the home cage, and the open field area was cleaned. The total distance and the average velocity in the area were measured.

IMMUNOHISTOCHEMISTRY

Rats were deeply anesthetized with the injection of pentobarbital (50 mg/kg, i.p.). All rats were perfused through the ascending aorta with 150 ml of 0.9% (w/v) saline followed by 50 ml of 4% (w/v) paraformaldehyde (Shanghai Xinran Biotechnology Co. Ltd.) and 0.2% (w/v) picric acid (Shanghai Xinran Biotechnology Co. Ltd.) in 0.1 M phosphate buffer (PB, pH 7.4) (Zuo et al., 2011; Kou et al., 2013a). After perfusion, lumbar segments of the spinal cord and the corresponding DRGs were removed, post-fixed with the same fresh fixative for 4 h and placed in 30% (w/v) sucrose solution (Shanghai Xinran Biotechnology Co. Ltd.) for 24 h at 4°C. The immunochemistry was performed as in our previous report (Kou et al., 2013b). Transverse sections of spinal cord (25 μm) and DRG (15 μm) were incubated in blocking solution (5% v/v normal goat serum) for 1 h at room temperature and then incubated overnight at 4°C with primary antibody: rabbit anti-CGRP (1:2000; Millipore, AB5920, CA, USA). The sections were then washed with 0.01 M phosphate buffered saline (PBS, pH 7.4) three times (10 min each). The sections were then incubated with Alexa594-conjugated donkey anti-rabbit IgG (1:1000; Invitrogen, A-21207, NY, USA) and fluorescein isothiocyanate (FITC)-IB4 (1:200; Vector Laboratories, FL-1201, CA, USA) for 2 h. Finally, the sections were rinsed with 0.01 M PBS, mounted onto clean glass slides, air-dried and cover slipped with a mixture of 0.05 M PBS containing 50% (v/v) glycerin and 2.5% (w/v) triethylenediamine. The sections were observed under a laser-scanning confocal microscope (FV-1000, Olympus, Tokyo, Japan).

NEUROTRACER INJECTION

The rats were deeply anesthetized by injection of pentobarbital, and the sciatic nerves on the left side were exposed. Then, 2 μl of 1% Alexa594-conjugated CTB (red fluorescence, Molecular Probes, C22842, CA, USA) was slowly injected with a 33-gauge Hamilton syringe into the proximal part of the sciatic nerve after it was exposed at the mid-thigh level. The incision was closed by suture. Three days after CTB injection, the rats were perfused. DRGs and lumbar segments were removed and processed as described for immunohistochemistry.

INTRATHECAL INJECTION

Intrathecal implantation was performed as in our previous reports (Mei et al., 2011; Kou et al., 2013a). A polyethylene (PE) tube (Intramedic®, Becton Dickinson and Company, NJ, USA) was inserted directly into the subarachnoid space of the lumbar enlargement (between L4 to L6) for drug injection. Under pentobarbital anesthesia, a midline incision from L6 to L3 was made along the back of the rat. A pre-measured length of PE-10 tube (I.D. 0.28 and O.D. 0.61 mm) was passed caudally into the lumbar enlargement at the level of the lumbar vertebra. Only rats judged to be neurologically normal and showing complete paralysis of the tail and bilateral hind legs after administration of 2% lidocaine (10 μl) intrathecally were used for the following experiments. After tube implantation, rats were allowed to recover for 2 days. The rats were intrathecally injected with either insulin (0.2 units) or saline (10 μl) through the catheter at 10:00–10:30 am every day for 14 days (**Figure 5**).

STATISTICAL ANALYSES

Immunohistochemical data were analyzed in accordance with previous reports (Fernyhough et al., 1989; Aizawa and Eggermont, 2006; Mei et al., 2011; Zuo et al., 2011; Kou et al., 2013a,b). For quantification of immunopositive cell profiles in the DRG and the spinal ventral horn, five sections from a series of every fourth serial section of the lumbar segments (L4–L6) of spinal cord (25 μm) and DRG (15 μm) were selected randomly from each animal. In each group, six rats were used for statistical analysis. All positively stained cells in the area were evaluated with ImageJ software (National Institutes of Health).[1] Statistical analysis was performed with one-way ANOVA with the Student–Newman–Kuels (SNK) *post hoc* test, using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). The changes in the small neurons among the total CTB-positive neurons in the DRG were analyzed by Cross-Tabulation with significance testing with Pearson's Chi-square. For analysis of immunopositive terminals in the spinal dorsal horn, the relative optical densities (ROD) of the immunostaining were analyzed with ImageJ software (Burgess et al., 2010). First, the integrated optical density (IOD) of the positive staining and background were acquired from five sections from each rat. Then, the ROD was calculated by subtracting the background from the IOD of the positive staining. The values for ROD were statistically analyzed among groups with SPSS. Data from immunofluorescence were expressed as fold change vs. the control group.

Analysis of the time-course of the behavioral tests between the saline- and insulin-treated groups was performed by two-factor (group and times) repeated measures analysis of variance (ANOVA). One-way ANOVA was employed for analyzing other data and was followed by the least significant difference (LSD) (equal variances assumed) or Dennett's T3 (equal variances not assumed) *post hoc* test. Data were expressed as the mean ± SD. Differences between groups were considered statistically significant at a value of $p < 0.05$.

RESULTS

STREPTOZOTOCIN (STZ)-INDUCED TYPE 1 DIABETIC RAT

Following a single *i.p.* injection of STZ (60 mg/kg), we monitored rats' blood glucose and body weight for 28 days. No difference in basal blood glucose concentration and body weight existed between STZ-treated rats and the control rats at the onset of the study (**Figure 1A**). However, compared with control rats, rats treated with STZ showed significant hyperglycemia at day 3 (23.75 ± 0.87 vs. 7.27 ± 0.30 mM in the control group, $p < 0.05$), which was maintained until day 28 (**Figure 1A**). Fourteen days after the onset of diabetes, body weight in the diabetic rats was significantly lower than in the control group (221.67 ± 6.00 vs. 336.67 ± 6.15 g in the control group, $p < 0.05$) and continued lower thereafter until day 28 (**Figure 1B**). These data show that rats injected with STZ developed type 1 diabetic features such as hyperglycemia and reduced body weight gain.

MECHANICAL ALLODYNIA AND IMPAIRED LOCOMOTOR ACTIVITY IN STREPTOZOTOCIN (STZ)-INDUCED TYPE 1 DIABETIC RATS

Fourteen days after STZ injection, the PWTs in diabetic rats were significantly lower than those in control rats when tested with *von* Frey filaments (14.17 ± 1.54 vs. 25.83 ± 0.83 g in the control group, $p < 0.05$, **Figure 1C**) and were further reduced at day 28 (6.67 ± 1.05 vs. 25.83 ± 1.54 g in the control group, $p < 0.05$, **Figure 1C**). The results indicate that, along with elevated blood glucose levels, the diabetic rats increasingly developed mechanical allodynia during days 14–28 (**Figure 1C**).

To determine whether STZ-induced diabetic rats also exhibited impaired locomotor activity, we assessed the total travel distance and average velocity in the open field test (**Figures 1D, E**). Significant reductions in total distance (102.54 ± 4.70 vs. 128.39 ± 6.68 m in control, $p < 0.05$, **Figure 1D**) and average velocity (11.39 ± 0.52 vs. 14.27 ± 0.74 cm/s in control, $p < 0.05$, **Figure 1E**) were observed in diabetic rats at day 28.

CHANGES IN ISOLECTIN B4 (IB4)-LABELED AND CALCITONIN GENE-RELATED PEPTIDE (CGRP)-IMMUNOREACTIVE STRUCTURES IN THE DORSAL ROOT GANGLION (DRG) AND SPINAL DORSAL HORN

To investigate whether the nonpeptidergic unmyelinated fibers and their cell bodies, and the peptidergic fibers and their cell bodies are involved in DPN, we examined the IB4-labeled and CGRP-immunoreactive (IR) structures in the DRG and spinal dorsal horn. In DRG, IB4-labeled and CGRP-IR cell

[1] http://rsbweb.nih.gov/ij/

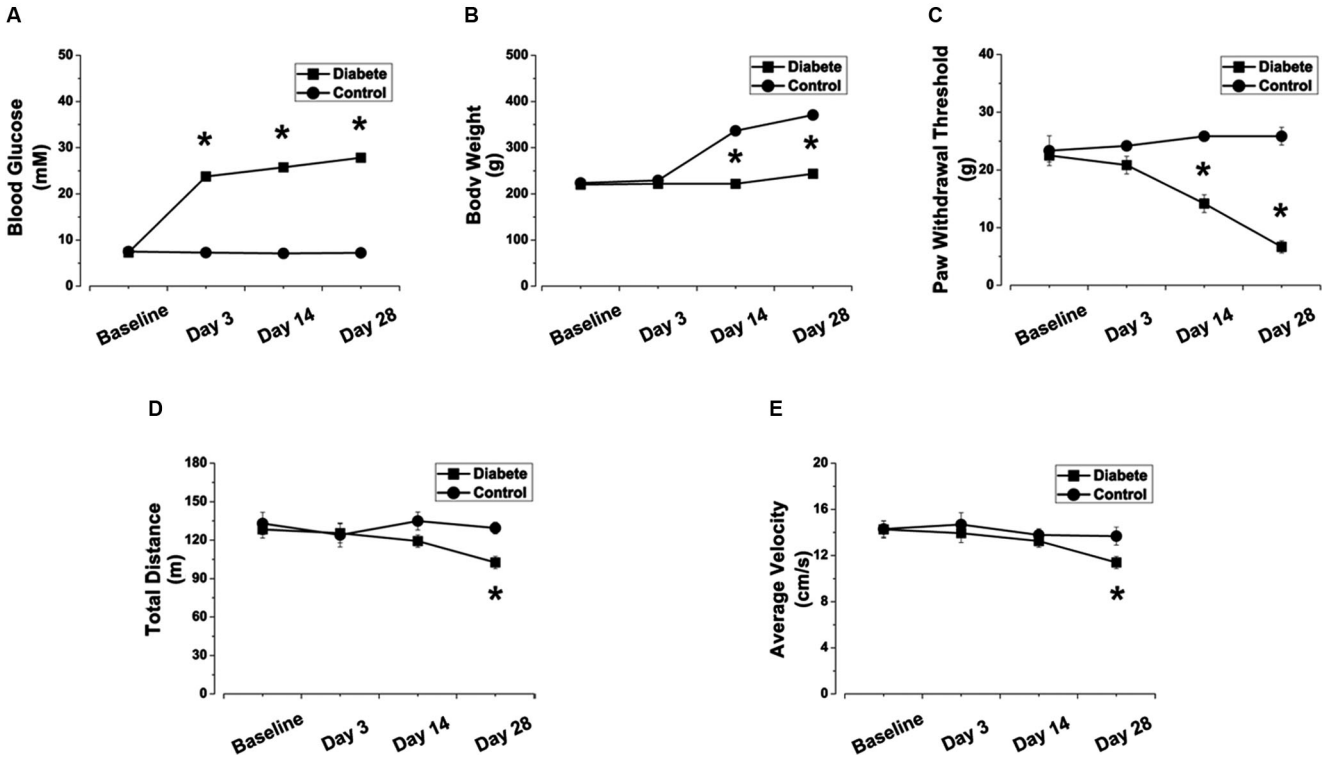

FIGURE 1 | STZ-induced diabetic rats exhibited elevated blood glucose concentration, reduced body weight gain, decreased PWTs, and impaired locomotor activity. Changes in blood glucose concentration **(A)**, body weight **(B)**, PWT **(C)**, total distance **(D)** and average velocity **(E)** in STZ-induced diabetic rats. Mean ± SD, n = 6–18 rats in each group. * $P <$ 0.05, comparing diabetic rats with non-diabetic control rats.

bodies are small (diameter ≤20 μm) to medium (20 μm < diameter ≤35 μm) neurons (Wang et al., 2010). Compared with controls, the number of IB4-labeled neurons decreased from day 14 (81.5% of control) to day 28 (73.5% of control) after onset of diabetes ($p < 0.05$, **Figures 2A, B**). The number of CGRP-IR neurons, however, significantly increased, measuring 125.3% of control at day 14 and 130.8% of control at day 28 ($p < 0.05$, **Figures 2A, C**). Similar changes occurred in the superficial laminae of the spinal dorsal horn, with IB4-labeled terminals decreasing (day 14, 83.4% of control, $p < 0.05$; day 28, 72.6% of control, $p < 0.05$, **Figures 3A, B**). CGRP-IR terminals were densely concentrated and increased in the inner part of lamina II ($p < 0.05$, **Figures 3A, C**). These data demonstrate that in both DRG and spinal dorsal horn, compared with the controls, IB4-labeled structures decreased and CGRP-IR structures increased in diabetic rats.

CHANGES OF CHOLERA TOXIN B (CTB)-LABELED STRUCTURES IN THE DORSAL ROOT GANGLION (DRG) AND SPINAL CORD

Consistent with a previous report (Wang et al., 2010), CTB-labeled elements were mainly large (diameter > 35 μm) neurons in controls. Numbers of CTB-labeled neurons markedly increased in the DRG, up to 119.8% of control at day 14 and 136.8% of control at day 28 ($p < 0.05$, **Figures 4A, B**). Notably, there were more small DRG neurons labeled with CTB in diabetic rats. In controls, 25.2% of CTB-labeled neurons were small neurons. However, the small CTB-labeled neurons increased in number from day 14 to day 28 in STZ-treated rats ($p < 0.05$, **Figures 4A, B**).

In spinal cord, CTB-labeling is present in both the dorsal and ventral horns of the spinal cord (**Figure 4A**). In the dorsal horn, CTB-labeled terminals were densely located in laminae III to IV, with some labeling in laminae I and II. In diabetic rats, CTB immunoreactivity was significantly increased at day 14 (120.1% of control, $p < 0.05$) and at day 28 (139.3% of control, $p <$ 0.05, **Figures 4A, C**). Importantly, compared with the controls, the diabetic rats showed denser immunoreactivity for CTB in the laminae III to IV of the spinal dorsal horn (**Figure 4A**). Numbers of CTB-labeled motor neurons in the ventral horn decreased significantly in diabetic rats (day 14, 83.2% of control, $p < 0.05$; day 28, 77.2% of control, $p < 0.05$, **Figures 4A, D**).

EFFECTS OF INTRATHECAL INJECTION OF INSULIN ON PAIN BEHAVIOR AND LOCOMOTOR ACTIVITY

To detect the effects of insulin at the early stages of DPN, either saline or insulin (0.2 units) was injected intrathecally once a day from day 14 to day 28 in STZ-induced diabetic rats. PWTs were tested on the day of STZ injection and at 3, 14, 21, and 28 days post-STZ injection (**Figure 5A**). Intrathecal injection

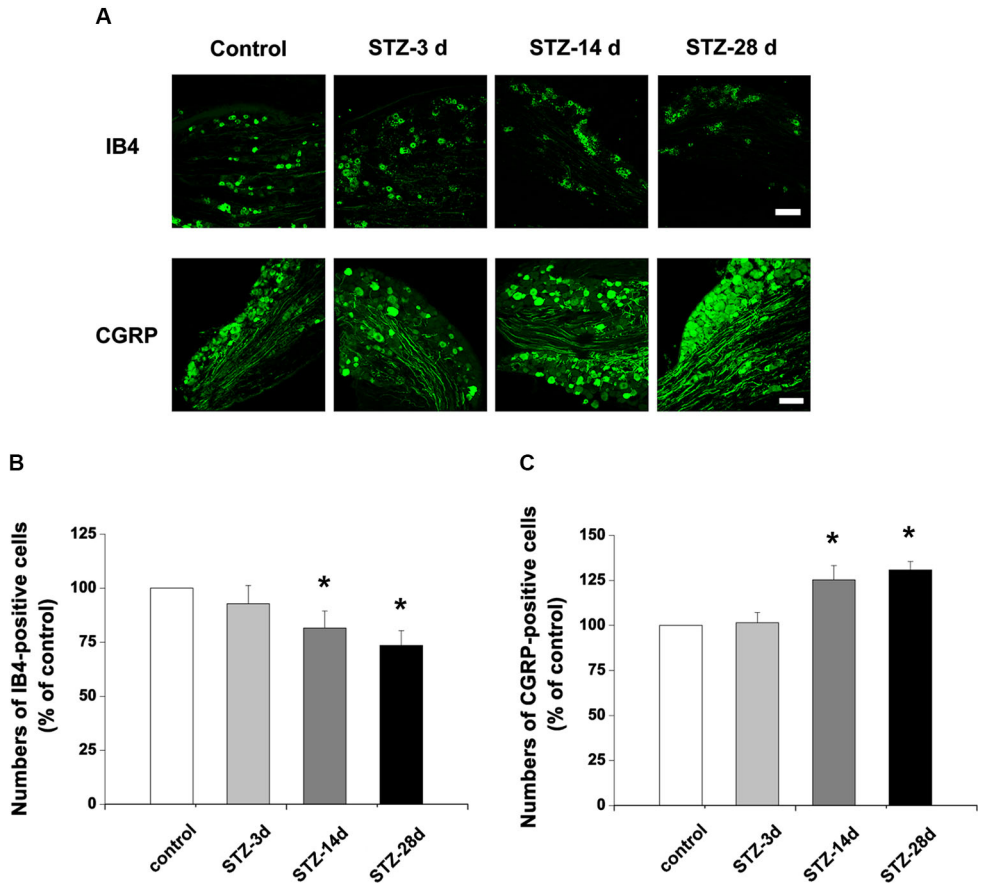

FIGURE 2 | Photographs showing IB4- and CGRP-stained neurons in the DRG. (A) Scale bar = 100 μm. Statistical analyses of the numbers of IB4-labeled **(B)** and CGRP-immunoreactive (IR) neurons **(C)** in different groups. Values are normalized, with the mean of the control group set as 100%. Mean ± SD, $n = 6$ rats in each group. * $P < 0.05$, comparing diabetic rats with non-diabetic control rats.

of insulin significantly alleviated allodynia in diabetic rats after 14 days ($p < 0.05$ vs. STZ rats-saline group), but could not completely attenuate the reduced PWTs ($p < 0.05$ vs. wild type-saline group, **Figure 5B**). Fourteen days of intrathecal insulin injection also prevented the decrease in total distance ($p < 0.05$ vs. STZ rats-saline group, $p > 0.05$ vs. wild type-saline group, **Figure 5C**) and average velocity ($p < 0.05$ vs. STZ rats-saline group, $p > 0.05$ vs. wild type-saline group, **Figure 5D**) in diabetic rats. These data indicate that insulin treatment at the early stages of DPN reduced the mechanical allodynia and impaired locomotor activity normally present in STZ-induced diabetic rats.

DISCUSSION

STZ-induced diabetic rodents are widely used for studying DPN (Mitsuhashi et al., 1993; Brussee et al., 2004; Zuo et al., 2011; Zguira et al., 2013). Our results show that STZ-induced diabetic rats exhibit hyperglycemia, decreased body weight gain and mechanical allodynia after STZ injection, as well as impaired locomotor activity at day 28, supporting the use of STZ-induced diabetic rats as an animal model for studying the mechanism of the early stages of type 1 DPN.

CHANGES IN ISOLECTIN B4 (IB4)-, CALCITONIN GENE-RELATED PEPTIDE (CGRP)- AND CHOLERA TOXIN B (CTB)-LABELED NEURONS IN THE DORSAL ROOT GANGLION (DRG)

DRG neurons receive a wide range of sensory information from nerve endings that are activated by mechanical, thermal, chemical and noxious stimuli (Delmas et al., 2011; Hughes et al., 2012). Nerve injury can induce marked changes in the expression of neuropeptides, neuromodulators, channels and related receptors in DRG neurons, resulting in a virtually new phenotype of DRG neurons (Hokfelt et al., 2000; Liu et al., 2000; Lai et al., 2004). For instance, after peripheral nerve injury, reduced IB4 binding was observed (Molander et al., 1996; Bennett et al., 1998). Importantly, in diabetic patients with DPN, there have been reports of abnormal function of unmyelinated afferents, suggesting that impairment in the receptive properties of unmyelinated nerves leads to DPN (Orstavik et al., 2006). Our results indicate that in DRG of diabetic rats, the number of IB4-labeled neurons decreased significantly from day 14, suggesting that decreased nonpeptidergic unmyelinated afferents might be one reason for early sensory dysfunction in diabetes.

In contrast to IB4, the number of CGRP-IR neurons increased from day 14 to day 28. It has been confirmed that CGRP is a

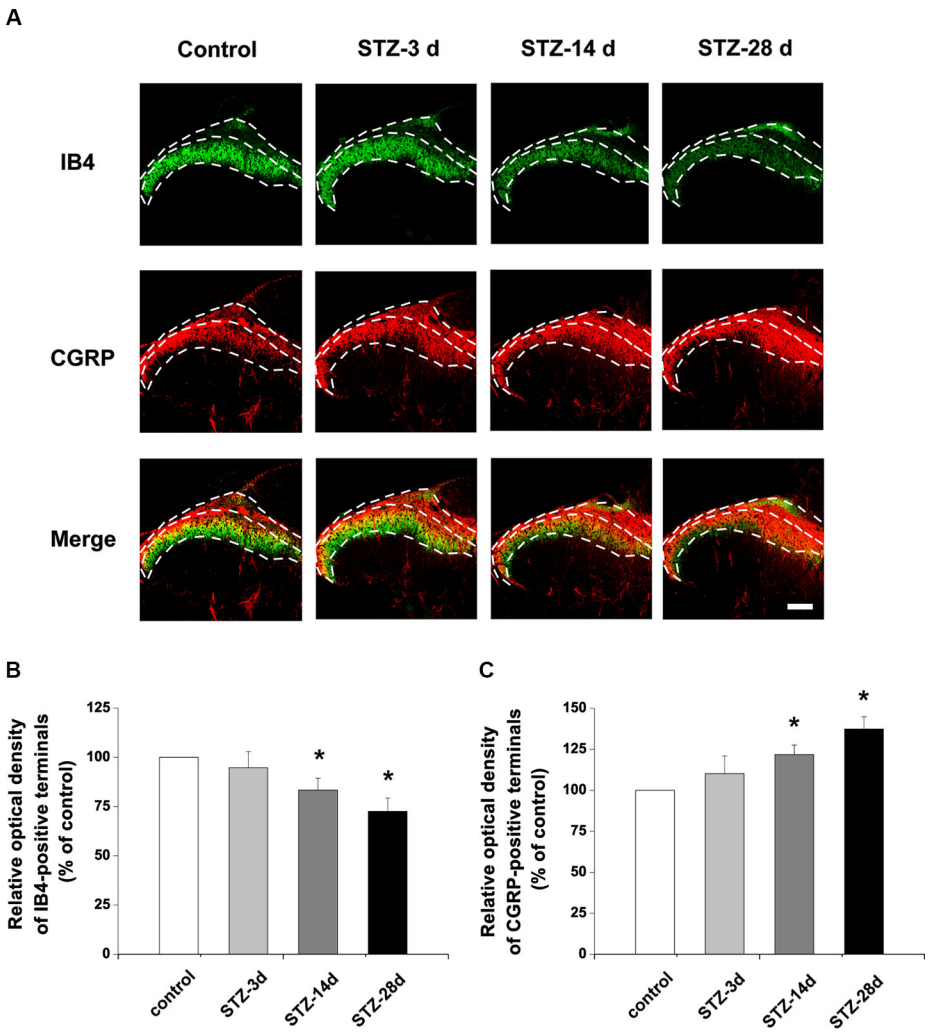

FIGURE 3 | Distribution and expression changes of IB4 labeling and CGRP immunoreactivity are observed in the spinal dorsal horn. Double fluorescent labeling for IB4 (green) and CGRP (red) shows that the expression of CGRP increased but IB4 staining decreased in diabetic rats **(A)**. Scale bar = 100 μm. Statistical analyses of the expression of IB4 **(B)** and CGRP **(C)** in different groups. Values are normalized, with the mean of the control group set as 100%. Mean ± SD, $n = 6$ rats in each group. * $P < 0.05$, comparing diabetic rats with non-diabetic control rats.

pain-related peptide and serves as a pain modulator, contributing to the augmented synaptic strength between DRG neurons and the spinal dorsal horn and leading to the generation of central sensitization in pain (Sun et al., 2003; Bird et al., 2006). Following peripheral inflammation or partial nerve injury, CGRP may be up-regulated and thus contribute to the generation of pain (Fehrenbacher et al., 2003; Scholz and Woolf, 2007). Our results demonstrate that CGRP expression increased in DRG neurons of diabetic rats from day 14 onward, suggesting that a subpopulation of peptidergic afferents might also be involved in painful DPN. Previous reports demonstrate that the CGRP expression in STZ-induced diabetic rodents either remains unchanged or decreases after 4 weeks (Diemel et al., 1994; Akkina et al., 2001). It is known that early intervention is very important for treating neuropathic complications in diabetes (Boulton et al., 2005), and rats treated with STZ developed mechanical allodynia beginning on day 14; therefore, we focused on the changes in neural circuits in the early stages (within 4 weeks). CGRP is a nerve growth factor (NGF)-dependent neuropeptide. In the early stages, the NGF production by DRG neurons increases in STZ-treated diabetic rats, but it gradually decreases after 4 weeks (Steinbacher and Nadelhaft, 1998). With the decreased support from NGF after 4 weeks, the expression of CGRP may decrease. The activation of transient receptor potential vanilloid subtype 1 (TRPV1) channel leads to the release of CGRP (Huang et al., 2008). The expression and function of TRPV1 is initially enhanced but is reduced in the later phase in diabetic rats (Pabbidi et al., 2008). Previous report also indicates similar changes in the early and late stages of human diabetes (Dyck et al., 2000). STZ-induced diabetes also shows two phases of pain sensitivity (Pabbidi et al., 2008). Therefore, our results suggest that the increased CGRP expression from day 14 to 28 might indicate the early changes in DPN.

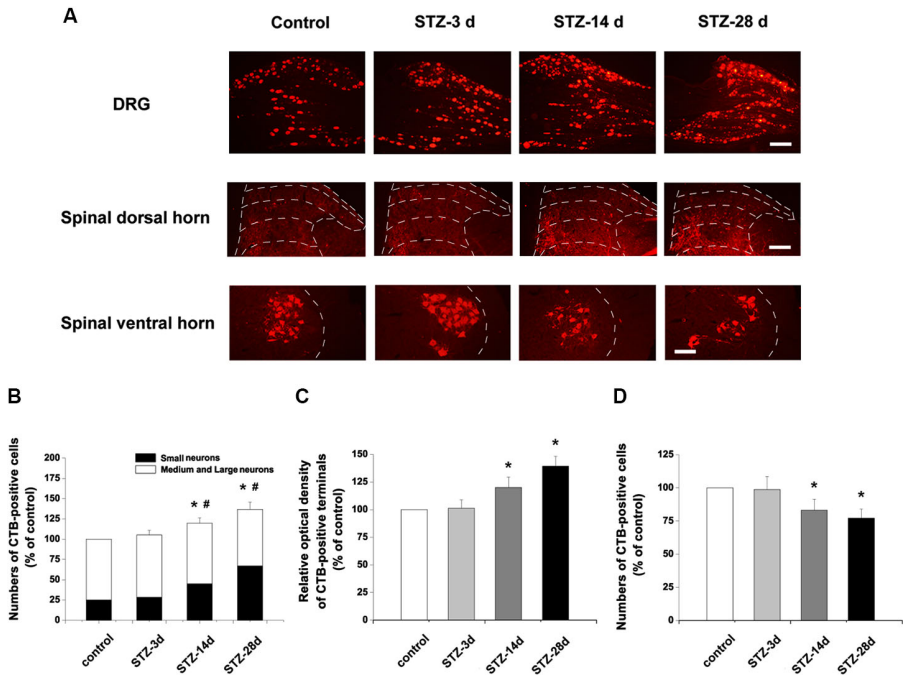

FIGURE 4 | Changes of CTB-labeled elements in the DRG and spinal dorsal and ventral horns. (A) Scale bar = 100 μm. Statistical analyses of CTB labeling in the DRG **(B)**, spinal dorsal horn **(C)** and spinal ventral horn **(D)**. Values are normalized, with the mean of the control group set as 100%. The proportions of small neurons to the total CTB-labeled DRG neurons are 25.2% in the control, 27.1% in STZ-3d, 37.7% in STZ-14d and 46.1% in STZ-28d groups. Mean ± SD, $n = 6$ rats for each group. * $P < 0.05$, comparing the numbers of CTB-labeled cells in diabetic rats with those in non-diabetic control rats. # $P < 0.05$, comparing the proportions of the small CTB-labeled DRG neurons in diabetic rats with non-diabetic control rats.

However, we could not exclude the possibility that other factors might be involved. For instance, differences in CGRP innervation in diabetic rats and mice have been demonstrated (Karanth et al., 1990; Christianson et al., 2003). Cutaneous CGRP-IR nerves are largely lost in diabetic mice, whereas they increase in STZ-induced diabetic rats. Further, the variations in the dosage of STZ and in the initial age of animals used in research, both of which are important for the development of diabetes, might also contribute to the differences among reports.

In diabetic rats, there was also a significant increase in the number of CTB-labeled neurons in the DRG. In normal rats, CTB-labeled cells are mainly large DRG neurons that send myelinated fibers to the spinal dorsal horn and are involved in proprioception (Tong et al., 1999; Shehab et al., 2004). Interestingly, in diabetic rats, CTB labeling was also found in many small neurons, indicating a shift in tracer uptake in small DRG neurons in diabetes. A similar change in CTB-labeling phenotype has been demonstrated in nerve injury (Tong et al., 1999). Therefore, these phenotypic changes in these subpopulations of DRG neurons might contribute to the sensory dysfunction in the early stages of DPN.

CHANGES IN ISOLECTIN B4 (IB4), CALCITONIN GENE-RELATED PEPTIDE (CGRP) AND CHOLERA TOXIN B (CTB) LABELING IN THE SPINAL DORSAL HORN

It has been shown that nociceptive signals are conveyed from the DRG to the CNS for integration, beginning in the spinal cord (Todd, 2010). Thus, the afferents in the spinal cord from the DRG are essential for understanding the mechanisms of DPN in the CNS. Our data also show that afferents in the dorsal horn, which showed IB4 labeling and CGRP immunoreactivity, were altered in diabetic rats. IB4 immunoreactivity decreased, whereas CGRP-positive terminals increased. Increased production of CGRP from primary sensory neurons is also present in other pathological pain conditions (Fehrenbacher et al., 2003; Scholz and Woolf, 2007). Therefore, the changes in IB4-labeled and CGRP-IR central axons originating from DRG neurons in diabetic rats might also contribute to painful DPN in the spinal dorsal horn. We also found that CTB-positive nerve endings increased in laminae III and IV in diabetic rats, and there was also nerve-injury-induced sprouting of myelinated afferent fibers from deeper laminae. (Tong et al., 1999; Tan et al., 2012). In addition, it is known that myelinated fibers normally respond to innocuous sensations such as touch and pressure (Woolf, 1995). Therefore, the reorganization of such structures in diabetes may contribute to the sustained pain and mechanical allodynia in diabetic rats. Combined with the data showing that more small DRG neurons in diabetic rats express CTB, it is possible that, at least in part, the increased sprouting of CTB-labeled fibers in diabetic rats is attributable to phenotypic changes in axons, resulting in the axons from small DRG neurons in spinal dorsal horn becoming CTB-positive. Moreover, the complicated physiological conditions displayed by diabetic rats, including hyperglycemia and hypoxia, might be possible reasons for the increased sprouting

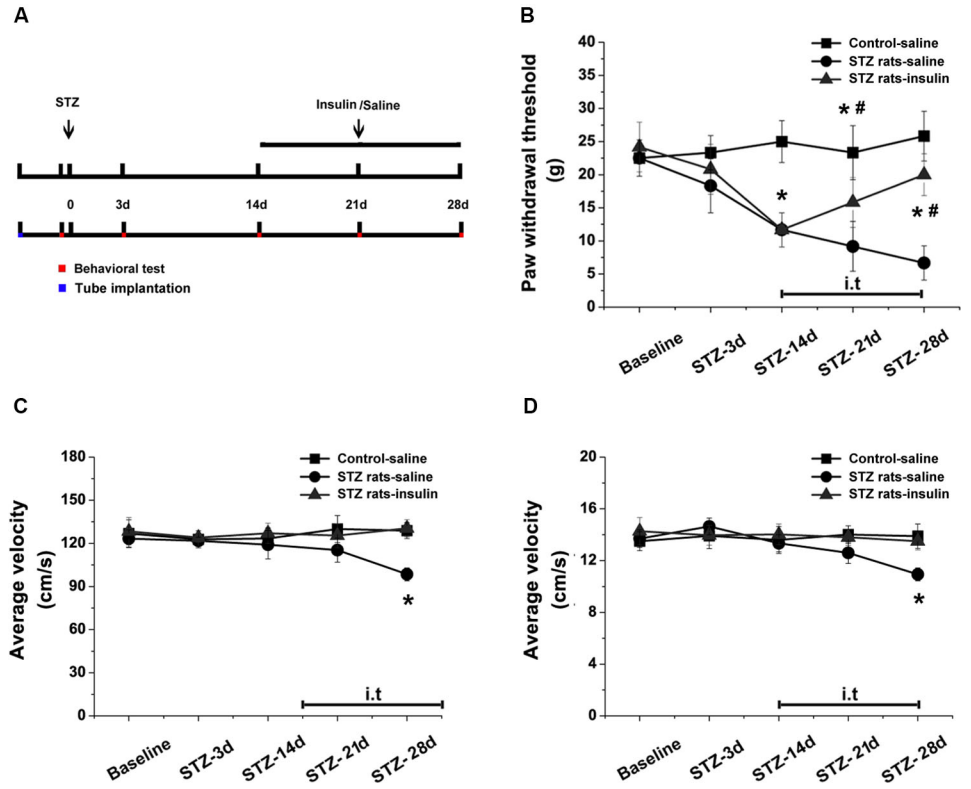

FIGURE 5 | Effects of intrathecal insulin on pain behavior and locomotor activity in diabetic rats compared with controls. PWTs and open field tests were measured before (baseline) and 3, 14, 21 and 28 days after STZ injection, with and without insulin application from day 14 to day 28 **(A)**. Intrathecal injection of insulin alleviated mechanical allodynia **(B)** and impaired locomotor activity (total distance, **(C)**, and average velocity, **(D)**, in the open field test) in diabetic rats after 14 days of administration. Mean ± SD, $n = 6$ rats in each group. * $P < 0.05$, comparing the STZ rats-insulin group with the STZ rats-saline group at corresponding time points. # $P < 0.05$, comparing the STZ rats-insulin group with the control-saline group at corresponding time points.

of myelinated terminals in the spinal dorsal horn. Thus, the changes in neural circuits from the peripheral nerves to the spinal dorsal horn might be involved in the progressive sensory defects in DPN.

CHANGES OF CHOLERA TOXIN B (CTB)-LABELED NEURONS IN THE SPINAL VENTRAL HORN

Motor diabetic neuropathy is a prominent complication in diabetes (Andersen and Mogensen, 1997; Andersen, 2012). Decline in nerve conduction velocity and motor function impairment are evident during early stages of diabetes in human patients (Dyck et al., 1986; Dyck and Giannini, 1996; Malik et al., 2001). The open field test data presented here show that locomotor activity, including total distance and average velocity, decreased at day 28 in diabetic rats. However, mechanical allodynia may also be impairing locomotor activity in diabetic rats. To clarify this issue, we injected CTB into the sciatic nerve to simultaneously observe the transportation of the neurotracer from the sciatic nerve to the spinal dorsal and ventral horns. This method could show the changes in sensory and motor regions at the same time points. CTB labeling in motor neurons decreased beginning on day 14 in diabetic rats, indicating that structural abnormalities occur in the CNS before motor dysfunction presents. These changes in the motor neural circuit from the peripheral nerves to the spinal ventral horn might lead to impaired motor activity in the early stages of DPN independent of mechanical allodynia.

A previous report demonstrates that motor neurons develop progressive features of distal loss of axonal terminals in chronic STZ-induced diabetic rats (Ramji et al., 2007). Although motor impairment might begin later than sensory defects in diabetes (Dyck et al., 1986; Dyck and Giannini, 1996; Malik et al., 2001), declines in motor nerve conduction velocity are evident beginning at 2 weeks in diabetic rats (Coppey et al., 2000), which is consistent with our results showing decreased CTB labeling in the spinal ventral horn on day 14. Therefore, the reduced CTB labeling at the early stages might suggest a defect in the transportation of CTB through the ventral root of the spinal nerve. However, because of the different neural structure, the blood-brain barrier might provide greater protection for the motor neurons in the spinal ventral horn than for sensory neurons in the DRG. The underlying relationship between the impaired motor activity and motor neural circuit requires further investigation.

INTRATHECAL INJECTION OF INSULIN ALLEVIATES MECHANICAL ALLODYNIA AND IMPAIRED LOCOMOTOR ACTIVITY

Because STZ-induced diabetic rats are insulin deficient, it was important to test whether insulin treatment would help to maintain normal neurotransmission, particularly because physiological concentrations of insulin directly enhance axon formation in the DRG and spinal cord (Fernyhough et al., 1993; Huang et al., 2005). Moreover, insulin modulates neurofilament and tubulin abundance, which is correlated with the induction of neurite elongation (Fernyhough et al., 1989). We therefore injected insulin for 14 days. Intrathecal injection of insulin significantly alleviated mechanical allodynia and impaired locomotor activity in diabetic rats, indicating that early intervention is important for treating DPN.

In summary, we have described the changes in the neural circuits in STZ-induced diabetic rats with progressive mechanical allodynia and impaired locomotor activity. The alterations in myelinated nerve fibers, unmyelinated nonpeptidergic nerve fibers, and peptidergic nerve fibers might be involved in the early stages of the development in DPN. The underlying mechanism of DPN might be addressed by the dysfunction of those subpopulations of afferents from the PNS to the CNS.

ACKNOWLEDGMENTS

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REFERENCES

Aizawa, N., and Eggermont, J. J. (2006). Effects of noise-induced hearing loss at young age on voice onset time and gap-in-noise representations in adult cat primary auditory cortex. *J. Assoc. Res. Otolaryngol.* 7, 71–81. doi: 10.1007/s10162-005-0026-3

Akkina, S. K., Patterson, C. L., and Wright, D. E. (2001). GDNF rescues nonpeptidergic unmyelinated primary afferents in streptozotocin-treated diabetic mice. *Exp. Neurol.* 167, 173–182. doi: 10.1006/exnr.2000.7547

Andersen, H. (2012). Motor dysfunction in diabetes. *Diabetes Metab. Res. Rev.* 28(Suppl. 1), 89–92. doi: 10.1002/dmrr.2257

Andersen, H., and Mogensen, P. H. (1997). Disordered mobility of large joints in association with neuropathy in patients with long-standing insulin-dependent diabetes mellitus. *Diabet. Med.* 14, 221–227. doi: 10.1002/(sici)1096-9136(199703)14:3<221::aid-dia338>3.0.co;2-k

Baron, R., Tolle, T. R., Gockel, U., Brosz, M., and Freynhagen, R. (2009). A cross-sectional cohort survey in 2100 patients with painful diabetic neuropathy and postherpetic neuralgia: differences in demographic data and sensory symptoms. *Pain* 146, 34–40. doi: 10.1016/j.pain.2009.06.001

Bastyr, E. J. 3rd, Price, K. L., and Bril, V. (2005). Development and validity testing of the neuropathy total symptom score-6: questionnaire for the study of sensory symptoms of diabetic peripheral neuropathy. *Clin. Ther.* 27, 1278–1294. doi: 10.1016/j.clinthera.2005.08.002

Bennett, D. L., Michael, G. J., Ramachandran, N., Munson, J. B., Averill, S., Yan, Q., et al. (1998). A distinct subgroup of small DRG cells express GDNF receptor components and GDNF is protective for these neurons after nerve injury. *J. Neurosci.* 18, 3059–3072.

Bird, G. C., Han, J. S., Fu, Y., Adwanikar, H., Willis, W. D., and Neugebauer, V. (2006). Pain-related synaptic plasticity in spinal dorsal horn neurons: role of CGRP. *Mol. Pain* 2:31. doi: 10.1186/1744-8069-2-31

Boulton, A. J., Vinik, A. I., Arezzo, J. C., Bril, V., Feldman, E. L., Freeman, R., et al. (2005). Diabetic neuropathies: a statement by the American Diabetes Association. *Diabetes Care* 28, 956–962. doi: 10.2337/diacare.28.4.956

Brussee, V., Cunningham, F. A., and Zochodne, D. W. (2004). Direct insulin signaling of neurons reverses diabetic neuropathy. *Diabetes* 53, 1824–1830. doi: 10.2337/diabetes.53.7.1824

Burgess, A., Vigneron, S., Brioudes, E., Labbe, J. C., Lorca, T., and Castro, A. (2010). Loss of human greatwall results in G2 arrest and multiple mitotic defects due to deregulation of the cyclin B-Cdc2/PP2A balance. *Proc. Natl. Acad. Sci. U S A* 107, 12564–12569. doi: 10.1073/pnas.0914191107

Christianson, J. A., Riekhof, J. T., and Wright, D. E. (2003). Restorative effects of neurotrophin treatment on diabetes-induced cutaneous axon loss in mice. *Exp. Neurol.* 179, 188–199. doi: 10.1016/s0014-4886(02)00017-1

Coppey, L. J., Davidson, E. P., Dunlap, J. A., Lund, D. D., and Yorek, M. A. (2000). Slowing of motor nerve conduction velocity in streptozotocin-induced diabetic rats is preceded by impaired vasodilation in arterioles that overlie the sciatic nerve. *Int. J. Exp. Diabetes Res.* 1, 131–143. doi: 10.1155/edr.2000.131

Delmas, P., Hao, J., and Rodat-Despoix, L. (2011). Molecular mechanisms of mechanotransduction in mammalian sensory neurons. *Nat. Rev. Neurosci.* 12, 139–153. doi: 10.1038/nrn2993

Diemel, L. T., Brewster, W. J., Fernyhough, P., and Tomlinson, D. R. (1994). Expression of neuropeptides in experimental diabetes; effects of treatment with nerve growth factor or brain-derived neurotrophic factor. *Brain Res. Mol. Brain Res.* 21, 171–175. doi: 10.1016/0169-328x(94)90391-3

Duce, I. R., and Keen, P. (1977). An ultrastructural classification of the neuronal cell bodies of the rat dorsal root ganglion using zinc iodide-osmium impregnation. *Cell Tissue Res.* 185, 263–277. doi: 10.1007/bf00220670

Dyck, P. J., and Giannini, C. (1996). Pathologic alterations in the diabetic neuropathies of humans: a review. *J. Neuropathol. Exp. Neurol.* 55, 1181–1193. doi: 10.1097/00005072-199612000-00001

Dyck, P. J., Kratz, K. M., Karnes, J. L., Litchy, W. J., Klein, R., Pach, J. M., et al. (1993). The prevalence by staged severity of various types of diabetic neuropathy, retinopathy and nephropathy in a population-based cohort: the Rochester Diabetic Neuropathy Study. *Neurology* 43, 817–824. doi: 10.1212/wnl.43.4.817

Dyck, P. J., Lais, A., Karnes, J. L., O'brien, P., and Rizza, R. (1986). Fiber loss is primary and multifocal in sural nerves in diabetic polyneuropathy. *Ann. Neurol.* 19, 425–439. doi: 10.1002/ana.410190503

Dyck, P. J., Larson, T. S., O'brien, P. C., and Velosa, J. A. (2000). Patterns of quantitative sensation testing of hypoesthesia and hyperalgesia are predictive of diabetic polyneuropathy: a study of three cohorts. Nerve growth factor study group. *Diabetes Care* 23, 510–517. doi: 10.2337/diacare.23.4.510

Eaton, S. E., Harris, N. D., Rajbhandari, S. M., Greenwood, P., Wilkinson, I. D., Ward, J. D., et al. (2001). Spinal-cord involvement in diabetic peripheral neuropathy. *Lancet* 358, 35–36. doi: 10.1016/S0140-6736(00)05268-5

Fehrenbacher, J. C., Taylor, C. P., and Vasko, M. R. (2003). Pregabalin and gabapentin reduce release of substance P and CGRP from rat spinal tissues only after inflammation or activation of protein kinase C. *Pain* 105, 133–141. doi: 10.1016/s0304-3959(03)00173-8

Fernyhough, P., Mill, J. F., Roberts, J. L., and Ishii, D. N. (1989). Stabilization of tubulin mRNAs by insulin and insulin-like growth factor I during neurite formation. *Brain Res. Mol. Brain Res.* 6, 109–120. doi: 10.1016/0169-328x(89)90044-2

Fernyhough, P., Willars, G. B., Lindsay, R. M., and Tomlinson, D. R. (1993). Insulin and insulin-like growth factor I enhance regeneration in cultured adult rat sensory neurones. *Brain Res.* 607, 117–124. doi: 10.1016/0006-8993(93)91496-f

Fischer, T. Z., and Waxman, S. G. (2010). Neuropathic pain in diabetes—evidence for a central mechanism. *Nat. Rev. Neurol.* 6, 462–466. doi: 10.1038/nrneurol.2010.90

Hokfelt, T., Broberger, C., Xu, Z. Q., Sergeyev, V., Ubink, R., and Diez, M. (2000). Neuropeptides–an overview. *Neuropharmacology* 39, 1337–1356. doi: 10.1016/s0028-3908(00)00010-1

Huang, T. J., Verkhratsky, A., and Fernyhough, P. (2005). Insulin enhances mitochondrial inner membrane potential and increases ATP levels through phosphoinositide 3-kinase in adult sensory neurons. *Mol. Cell. Neurosci.* 28, 42–54. doi: 10.1016/j.mcn.2004.08.009

Huang, W., Wang, H., Galligan, J. J., and Wang, D. H. (2008). Transient receptor potential vanilloid subtype 1 channel mediated neuropeptide secretion and depressor effects: role of endoplasmic reticulum associated Ca2+ release receptors in rat dorsal root ganglion neurons. *J. Hypertens.* 26, 1966–1975. doi: 10.1097/hjh.0b013e328309eff9

Hughes, J. P., Chessell, I., Malamut, R., Perkins, M., Backonja, M., Baron, R., et al. (2012). Understanding chronic inflammatory and neuropathic pain. *Ann. N Y Acad. Sci.* 1255, 30–44. doi: 10.1111/j.1749-6632.2012.06561.x

Karanth, S. S., Springall, D. R., Francavilla, S., Mirrlees, D. J., and Polak, J. M. (1990). Early increase in CGRP- and VIP-immunoreactive nerves in the skin of streptozotocin-induced diabetic rats. *Histochemistry* 94, 659–666. doi: 10.1007/bf00271994

Karanth, S. S., Springall, D. R., Kuhn, D. M., Levene, M. M., and Polak, J. M. (1991). An immunocytochemical study of cutaneous innervation and the distribution of neuropeptides and protein gene product 9.5 in man and commonly employed laboratory animals. *Am. J. Anat.* 191, 369–383. doi: 10.1002/aja.1001910404

Kou, Z. Z., Li, C. Y., Tang, J., Hu, J. C., Qu, J., Liao, Y. H., et al. (2013a). Down-regulation of insulin signaling is involved in painful diabetic neuropathy in type 2 diabetes. *Pain Physician* 16, E71–E83.

Kou, Z. Z., Qu, J., Zhang, D. L., Li, H., and Li, Y. Q. (2013b). Noise-induced hearing loss is correlated with alterations in the expression of GABAB receptors and PKC gamma in the murine cochlear nucleus complex. *Front. Neuroanat.* 7:25. doi: 10.3389/fnana.2013.00025

Lai, J., Porreca, F., Hunter, J. C., and Gold, M. S. (2004). Voltage-gated sodium channels and hyperalgesia. *Annu. Rev. Pharmacol. Toxicol.* 44, 371–397. doi: 10.1146/annurev.pharmtox.44.101802.121627

Liu, C. N., Wall, P. D., Ben-Dor, E., Michaelis, M., Amir, R., and Devor, M. (2000). Tactile allodynia in the absence of C-fiber activation: altered firing properties of DRG neurons following spinal nerve injury. *Pain* 85, 503–521. doi: 10.1016/s0304-3959(00)00251-7

Malik, R. A., Veves, A., Walker, D., Siddique, I., Lye, R. H., Schady, W., et al. (2001). Sural nerve fibre pathology in diabetic patients with mild neuropathy: relationship to pain, quantitative sensory testing and peripheral nerve electrophysiology. *Acta Neuropathol.* 101, 367–374.

Mei, X. P., Zhou, Y., Wang, W., Tang, J., Zhang, H., Xu, L. X., et al. (2011). Ketamine depresses toll-like receptor 3 signaling in spinal microglia in a rat model of neuropathic pain. *Neurosignals* 19, 44–53. doi: 10.1159/000324293

Michael, G. J., and Priestley, J. V. (1999). Differential expression of the mRNA for the vanilloid receptor subtype 1 in cells of the adult rat dorsal root and nodose ganglia and its downregulation by axotomy. *J. Neurosci.* 19, 1844–1854.

Mitsuhashi, T., Nakayama, H., Itoh, T., Kuwajima, S., Aoki, S., Atsumi, T., et al. (1993). Immunochemical detection of advanced glycation end products in renal cortex from STZ-induced diabetic rat. *Diabetes* 42, 826–832. doi: 10.2337/diabetes.42.6.826

Molander, C., Wang, H. F., Rivero-Melian, C., and Grant, G. (1996). Early decline and late restoration of spinal cord binding and transganglionic transport of isolectin B4 from Griffonia simplicifolia I after peripheral nerve transection or crush. *Restor. Neurol. Neurosci.* 10, 123–133. doi: 10.3233/RNN-1996-10301

Orstavik, K., Namer, B., Schmidt, R., Schmelz, M., Hilliges, M., Weidner, C., et al. (2006). Abnormal function of C-fibers in patients with diabetic neuropathy. *J. Neurosci.* 26, 11287–11294. doi: 10.1523/jneurosci.2659-06.2006

Pabbidi, R. M., Yu, S. Q., Peng, S., Khardori, R., Pauza, M. E., and Premkumar, L. S. (2008). Influence of TRPV1 on diabetes-induced alterations in thermal pain sensitivity. *Mol. Pain* 4:9. doi: 10.1186/1744-8069-4-9

Quan-Xin, F., Fan, F., Xiang-Ying, F., Shu-Jun, L., Shi-Qi, W., Zhao-Xu, L., et al. (2012). Resolvin D1 reverses chronic pancreatitis-induced mechanical allodynia, phosphorylation of NMDA receptors and cytokines expression in the thoracic spinal dorsal horn. *BMC Gastroenterol.* 12:148. doi: 10.1186/1471-230x-12-148

Ramji, N., Toth, C., Kennedy, J., and Zochodne, D. W. (2007). Does diabetes mellitus target motor neurons? *Neurobiol. Dis.* 26, 301–311. doi: 10.1016/j.nbd.2006.11.016

Scholz, J., and Woolf, C. J. (2007). The neuropathic pain triad: neurons, immune cells and glia. *Nat. Neurosci.* 10, 1361–1368. doi: 10.1038/nn1992

Shehab, S. A., Spike, R. C., and Todd, A. J. (2004). Do central terminals of intact myelinated primary afferents sprout into the superficial dorsal horn of rat spinal cord after injury to a neighboring peripheral nerve? *J. Comp. Neurol.* 474, 427–437. doi: 10.1002/cne.20147

Steinbacher, B. C. Jr., and Nadelhaft, I. (1998). Increased levels of nerve growth factor in the urinary bladder and hypertrophy of dorsal root ganglion neurons in the diabetic rat. *Brain Res.* 782, 255–260. doi: 10.1016/s0006-8993(97)01287-0

Sun, R. Q., Lawand, N. B., and Willis, W. D. (2003). The role of calcitonin gene-related peptide (CGRP) in the generation and maintenance of mechanical allodynia and hyperalgesia in rats after intradermal injection of capsaicin. *Pain* 104, 201–208. doi: 10.1016/s0304-3959(03)00008-3

Tan, A. M., Chakrabarty, S., Kimura, H., and Martin, J. H. (2012). Selective corticospinal tract injury in the rat induces primary afferent fiber sprouting in the spinal cord and hyperreflexia. *J. Neurosci.* 32, 12896–12908. doi: 10.1523/jneurosci.6451-11.2012

Tesfaye, S., Boulton, A. J., Dyck, P. J., Freeman, R., Horowitz, M., Kempler, P., et al. (2010). Diabetic neuropathies: update on definitions, diagnostic criteria, estimation of severity and treatments. *Diabetes Care* 33, 2285–2293. doi: 10.2337/dc10-1303

IJzerman, T. H., Schaper, N. C., Melai, T., Meijer, K., Willems, P. J., and Savelberg, H. H. (2012). Lower extremity muscle strength is reduced in people with type 2 diabetes, with and without polyneuropathy and is associated with impaired mobility and reduced quality of life. *Diabetes Res. Clin. Pract.* 95, 345–351. doi: 10.1016/j.diabres.2011.10.026

Todd, A. J. (2010). Neuronal circuitry for pain processing in the dorsal horn. *Nat. Rev. Neurosci.* 11, 823–836. doi: 10.1038/nrn2947

Tong, Y. G., Wang, H. F., Ju, G., Grant, G., Hokfelt, T., and Zhang, X. (1999). Increased uptake and transport of cholera toxin B-subunit in dorsal root ganglion neurons after peripheral axotomy: possible implications for sensory sprouting. *J. Comp. Neurol.* 404, 143–158. doi: 10.1002/(sici)1096-9861(19990208)404:2<143::aid-cne1>3.3.co;2-r

Wang, H. B., Zhao, B., Zhong, Y. Q., Li, K. C., Li, Z. Y., Wang, Q., et al. (2010). Coexpression of delta- and mu-opioid receptors in nociceptive sensory neurons. *Proc. Natl. Acad. Sci. U S A* 107, 13117–13122. doi: 10.1073/pnas.1008382107

Woolf, C. J. (1995). Somatic pain–pathogenesis and prevention. *Br. J. Anaesth.* 75, 169–176. doi: 10.1093/bja/75.2.169

Yasuda, H., Terada, M., Maeda, K., Kogawa, S., Sanada, M., Haneda, M., et al. (2003). Diabetic neuropathy and nerve regeneration. *Prog. Neurobiol.* 69, 229–285. doi: 10.1016/S0301-0082(03)00034-0

Zguira, M. S., Vincent, S., Le Douairon Lahaye, S., Malarde, L., Tabka, Z., and Saiag, B. (2013). Intense exercise training is not effective to restore the endothelial NO-dependent relaxation in STZ-diabetic rat aorta. *Cardiovasc. Diabetol.* 12:32. doi: 10.1186/1475-2840-12-32

Zuo, Z. F., Wang, W., Niu, L., Kou, Z. Z., Zhu, C., Zhao, X. H., et al. (2011). RU486 (mifepristone) ameliorates cognitive dysfunction and reverses the down-regulation of astrocytic N-myc downstream-regulated gene 2 in streptozotocin-induced type-1 diabetic rats. *Neuroscience* 190, 156–165. doi: 10.1016/j.neuroscience.2011.06.025

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Origin and neurochemical properties of bulbospinal neurons projecting to the rat lumbar spinal cord via the medial longitudinal fasciculus and caudal ventrolateral medulla

Zilli Huma, Amy Du Beau, Christina Brown and David J. Maxwell*

Spinal Cord Group, Institute of Neuroscience and Psychology, College of Medicine, Veterinary Medicine and Life Sciences, University of Glasgow, Glasgow, UK

Edited by:
Brian R. Noga, University of Miami, USA

Reviewed by:
Alan Roberts, University of Bristol, UK
Marie-Claude Perreault, Emory University, USA

Correspondence:
David J. Maxwell, Spinal Cord Group, Institute of Neuroscience and Psychology, College of Medicine, Veterinary Medicine and Life Sciences, University of Glasgow, West Medical Building, Glasgow G12 8QQ, UK
e-mail: david.maxwell@glasgow.ac.uk

Bulbospinal systems (BS) originate from various regions of the brainstem and influence spinal neurons by classical synaptic and modulatory mechanisms. Our aim was to determine the brainstem locations of cells of origin of BS pathways passing through the medial longitudinal fasciculus (MLF) and the caudal ventrolateral medulla (CVLM). We also examined the transmitter content of spinal terminations of the CVLM pathway. Six adult rats received Fluorogold (FG) injections to the right intermediate gray matter of the lumbar cord (L1–L2) and the b-subunit of cholera toxin (CTb) was injected either into the MLF or the right CVLM (3 animals each). Double-labeled cells were identified within brainstem structures with confocal microscopy and mapped onto brainstem diagrams. An additional 3 rats were injected with CTb in the CVLM to label axon terminals in the lumbar spinal cord. Double-labeled cells projecting via the MLF or CVLM were found principally in reticular regions of the medulla and pons but small numbers of cells were also located within the midbrain. CVLM projections to the lumbar cord were almost exclusively ipsilateral and concentrated within the intermediate gray matter. Most (62%) of terminals were immunoreactive for the vesicular glutamate transporter 2 while 23% contained the vesicular GABA transporter. The inhibitory subpopulation was glycinergic, GABAergic or contained both transmitters. The proportions of excitatory and inhibitory axons projecting via the CVLM to the lumbar cord are similar to those projecting via the MLF. Unlike the MLF pathway, CVLM projections are predominantly ipsilateral and concentrated within intermediate gray but do not extend into motor nuclei or laminia VIII. Terminations of the CVLM pathway are located in a region of the gray matter that is rich in premotor interneurons; thus its primary function may be to coordinate activity of premotor networks.

Keywords: brainstem, spinal cord, descending system, neurotransmitters, motor control, tract-tracing, confocal microscopy, neuroanatomy

Anatomical Abbreviations: 4v, 4th ventricle; 5n, motor trigeminal nucleus; 7n, facial nucleus; 12n, hypoglossal nucleus; CIC, central nucleus of the inferior colliculus; Cu, cuneate nucleus; CVLM, caudal ventrolateral medulla; DpMe, deep mesencephalic nucleus; Gi, gigantocellular reticular nucleus; Gr, gracile nucleus; IO, inferior olive; IRt, intermediate reticular nucleus; LDTg, laterodorsal tegmental nucleus; LPAG, lateral periaqueductal gray; LPGi, lateral paragigantocellular nucleus; LRt, lateral reticular nucleus; MdV, medullary reticular nucleus; Mlf, medial longitudinal fasciculus; MVe, medial vestibular nucleus; MVPO, medioventral periolivary nucleus; PaR, pararubral nucleus; PBP, parabrachial pigmented nucleus of the VTA; PCRt, parvicellular reticular nucleus; PnC, pontine reticular nucleus, caudal part; PnO, pontine reticular nucleus, oral part; PPTg, pedunculopontine tegmental nucleus; PPy, parapyramidal nucleus; Py, pyramid; RMg, raphe magnus nucleus; Rob, raphe obscurus nucleus; RPa, raphe pallidus nucleus; RR, retrorubral nucleus; RtTg, reticulotegmental nucleus; Sol, nucleus of the solitary tract; SPO, superior paraolivary nucleus; SPTg, subpeduncular tegmental nucleus; subCD, subcoeruleus nucleus; tz, trapezoid body; VLPAG, ventrolateral periaqueductal gray.
Other Abbreviations: CTb, cholera toxin B subunit; CST, DAB, 3,3′-diaminobenzidine; GAD-67, glutamic acid decarboxylase 67 isoform; GLY T2, glycine transporter 2, HRP, horseradish peroxidise; IgG, immunoglobulin gamma; PB, Phosphate buffer; PBS, Phosphate buffer saline; PBST, phosphate buffer saline containing 0.3% Trition X-100; RST, reticulospinal tract; SD, standard deviation; VGAT, vesicular GABA transporter, VGLUT, vesicular glutamate transporter.

INTRODUCTION

Bulbospinal (BS) systems are composed of heterogeneous pathways that originate from the brainstem. They influence a variety of spinal networks including those concerned with motor control, sensory input (including pain) and autonomic function. It is likely that one of the functions of BS systems is to coordinate the activity of spinal networks involved in these various processes (Holstege, 1996; Hardy et al., 1998; Tavares and Lima, 2002). The neurons that give rise to BS pathways project to the spinal cord via fiber tracts in the medulla (Mitani et al., 1988). These tracts include the MLF, which contains axons principally involved in motor control (Jankowska et al., 2007; Jankowska, 2008) and axonal systems within the caudal ventrolateral medulla (CVLM) which have a role in sensory and autonomic control in addition to an influence on motor activity (Tavares and Lima, 2002). Some classes of bulbospinal neuron which arise principally from the medullary reticular formation are responsible for conveying signals from motor centers in the brain which include the mesencephalic locomotor center and the primary

motor cortex (Matsuyama et al., 2004; Jordan et al., 2008). These neurons have complex effects on motor function; they are not only involved in the maintenance of posture but may also have a role in goal-directed activities such as reaching (Drew et al., 2004; Riddle et al., 2009). The descending axons of these cells innervate wide areas of the spinal gray matter and individual axons give rise to collaterals that terminate at many segmental levels (Peterson et al., 1975; Matsuyama et al., 1999; Reed et al., 2008). Therefore an individual BS cell has the capacity to influence a wide range of spinal neurons and, on this basis, it has been suggested that these neurons are components of a system that is responsible for integrating "common neuronal elements" in order to produce a variety of coordinated motor patterns (Drew et al., 2004; Matsuyama et al., 2004). Although the action of BS neurons on their spinal targets is predominantly excitatory (Jankowska et al., 2007; Jankowska, 2008) immunocytochemical evidence shows that some BS axons also contain inhibitory neurotransmitters (Holstege, 1996; Du Beau et al., 2012; Hossaini et al., 2012). Therefore these systems can have monosynaptic inhibitory actions on spinal neurons in addition to direct excitatory actions. Such actions could serve to coordinate motor output by facilitating or depressing specific components of motor networks.

During a series of experiments to label spinal cells projecting to the lateral reticular nucleus we observed anterogradely labeled terminals in the lumbar spinal cord that had ipsilateral projections and terminated predominantly in the intermediate gray matter. Although these injections were focused upon the CVLM it was not clear if this was the origin of this pathway as the b subunit of cholera toxin (CTb) we used to label descending axons is taken up by axons of passage in addition to cell bodies and axon terminals (Chen and Aston-Jones, 1995). Electrical stimulation of the CVLM can evoke responses in spinal neurons (Tavares and Lima, 2002) but electrical stimulation not only activates cells but also axons of passage and therefore the neurons that mediate these effects may be located at some distance from the site of stimulation. Although a number of studies have documented the origins of BS cells within structures of the rat brainstem (Leong et al., 1984; Zemlan et al., 1984; Rye et al., 1988; Reed et al., 2008; Hossaini et al., 2012), the exact locations of cells with axons that project via the MLF are still largely unknown. Furthermore the locations of cells that give rise to the pathways passing through the CVLM are completely unknown. In view of the limited anatomical information available concerning these systems, our primary aim was to determine the locations of cells that give rise to these pathways. We exploited the propensity for CTb to be taken up by axons of passage by injecting it into MLF or the CVLM and, in the same experiments, we injected Fluorogold (FG) into the spinal cord. Hence we were able to map the locations of double-labeled cells in the brainstem that project to the spinal cord via these two routes.

Bulbospinal systems influence spinal neurons by means of classical synaptic mechanisms but also have a modulatory function which can be direct or indirect via spinal interneurons (e.g., see Jordan et al., 2008). A variety of neurotransmitters and neuromodulators have been associated with these pathways including glutamate, GABA, glycine, monoamines, and peptides. In a previous study (Du Beau et al., 2012) we investigated transmitter phenotypes of lumbar spinal terminations of the BS systems that passes through the MLF. Stereotaxic injections of CTb within the MLF revealed terminals in the lumbar spinal cord that were concentrated within the intermediate gray matter and the ventral horn. Although the majority (59%) of axon terminals in the lumbar spinal cord were glutamatergic, a sizable minority were inhibitory (20%) and these could be subdivided into those that are GABAergic (7%), those that are glycinergic (9%) and those that contained both transmitters (3%). None of the terminals contained serotonin and there was also a significant population (18%) that did not show immunoreactivity for any of the transmitters tested. A secondary aim of the study therefore was to determine the types of neurotransmitters associated with the CVLM pathway.

Table 1 | Interaural stereotaxic coordinates used to target the medial longitudinal fascicle (MLF) and caudal ventrolateral medulla (CVLM) From Paxinos and Watson (2005).

	Anterior-posterior	Medial-lateral	Dorsal-ventral
MLF	−3.8 mm	−0.1 mm	+0.8 mm
CVLM	−4.8 mm	−1.8 mm	−0.4 mm

Table 2 | Antibodies used in the study.

	Primary antibody combination	Primary antibody concentration	Supplier	Secondary antibodies
A	gt. CTb	1:5000	List Biological Laboratories, Campell, CA	Rh.Red
	rbt FG	1:5000	Chemicon/Millipore,CA,USA	Alexa488
B	mo. CTb	1:250	A. Wikström, University of Gothenburg	Rh.Red
	gp VGLUT1	1:5000	Millipore, Harlow, UK	Alexa488
	gp VGLUT2	1:5000	Millipore, Harlow, UK	Dylight 649
C	mo. CTb	1:250	A. Wikström, University of Gothenburg	Rh.Red
	rbt VGAT	1:1000	Synaptic System, Göttingen, Germany	Alexa488
	gp VGLUT1	1:5000	Millipore, Harlow, UK	Dylight 649
	gp VGLUT2	1:5000	Millipore, Harlow, UK	Dylight 649
D	gt. CTb	1:5000	List Biological Laboratories, Campell, CA	Rh.Red
	rbt GLYT2	1:1000	Millipore, Harlow, UK	Alexa488
	mo GAD67	1:1000	Millipore, Harlow, UK	Dylight 649
E	mo. CTb	1:250	A. Wikström, University of Gothenburg	Rh.Red
	rbt Serotonin	1:100	Affiniti, Exeter, UK	Alexa488

gt, goat; rbt, rabbit; mo, mouse; gp, guinea pig; FG, Fluorogold; CTb, the b subunit of cholera toxin; VGLUT1, vesicular glutamate transporter 1; VGLUT2, vesicular glutamate transporter 2; VGAT, vesicular GABA transporter; GLYT2, glycine transporter 2; GAD67, 67 isoform of glutamate decarboxylase. In Experiment C tissue was incubated in a mixture of VGLUT1+2 antibodies.

MATERIALS AND METHODS

SURGICAL PROCEDURES

In these experiments the MLF or CVLM was injected with the b subunit of cholera toxin (Sigma-Aldrich, Co., Poole, UK) which is a retrograde and anterograde tracer and the spinal cord was injected with Fluorogold (Fluorochrome, LLC, USA) which is a retrograde tracer that is primarily taken up by axon terminals. All animal procedures were carried out according to British Home Office legislation and were approved by the Glasgow University Ethical Review Committee. Nine adult male Sprague Dawley rats (Harlan, Bicester, UK) weighing between 250 and 350 g were anesthetized with isoflurane (up to 4% in oxygen), placed in a stereotaxic frame and maintained under deep anaesthesia. The skin at the back of the head was cut in the midline to expose the skull and a small burr hole was then made. The stereotaxic coordinates for injections are given in **Table 1** (see Paxinos and Watson, 2005). A glass micropipette with a tip diameter of 20 μm filled with 1% CTb in distilled water was aligned with the burr hole and inserted into the brain. CTb (200 nl) was injected by pressure with a Pico-Injector (10 ms pulses at 20 psi; World Precision Instruments, Sarasota, USA) into the right MLF (3 animals) or the right CVLM (6 animals: 3 for spinal injections). At the conclusion of surgery, the scalp was sutured and animals were placed in an incubator to assist recovery.

Following a period of 48 h, six animals (three MLF and three CVLM) were re-anesthetized with isoflurane and placed in a spinal frame. The thirteenth thoracic vertebra was identified according to the location of the last rib and a small dorsal midline incision was made at this level. A hole with a diameter of 1 mm was made adjacent to the midline in the laminar surface of the caudal part of the Th13 or L1 vertebrae to expose the dorsal surface of L1 or L2 segments of the spinal cord. Unilateral spinal injections of 50 nl were made with glass micropipettes containing 4% FG in distilled water. The tip of the injection pipette (20 μm in diameter) was inserted into the spinal cord to a depth of up to 1.5 mm from the surface at an angle of 15° to target the intermediate gray matter of the right side of the spinal cord. The wound was sutured and animals recovered uneventfully.

PERFUSION AND FIXATION

Following a 7 day survival period from initial brain injections, rats were anaesthetized with pentobarbitone (1 ml i.p.) and perfused through the left ventricle with mammalian Ringer's solution followed by one litre of a fixative containing 4% formaldehyde in 0.1 M phosphate buffer (PB; pH 7.4) at room temperature. Spinal cords and brains were removed and post-fixed for 8 h at 4°C and were cut into 60 μm thick transverse sections with a Vibratome (Oxford Instruments, Technical Products International, Inc., USA). All sections were treated with an aqueous solution of 50% ethanol for 30 min to aid complete antibody penetration.

TISSUE PROCESSING

In experiments to examine spinally projecting cells via the MLF and CVLM, the brainstem was divided into the medulla, pons and midbrain by using a razor blade to cut the medullary-pontine junction in the coronal plane to separate the medulla and pons and similarly to separate the pons from the midbrain by cutting at a level just inferior to the inferior colliculi. Sections from these three regions were reacted with solutions of primary antibodies to identify CTb and FG for 48 h (See **Table 2A** for details). Subsequently they were incubated in secondary antibodies coupled to fluorphores for 3 h and mounted on glass slides with antifade medium, (Vectashield; Vector Laboratories, Peterborough, UK). Spinal injection sites containing FG were examined with UV epifluorescence and photographed whereas brainstem injection sites were visualized by using 3,3′-diaminobenzidine (DAB) as a chromogen. Sections were incubated in goat anti-CTb for 48 h followed by biotinylated anti-goat IgG for 3 h at room temperature. They were then incubated in avidin-horseradish peroxidase (HRP) for 1 h and hydrogen peroxide plus DAB was applied for a period of approximately 15 min to reveal immunoreactivity.

In experiments to examine descending axons, brainstem injection sites were processed as described above. Spinal sections

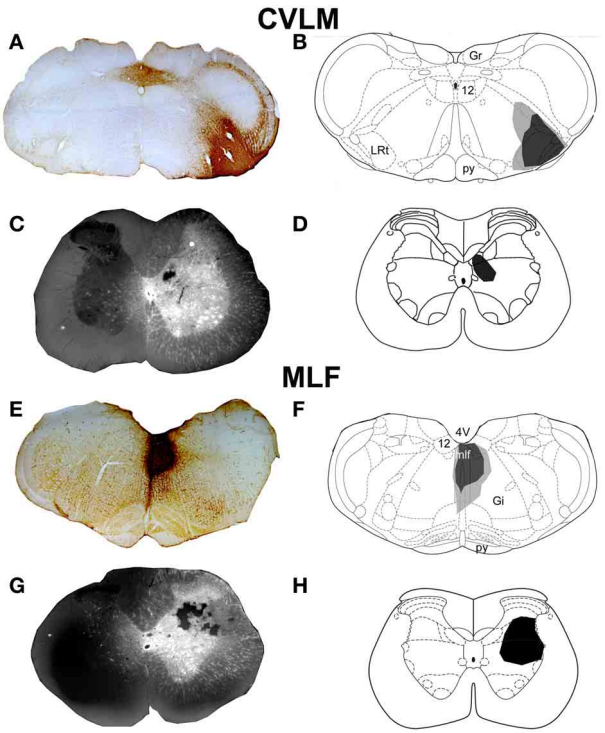

FIGURE 1 | CVLM and MLF injection sites with associated spinal injection sites. (A–D) Shows an experiment to double-label cells with axons passing through the CVLM and **(E–H)** are taken from an experiment to label axons of cells passing through the MLF. **(A,E)** Show CTb injection sites in coronal sections of the medulla. **(B,F)** Show injection sites mapped onto outline diagrams (Paxinos and Watson, 2005). Black, the core and gray, spread of CTb. **(C,G)** Fluorescent images superimposed upon dark field images of the same sections illustrating Fluorogold injection sites at L1–2. Drawings showing the locations of spinal injections (black) are shown in **(D,H)**. Note the spread of Fluorogold within the gray matter surrounding the injection. 4V, 4th ventricle; 12, hypoglossal nucleus; CVLM, caudal ventrolateral medulla; Gi, gigantocellular reticular nucleus; Gr, gracile nucleus; LRt, lateral reticular nucleus; MLF, medial longitudinal fascicle; py, pyramid.

from L3–5 segments were incubated in a combination of antibodies (see **Tables 2B–E** for details) against: (1) CTb, VGLUT1, and VGLUT2; (2) CTb, a mixture of VGLUT1+2 antibodies and VGAT; (3) CTb, glutamic acid decarboxylase 67 (GAD67) and the glycine transporter 2 (GLYT2) or (4) CTb and serotonin (5-HT). Thereafter the sections were washed in PBS, incubated in secondary antibodies coupled to fluorphores for 3 h and had a final wash with PBS before they were mounted on glass slides.

DATA ACQUISITION AND ANALYSIS

To document the locations of double-labeled cells in the brainstem, transmitted light images of all incubated sections were captured digitally using a x1 lens (AxioVision 4.8 software Carl Zeiss, Inc, Germany). These sections were used to identify the various levels of the brainstem according to the rat brain atlas of Paxinos and Watson (2005). The sections were scanned and montaged using a confocal microscope (LSM 710, Zeiss, Germany: magnification 20× lens, zoom factor 0.6 at

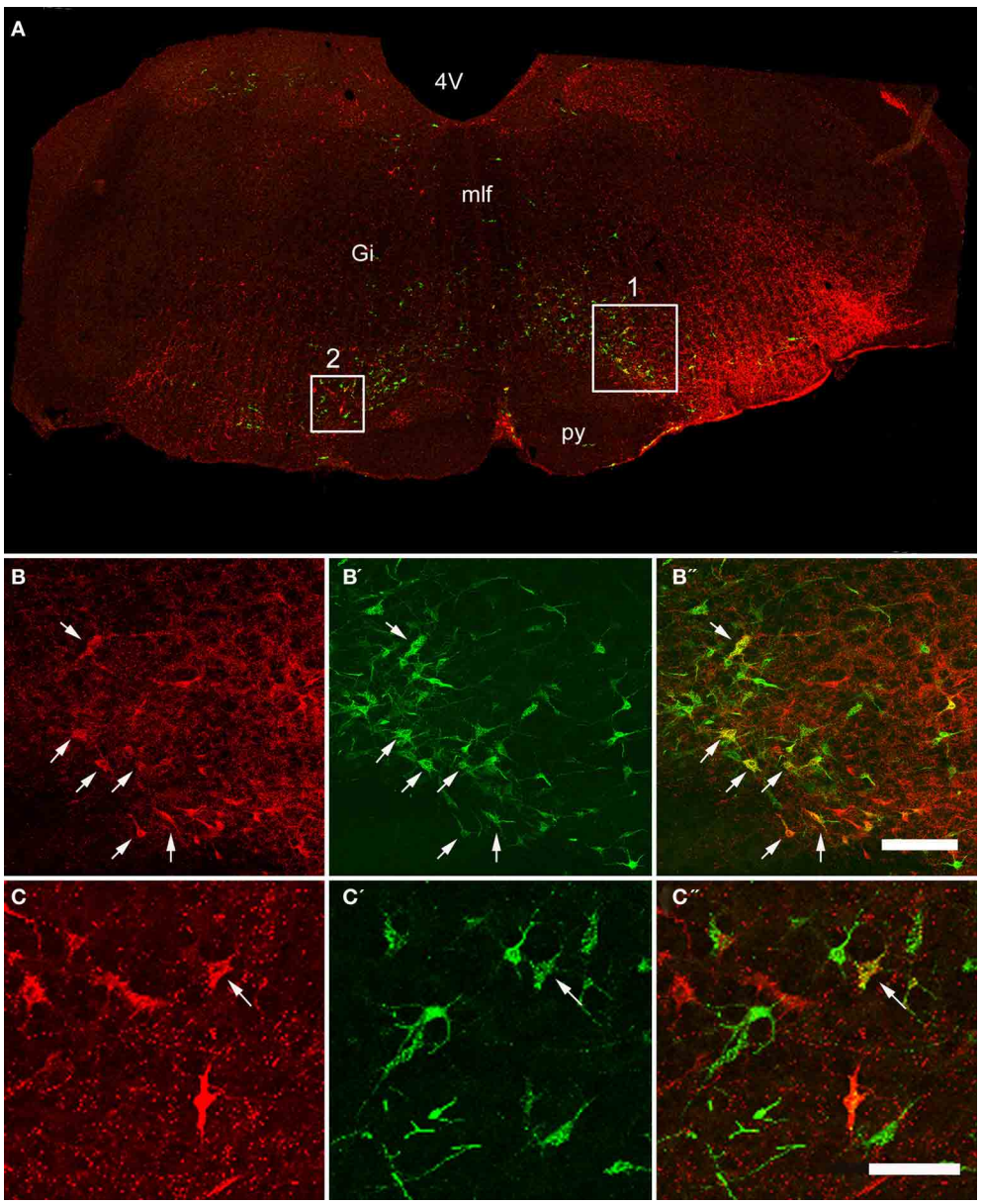

FIGURE 2 | Confocal images of double-labeled cells in the Medulla. **(A)** Shows a montage of a coronal section through the medulla of an animal that had received a CVLM and spinal injection, (both on the right side). Areas 1 and 2 within the boxes are shown at higher magnification in series **(B,C)** respectively which are short projected sequences of confocal images showing cells labeled from the CVLM **(B,C)**, the spinal cord **(B′,C′)** and merged images showing yellow double-labeled cells **(B″,C″)**. Arrows indicate Double-labeled cells. 4V, 4th ventricle, Gi, gigantocellular reticular nucleus, mlf, medial longitudinal fasciculus, py, pyramid. Scale bars = 100 μm.

z-steps of 1 μm intervals). Spinally projecting cells were identified by the presence of FG whereas cells with axons passing through the MLF or CVLM appeared red and cells containing both FG and CTb were yellow. Scans of entire brainstem sections at representative levels (approximately Bregma anterior-posterior levels: −14; −13; −12; −9; −8; −6) were taken. Confocal images were analyzed with Neurolucida for Confocal software (Microbrightfield Inc, Colchester, VT, USA) in order to estimate numbers of cells contained within brainstem structures. A counting grid (100 × 100 μm) was applied to each section and cells were counted within 2–5 adjacent sections (depending on the size of the structure) from each brainstem level for the two groups of animals. Images were exported to Adobe Photoshop so that locations of cells could be plotted onto outline diagrams of the brainstem (Paxinos and Watson, 2005). Cell counts are presented as averaged numbers of double-labeled cells per structure for the three animals in each group. This was done for cells contralateral and ipsilateral to spinal injections with the exception of cells within midline structures such as raphe nuclei or the MLF where data on both sides were pooled.

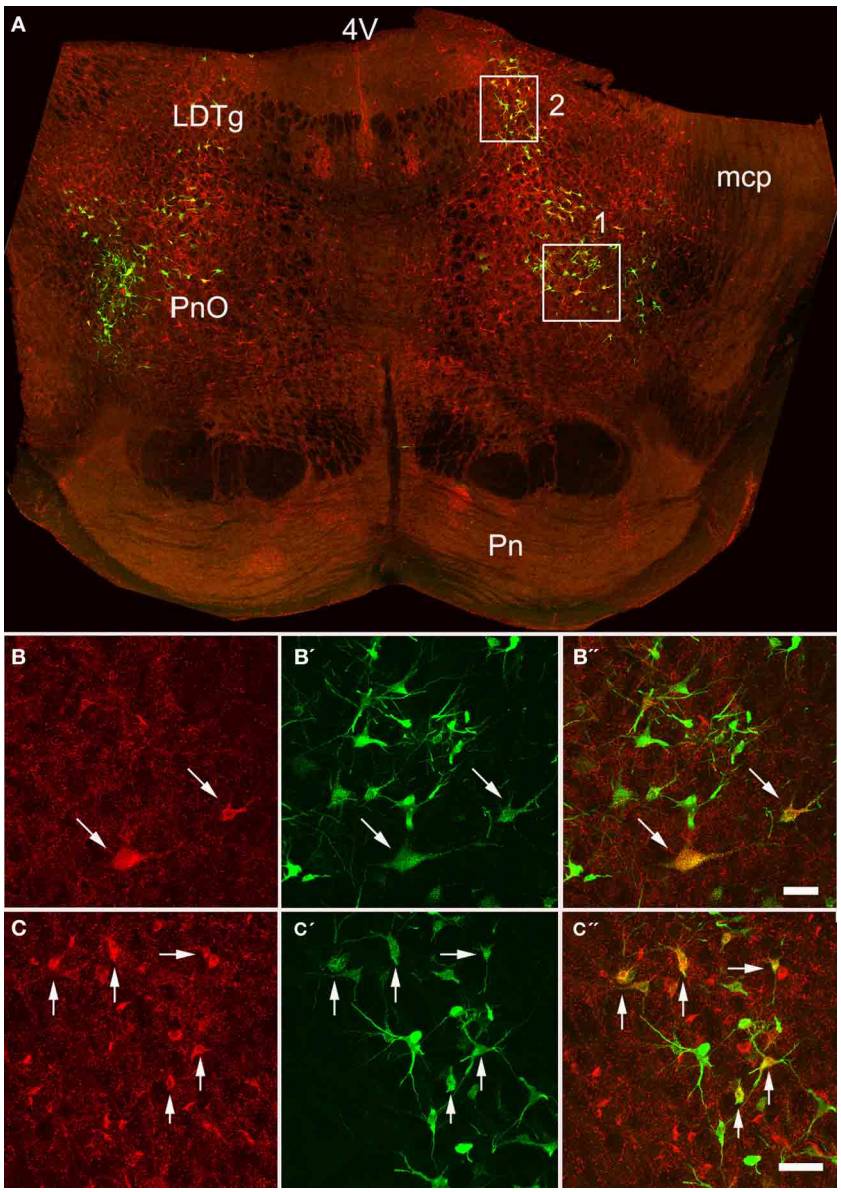

FIGURE 3 | Confocal images of double-labeled cells in the pons. **(A)** Shows a montage of a coronal section through the pons of an animal that had received a MLF and spinal injection, (both on the right side). Areas 1 and 2 within the boxes are shown at higher magnification in series **(B,C)** respectively which are short projected sequences of confocal images showing cells labeled from the MLF **(B,C)**, the spinal cord **(B′,C′)** and merged images showing yellow double-labeled cells **(B″,C″)**. 4V, 4th ventricle; LDTg, laterodorsal tegmental nucleus, mcp, middle cerebellar peduncle, Pn, pontine nuclei, PnO, pontine reticular nucleus, oral part. Arrows indicate Double-labeled cells. Scale bars = 50 μm.

Table 3 | Location of double-labeled cells in the brainstem following Fluorogold injections into the right lumbar cord and injections of cholera toxin in the MLF or right CVLM.

	MLF		CVLM	
	IPSI	CONTRA	IPSI	CONTRA
MEDULLA				
Gi	69.7	6.7	52.7	41.7
IO	7.7	18.3	37.7	20.0
LPGi	0.0	1.0	24.7	12.7
MdV	7.3	0.0	22.0	8.3
Mlf		17.0		16.3
5n	0.0	0.0	4.3	4.3
7n	0.0	0.0	6.0	2.7
RMg		14.3		13.3
Rob		3.3		14.3
RPa		0.0		20.0
RR	2.7	0.0	0.0	0.0
Cu	0.0	0.0	0.0	0.7
LPAG	0.0	0.0	0.3	0.0
LRt	0.0	0.0	5.3	0.0
MVe	9.3	0.0	3.0	5.0
PPy	0.0	0.0	1.3	1.3
Sol	0.0	6.3	0.0	4.7
SPO	0.0	0.0	3.0	1.0
PONS				
PnO	16.0	13.0	13.3	6.3
PnC	13.0	2.0	4.0	7.5
SubCD	0.0	0.0	5.7	7.3
DpMe	2.7	10.7	0.0	0.0
MVPO	0.0	0.0	1.3	0.3
LDTg	2.0	0.0	0.0	0.0
RtTg	6.0	0.0	0.0	0.0
SPTg	2.0	1.0	0.0	0.0
IRt	0.0	1.3	5.3	0.0
PCRt	0.0	0.0	0.7	2.3
PPTg	6.0	0.0	6.0	2.7
Tz	0.0	0.0	0.0	1.7
MIDBRAIN				
PaR	0.0	0.0	1.3	0.3
PBP	0.0	0.0	8.5	0.0
CIC	0.0	0.0	0.0	4.3
VLPAG	1.3	7.3	0.0	0.0

The values show averaged counts per structure for each of the three animals in MLF and CVLM experiments. IPSI, ipsilateral to spinal injection; CONTRA, contralateral to spinal injection. 5n, motor trigeminal nucleus; 7n, facial nucleus; CIC, central nucleus of the inferior colliculus; Cu, cuneate nucleus; DpMe, deep mesencephalic nucleus; Gi, gigantocellular reticular nucleus; IO, inferior olive; IRt, intermediate reticular nucleus,; LDTg, laterodorsal tegmental nucleus; LPAG,lateral periaqueductal gray; LPGi, lateral paragigantocellular nucleus; LRt,lateral reticular nucleus; MdV, medullary reticular nucleus; Mlf, medial longitudinal fasciculus; MVe, medial vestibular nucleus, MVPO, medioventral periolivary nucleus; PaR, pararubral nucleus; PBP, parabrachial pigmented nucleus of the VTA; PCRt, parvicellular reticular nucleus; PnC, pontine reticular nucleus, caudal part; PnO, pontine reticular nucleus, oral part; PPTg, pedunculopontine tegmental nucleus; PPy, parapyramidal nucleus; RMg, raphe magnus nucleus; ROb, raphe obscurus nucleus, RPa, raphe pallidus nucleus; RR, retrorubral nucleus; RtTg, reticulotegmental nucleus; Sol, nucleus of the solitary tract; SPO, superior paraolivary nucleus; SPTg, subpeduncular tegmental nucleus; subCD, subcoeruleus nucleus; tz, trapezoid body; VLPAG, ventrolateral periaqueductal gray.

For axons labeled by CVLM injections, confocal microscope images were acquired from a minimum of six sections per animal. Fields containing CTb-labeled axon terminals were scanned by using a x40 oil-immersion lens with a zoom factor of 2 at 0.5 μm intervals. For each section three fields with a 100 × 100 μm scanning area were obtained from different regions of the gray matter. Stacks of images were analyzed with Neurolucida for Confocal software (MBF Bioscience, Colchester, VT, USA). Image stacks were initially viewed so that only CTb immunoreactivity was visible. All CTb labeled terminals within the scanning box from each animal were used for analysis. The terminals were then examined in the blue and green channels in order to assess expression of transmitter-related markers. The percentage of double-labeled CTb terminals as a proportion of the total number of CTb terminals was calculated for each animal. This value was averaged for the three animals and expressed as the mean percentage ± the standard deviation (SD).

RESULTS

INJECTION SITES FOR DOUBLE-LABELING EXPERIMENTS

Representative examples of injection sites are shown in **Figure 1**. MLF injections were centered upon the right medial longitudinal fascicle. We attempted to minimize the extent of MLF injections but there was always some spread of CTb into the contralateral MLF. In addition there was also spread into the raphe obscuris, paramedian reticular nucleus and tectospinal tract. The rostro-caudal spread of these injections was ±1.38 mm on average from the location of the injection. CVLM injections were centered upon the lateral reticular nucleus but spread into surrounding structures including the parvicellular reticular nucleus, the internal reticular nucleus and the nucleus ambiguous; however they did not encroach on the rubrospinal tract. The rostro-caudal spread of CTb in CVLM injections extended over a distance of ±0.65 mm on average. Spinal injections were confined to L1–2 and injection sites were present in the intermediate gray matter in all experiments but the precise location of each injection varied. Considerable spread of Fluorogold was observed within the gray matter ipsilateral to injection sites but there was no spread to the contralateral gray matter. The rostro-caudal spread of FG within the spinal gray matter was ±0.27 mm on average on each side of the injection site.

DISTRIBUTION OF CELLS IN BRAINSTEM

Double-labeled cells were found within the medulla and pons for CVLM and MLF injections (**Figures 2**, **3**) and small numbers of additional cells were found in the midbrain (69 out of a total of 2033 cells). For MLF injections the average percentage (±SD) of double-labeled cells found in the medulla for the three animals was 68 ± 5.9% (494/722 cells in total for all 3 animals) and the equivalent value for CVLM injections was 84 ± 10.2% (1040/1242 cells in total). In each experiment there was some variation in the distribution of cells as a consequence of inevitable differences in brainstem and spinal injections but consistencies in the distribution of cells were apparent across all animals in each experimental group. A full list of all structures containing double-labeled cells (average number per structure) is given in **Table 3**.

MEDULLA

Distributions of cells in the medulla for CVLM and MLF injections are shown in **Figure 4**. For MLF injections, the majority of double-labeled cells were found ipsilateral to the spinal injection (394 vs. 97 contralateral cells). The greatest numbers of double-labeled cells were found in the gigantocellular reticular nucleus (Gi), the inferior olivary complex (IO), medullary reticular nucleus, dorsal part (MdD), the MLF and the raphe magnus and obscurus (RMg, ROb). Small numbers of cells were noted in a variety of other structures (**Table 3**), including the nucleus of the solitary tract (sol) and the medial vestibular nucleus (MVe). Double-labeled cells following CVLM injections were also found predominantly ipsilateral to spinal injections (673 vs. 307 contralateral cells) and were present in many of the same structures observed for the MLF except that many more cells were located within the lateral paragigantocellular nucleus (LPGi) and the raphe pallidus (RPa) than were found for MLF injections. Additional cells were also present in sol and MVe along with the facial nucleus (7n) and the lateral reticular nucleus (LRt).

PONS

Pontine cell distributions are shown in **Figure 5**. The majority of double-labeled cells in both experimental groups were found ipsilateral to spinal injections (143 vs. 84 for MLF and 109 vs. 84 for CVLM) and, in both groups, they were present in the oral and caudal part of the pontine reticular nucleus, (PnO and PnC). For MLF injections cells were also observed in the deep mesencephalic nucleus (DpMe) the reticulotegmental nucleus (RtTg) and the pedunculopontine tegmental nucleus (PPTg) and for CVLM injections cells were found in the dorsal subcoeruleus (subCD). Full details of other structures containing cells are given in **Table 3**.

MIDBRAIN

Small numbers of double-labeled cells were found in the midbrain (**Figure 6**). For CVLM injections, they were concentrated in two areas: the pararubral nucleus (PaR) and parabrachial pigmented nucleus of the ventral tegmental area (PbP). The only midbrain region containing cells following MLF injections was the ventrolateral periaqueductal gray (VLPAG).

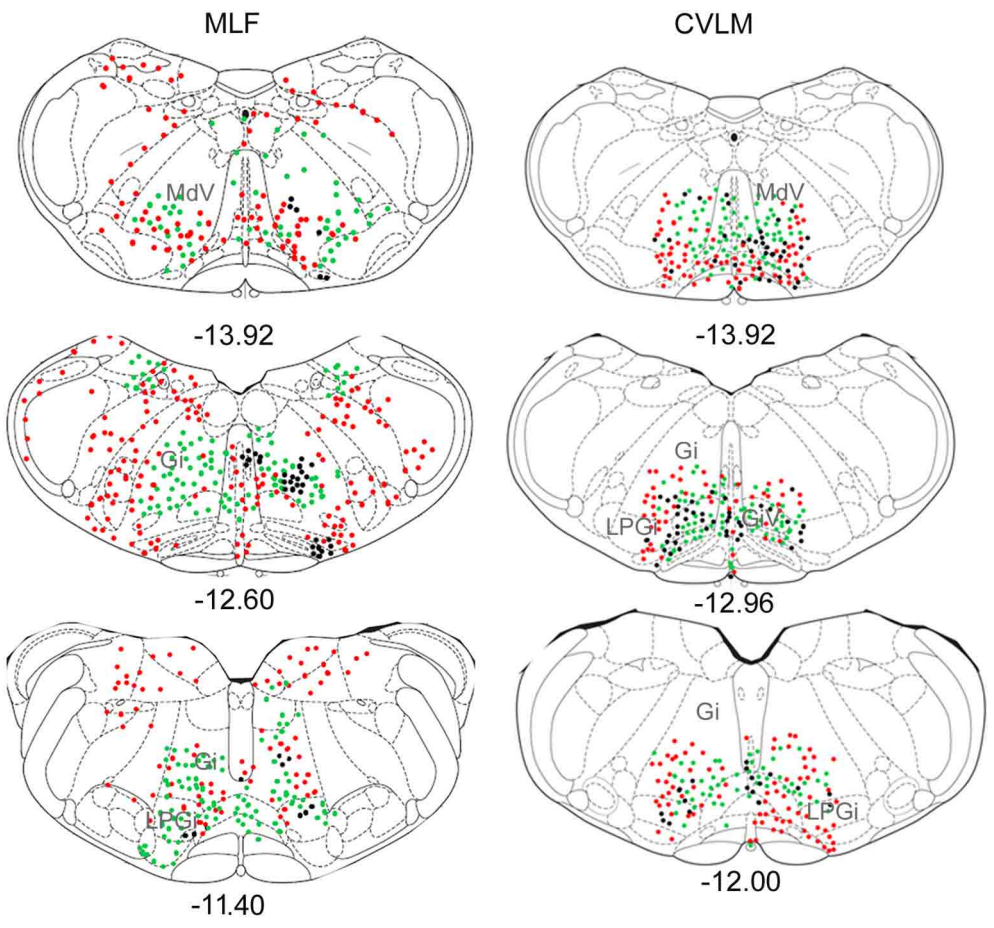

FIGURE 4 | Distribution of cells in medulla. Outline diagrams of coronal sections through the medulla at three levels (anterior-posterior coordinates according to Bregma) showing the locations of cells following spinal injections in the right intermediate gray matter and injections within the MLF or right CVLM. Black circles represent double-labeled cells; green circles represent spinally-projecting cells; and red circles represent cells labeled from MLF or CVLM. Gi, gigantocellular reticular nucleus; LPGi, lateral paragigantocellular nucleus; MdD, medullary reticular nucleus.

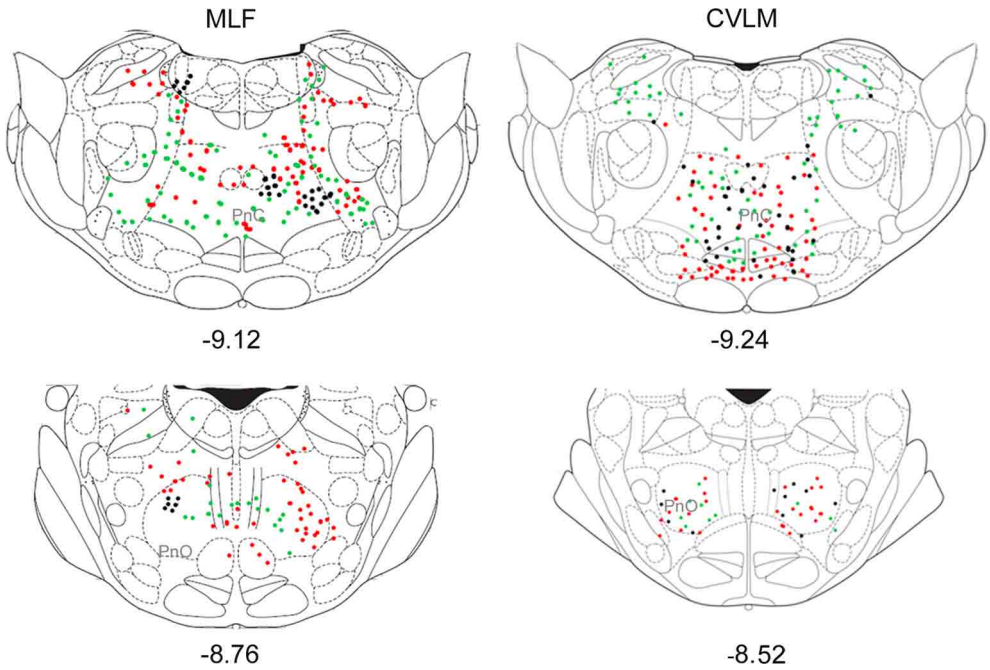

FIGURE 5 | Distribution of cells in pons. Outline diagrams of coronal sections through the pons at two levels (anterior-posterior coordinates according to Bregma) showing the locations of cells following spinal injections in the right intermediate gray matter and injections within the MLF or right CVLM. Black circles represent double-labeled cells; green circles represent spinally-projecting cells; and red circles represent cells labeled from MLF or CVLM. PnC, pontine reticular nucleus, caudal part; PnO, pontine reticular nucleus, oral part.

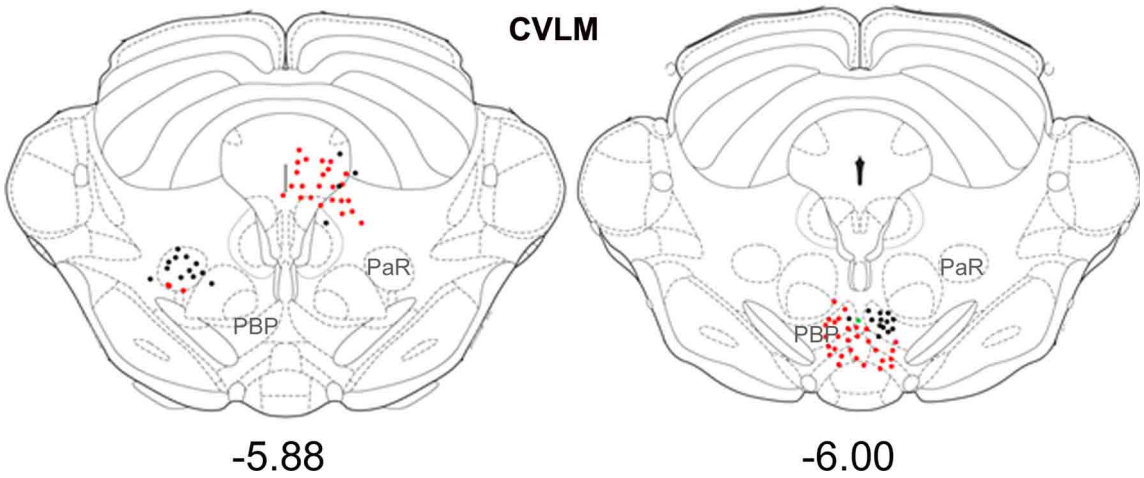

FIGURE 6 | Distribution of cells in midbrain. Outline diagrams of coronal sections through the midbrain at two levels (Bregma anterior-posterior coordinates) showing the locations of cells following spinal injections and injections within the left CVLM. Black circles represent double-labeled cells; green circles represent spinally-projecting cells; and red circles represent cells labeled from CVLM. PaR, pararubral nucleus; PBP, parabrachial pigmented nucleus of the ventral tegmental area.

AXON TERMINALS LABELED FROM THE CVLM

CVLM Injection sites (**Figures 7A,B**) were similar to those shown above for double-labeling experiments. Axon terminals were found throughout lumbar segments principally ipsilateral to the injection site (**Figures 7C–F**). The majority of terminals were concentrated within the intermediate gray matter (laminae V, VI, VII, and X). Few terminals were present in the dorsal horn above lamina V and in the ventral horn including motor nuclei and lamina VIII.

TRANSMITTER PHENOTYPES

Data concerning the immunoreactivity of terminals in L3, 4, and 5 which were labeled from the CVLM are given in **Table 4**. The majority of terminals were found to contain VGLUT2

(average ± *SD* 62.2 ± 3.13%). **Figures 8A–D** shows examples of terminals in the intermediate gray matter that are immunoreactive for VGLUT2 but not VGLUT1. **Figures 8E–H** also shows that some terminals are not immunoreactive for either VGLUT1 or VGLUT2 but contain VGAT (22.7 ± 2.2%). Further investigation of inhibitory terminals (**Figures 9A–D**) showed that they consisted of three types: (1) those immunoreactive for GLYT2 (12.5 ± 1.5%); (2) those immunoreactive for GAD 67 (8.7 ± 0.3%); (3) those that contained both markers (2.9 ± 1.0%). In addition a substantial number of terminals were not immunoreactive for VGLUT or VGAT (approximately 12%). None of these terminals were immunoreactive for serotonin (**Figure 9E**).

DISCUSSION

In this study we have shown that the cells that form pathways from the brainstem to the lumbar spinal cord passing through the MLF and CVLM, for the most part, have overlapping spatial distributions. The vast majority of cells in both pathways originate from reticular areas of the brainstem such as Gi, LPGi, and MdV in the medulla and PnO and PnC in the pons. In addition, both pathways contain raphe-spinal neurons and spinally projecting cells located within the inferior olivary complex. Double-labeled cells were found ipsilateral and contralateral to spinal injection sites, but there was a tendency for greater numbers of cells to be located ipsilaterally. Therefore both pathways contain a mixture of crossed and uncrossed axonal projections. Differences between the two pathways are subtle; the CVLM pathway projects predominantly to the ipsilateral gray matter of the spinal cord whereas a proportion of BS axons which pass through the MLF innervate both sides of the spinal gray matter (Nyberg-Hansen, 1965; Peterson et al., 1975; Martin et al., 1985; Mitani et al., 1988; Matsuyama et al., 1993, 1999, 2004). The most significant difference is that the CVLM pathway has few terminations in the ventral horn whereas many MLF fibers terminate within motor nuclei and are often concentrated within lamina VIII (Jones and Yang, 1985; Matsuyama et al., 2004; Du Beau et al., 2012).

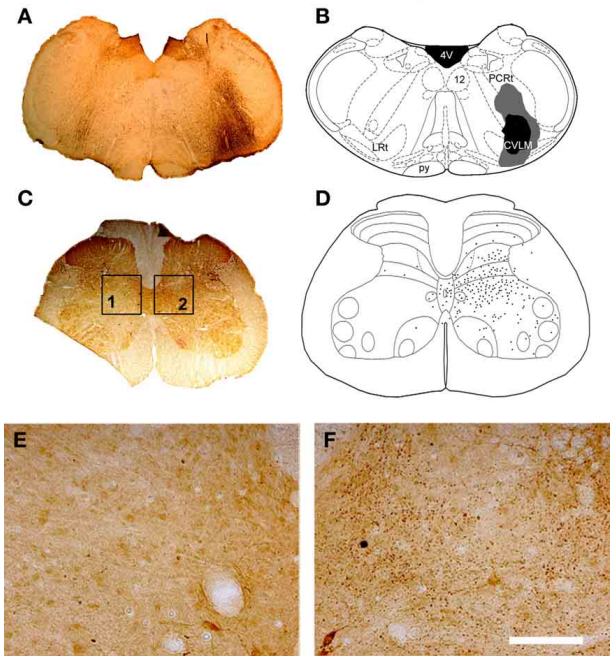

FIGURE 7 | CVLM injections and distribution of terminals in lumbar spinal cord. (A) Shows a light micrograph of a transverse section through the medulla illustrating a CTb injection site. Injection sites were mapped onto outline diagrams of the medulla **(B)** obtained from the stereotaxic atlas of Paxinos and Watson (2005). The black area is the core of the injection and the gray area represents the spread of CTb. Plate **(C)** shows a transverse section through L4 that was reacted to reveal CTb-labeled terminals. **(D)** The distribution of terminals was mapped onto outline diagrams of the spinal cord. Note the predominance of terminals ipsilateral to the injection site which are concentrated in the intermediate gray matter. **(E,F)** Are magnified views of boxes 1 and 2 of plate **(C)**. Note the scarcity of terminals contralateral to the injection site **(E)** and the abundance of terminals on the ipsilateral side **(F)**. Scale bar **E** and **F** = 100 μm. 4V, 4th ventricle; 12, hypoglossal nucleus; CVLM, caudal ventrolateral medulla; LRt, lateral reticular nucleus; PCRt, parvicellular reticular nucleus; py, pyramid.

TECHNICAL CONSIDERATIONS

The b subunit of cholera toxin has been used extensively as a retrograde tracer (Chen and Aston-Jones, 1995) but is transported in the anterograde as well as the retrograde direction (Ericson and Blomqvist, 1988). In common with other tracers, it is taken up by axons of passage in the CNS (Chen and Aston-Jones, 1995) and

Table 4 | Percentages of immunoreactive terminals in the lumbar spinal cord labeled by CVLM injections.

Animal	No. terminals	VGLUT1	VGLUT2
1	1042	0.38	64.59
2	903	1.44	63.34
3	936	0.64	58.65
Mean%		**0.82**	**62.20**
±*SD*		0.55	3.13
Animal	**No. terminals**	**VGLUT1+2**	**VGAT**
1	334	65.37	23.13
2	997	72.34	20.47
3	288	57.01	24.71
Mean%		**64.91**	**22.77**
±*SD*		7.68	2.15

Animal	No. terminals	GLYT2	GAD67	GLY/GAD
1	1220	11.30	8.85	2.77
2	1818	12.11	8.33	2.02
3	885	14.13	8.83	3.92
Mean%		**12.51**	**8.67**	**2.91**
±*SD*	–	1.46	0.29	0.96

The table shows the total number of terminals analyzed per animal for each of the experiments, the percentages of terminals immunoreactive for each of the markers tested and the mean percentage (shown in bold) ± the standard deviation for each marker. VGLUT1, vesicular glutamate transporter 1; VGLUT2, vesicular glutamate transporter 2; VGAT, vesicular GABA transporter; GLYT2, glycine transporter 2; GAD67, 67 isoform of glutamate decarboxylase. VGLUT1+2, tissue incubated in a mixture of both antibodies.

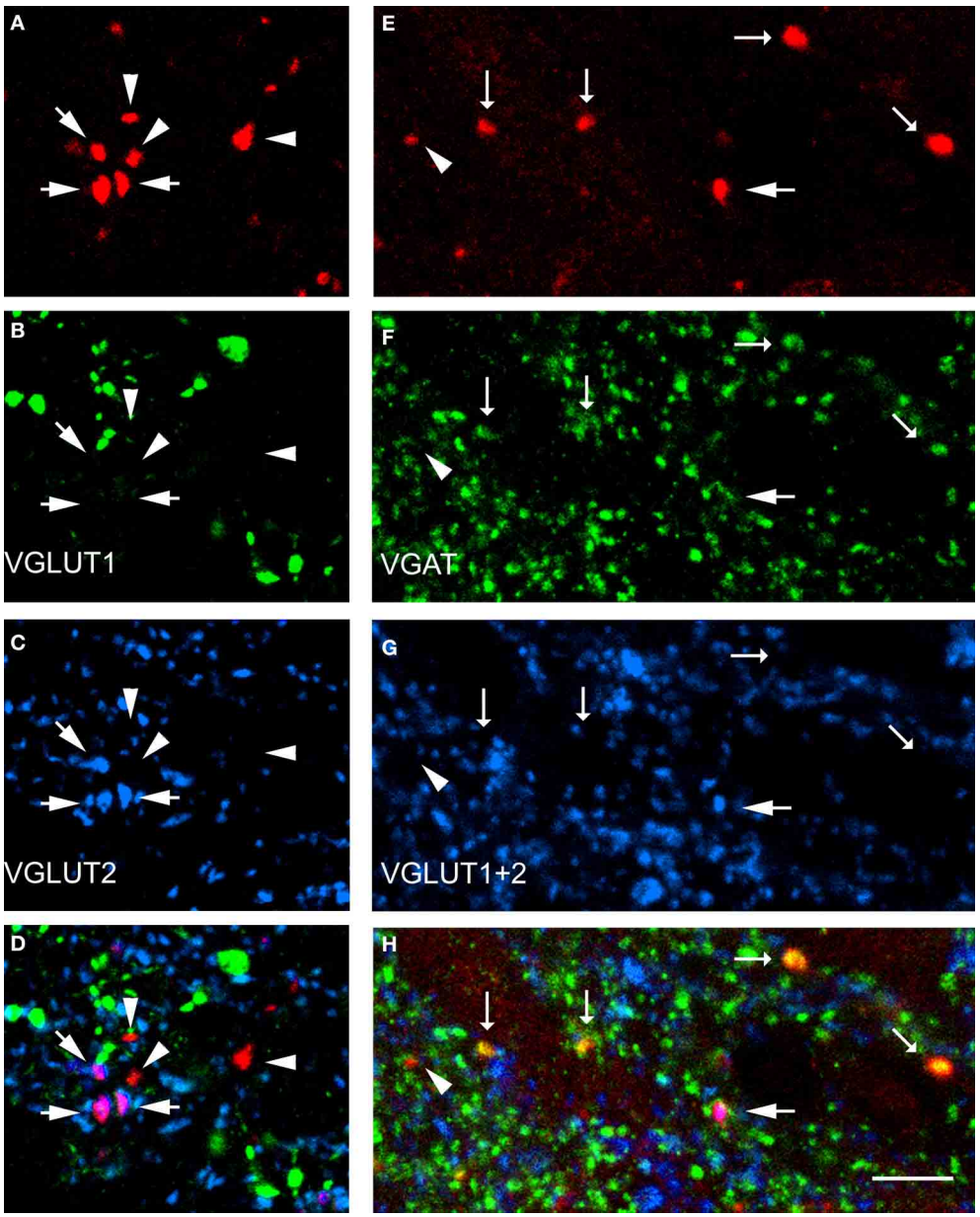

FIGURE 8 | Transmitter phenotypes of spinal axons projecting from the CVLM. (A–D) Single optical sections of **(A)** CTb labeled terminals in the same optical plane for vesicular glutamate transporters (VGLUT)1 and 2 **(B,C)** and a merged image **(D)**. Arrows indicate VGLUT2-immunoreactive terminals and arrow heads indicate terminals unlabeled for both markers. **(E–H)** A similar series where tissue was reacted with a mixture of VGLUT1 and 2 antibodies and the vesicular GABA transporter (VGAT; **F,G**). Large arrows indicate VGLUT-immunoreactive terminals, small arrows indicate VGAT-immunoreactive terminals and the arrowhead indicates a terminal that is not immunoreactive for either marker. Scale bar = 10 μm.

we have exploited this property to label cells whose axons pass through the CVLM and MLF in order to determine the origin of spinally-projecting cells with axons in the two pathways. However, it is almost impossible to label only one side of the MLF as a consequence of the inevitable spread of tracer across the midline and our sample of spinally-projecting MLF cells is likely to be composed of cells with axons that travel through left or right sides of the MLF whereas CVLM double-labeled cells will be exclusively unilateral with axons passing through the right CVLM only.

To compensate for variation between injection sites we attempted to identify consistent patterns of cell distribution for the three animals within each group quantitatively by averaging the numbers of double-labeled cells found within each structure and thus extracted the principal structures associated with spinally-projecting cells. Borders of many brainstem structures are not well-defined and therefore some cells may have been wrongly allocated by the mapping procedure we used which depended upon superimposing stylized diagrams

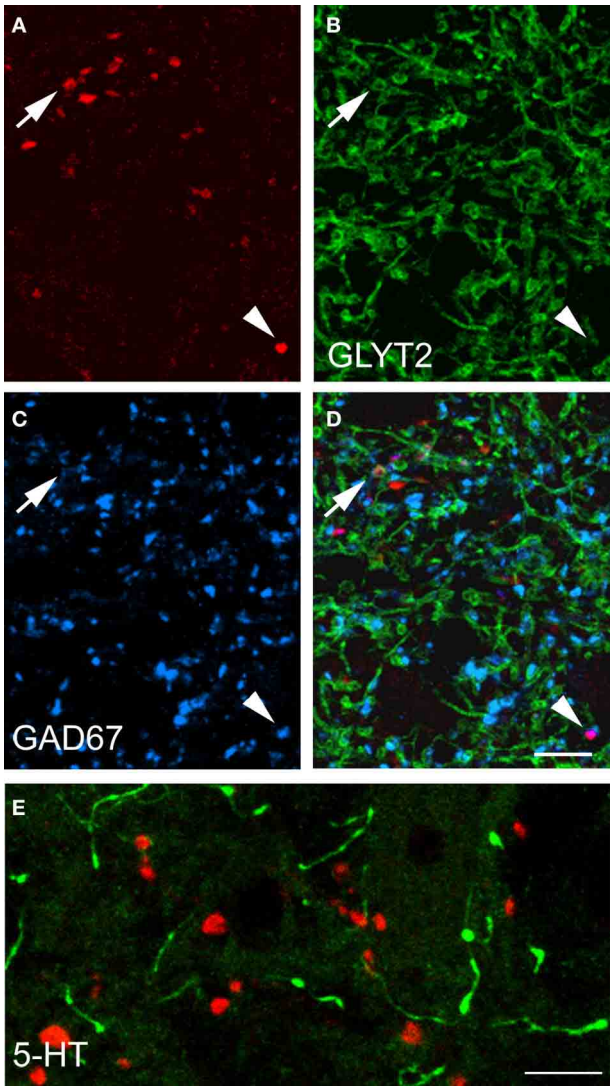

FIGURE 9 | Transmitter phenotypes of spinal axons projecting through the CVLM. (A–D) Single optical sections of **(A)** CTb labeled terminals shown in the same plane for immunoreactivity for the glycine transporter T2 (GLYT2) and the 67 isoform of glutamate decarboxylase (GAD67; **B,C**) A merged image is shown in **(D)**. The arrow indicates a GLYT2-immunoreactive terminal and the arrowhead is a GAD67 labeled terminal. **(E)** Shows immunoreactivity for serotonin (5-HT). Note the absence of 5-HT immunoreactivity (green) in CTb-labeled axon terminals (red). Scale bars = 10 μm.

(Paxinos and Watson, 2005) upon coronal sections at various levels of the brainstem. For example cells attributed to the mlf may have belonged to ROb or Gi. Another complication was that as we were injecting CTb into the brainstem itself, the area in the immediate vicinity of the injection site could not be used to identify double-labeled cells. This is possibly why no cells could be identified within RPa following MLF injections.

We also used the CTb tracing technique to label axon terminals in lumbar segments that originate or pass through the CVLM. The merits of this technique were discussed in a previous publication (Du Beau et al., 2012). A potential problem with the CTb method, however is that it may not label unmyelinated axons anterogradely. This could be the explanation of why we saw many cells in raphe nuclei following retrograde labeling but no serotonin immunoreactive terminals following anterograde labeling (see Du Beau et al., 2012 for a fuller Discussion). Finally, in addition to anterograde labeling of descending axons, CTb injections in the CVLM also label spinomedullary neurons retrogradely; hence some of the terminals we observed could have arisen from collateral axons of these cells. However, similar numbers of spinomedullary cells are labeled on both sides of the cord but very few terminals are present in the contralateral gray matter following CVLM injections which contrasts with the large numbers of terminals found in the ipsilateral gray matter. For this reason we consider it unlikely that our sample of terminals was contaminated by significant numbers of terminals from collateral axons of ascending neurons.

COMPARISON WITH OTHER STUDIES

Previous studies have used lesion techniques, spinal injections of tracer substances or combinations of tract-tracing and spinal lesions to determine anatomical locations of cells of origin of bulbospinally-projecting pathways in a variety of species including rat (Leong et al., 1984; Zemlan et al., 1984; Jones and Yang, 1985; Rye et al., 1988; Reed et al., 2008), mouse (Terashima et al., 1984; VanderHorst and Ulfhake, 2006) cat (Torvik and Brodal, 1957; Kuypers and Maisky, 1975; Basbaum and Fields, 1979; Mitani et al., 1988), opossum (Martin et al., 1979) and monkey (Kneisley et al., 1978). Studies of brainstem cells following lumbar injections in the rat (Leong et al., 1984; Reed et al., 2008) show similar distribution patterns to the Fluorogold-labeled cells documented in the present study. An unexpected finding was that a major component of both pathways appears to originate from cells within the inferior olivary complex. Although the older literature often refers to the olivo-spinal tract, the existence of this pathway has been a matter of contention (e.g., see Brodal et al., 1950; cf Voneida, 1967). Nevertheless, Leong et al. (1984) also reported the presence of spinally projecting cells within the IO and it remains an open question as to whether the pathway exists. Zemlan et al. (1984) observed large clusters of spinally-projecting cells just dorsal to the IO in rats following injections of HRP into T10. As it is difficult to define the exact borders of brainstem nuclei in many cases (see Technical issues above) it is possible that some of the cells we plotted as IO cells belong to adjacent reticular nuclei and were misattributed or perhaps even misplaced anatomically (see **Figure 2A**).

In addition to reticulospinal components mentioned above, the other major components of these pathways originated from raphe nuclei; an observation which is consistent with many previous reports (e.g., see Bowker et al., 1981). Small numbers of cells were also noted within structures previously reported to have projections to the spinal cord including the nucleus of the solitary tract (Loewy and Burton, 1978), the medial vestibular nucleus (Bankoul and Neuhuber, 1992) the sub coeruleus, which is a source of noradrenergic fibers (Kuypers and Maisky, 1975; Hancock and Fougerousse, 1976; VanderHorst and Ulfhake, 2006) and the parabrachial pigmented nucleus of the ventral

tegmental area (Hancock and Fougerousse, 1976), which is likely to be a source of dopaminergic fibers (Hasue and Shammah-Lagnado, 2002; VanderHorst and Ulfhake, 2006).

COMPARISON OF FINDINGS FOR MLF AND CVLM

Not only do the major elements of the MLF and CVLM pathways originate principally from similar brainstem structures but both pathways have similar proportions of excitatory and inhibitory components (Du Beau et al., 2012). The majority of axon terminals in both pathways contain VGLUT2 and thus are mostly excitatory (58 and 62%; MLF and CVLM respectively) but there is also a significant inhibitory component in both pathways that consists of purely GABAergic terminals (7 and 9%), purely glycinergic terminals (9 and 13%) and a small group that are both GABAergic and glycinergic (3 and 3%). Components of the BS pathways are found in the ventromedial, dorsolateral and ventrolateral white matter of the spinal cord and there is evidence that these different pathways originate from distinct regions of the brainstem and may be highly ordered (Nyberg-Hansen, 1965; Kuypers and Maisky, 1977; Basbaum et al., 1978; Jones and Yang, 1985; Martin et al., 1985; Mitani et al., 1988; VanderHorst and Ulfhake, 2006). Our study indicates that the MLF and CVLM pathways contain axons of cells originating from both pontine and medullary locations. According to the atlas of Paxinos and Watson (2005) the MLF begins to form its medial and lateral components at roughly −5.7 mm anterior-posterior to the interaural zero point. As our CVLM injections were made at −4.8 mm it is unlikely that lateral components of the MLF pathway were labeled and therefore axons passing through the CVLM presumably form an additional pathway (see also Mitani et al., 1988). However, we cannot exclude the possibility that some of the double-labeled cells we observed give rise to bifurcating axons which pass through both structures.

FUNCTIONAL IMPLICATIONS

Bulbospinal pathways are known to have monosynaptic actions on motoneurons and premotor interneurons (Grillner and Lund, 1968; Floeter et al., 1993; Gossard et al., 1996; Bannatyne et al., 2003; Jankowska et al., 2003; Riddle et al., 2009; Galea et al., 2010) and they also have profound influences on sensory systems (especially nociceptive pathways), respiration and autonomic activity (Basbaum and Fields, 1979; Hardy et al., 1998; Tavares and Lima, 2002). These pathways form extensive termination patterns within the spinal gray matter. Individual SB cells can have axons that innervate several segmental levels (Huisman et al., 1981; Cavada et al., 1984; Matsuyama et al., 2004; Reed et al., 2008) and provide input to extensive areas of the gray matter, with some axon collaterals projecting to both sides of the cord (Nyberg-Hansen, 1965; Peterson et al., 1975; Martin et al., 1985; Holstege, 1996; Matsuyama et al., 2004). Therefore these systems appear to be ideally suited to coordinate activity of diverse neuronal networks on both sides of the cord located at different segmental levels. They also contain a multiplicity of neurotransmitters and neuromodulators, including inhibitory and excitatory amino acids, monoamines and peptides (Holstege, 1996; Grillner et al., 2000; VanderHorst and Ulfhake, 2006; Jordan et al., 2008) and thus have the capacity to facilitate or depress network activity via direct and indirect inhibitory and excitatory synaptic actions on spinal neurons along with modulatory effects.

The moot question is what is the function of the CVLM pathway given its apparent similarities to the MLF pathway? A clue to the function of this pathway is provided by its anatomical organization: generally it innervates a more circumscribed region of the midlumbar spinal gray matter than the MLF pathway and, unlike the MLF pathway, does not have many terminations in motor nuclei and lamina VIII. Also very few axons passing through the CVLM cross to form terminals in the contralateral gray matter and therefore the influence of this lateral pathway appears to be more restricted than the MLF pathway. In a study of CVLM terminations in the thoracic spinal cord, Hardy et al. (1998) reported that in addition to the ipsilateral pathway, which terminates principally on sympathetic preganglionic neurons, there is a contralateral component that specifically targets phrenic motoneurons. These authors suggested that a possible function of the CVLM pathway is to coordinate respiratory and autonomic function. In midlumbar segments, CVLM axons terminate predominantly in the deep dorsal horn and intermediate gray matter and are ideally located to influence premotor interneurons that are also found in this region (Jovanovic et al., 2010; Coulon et al., 2011; Tripodi et al., 2011). Thus the function of the CVLM pathway may be to harmonize motor activity with respiratory and autonomic activity to produce the coordinated output of these systems required for physical exercise.

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REFERENCES

Bankoul, S., and Neuhuber, W. L. (1992). A direct projection from the medial vestibular nucleus to the cervical spinal dorsal horn of the rat, as demonstrated by anterograde and retrograde tracing. *Anat. Embryol. (Berl.)* 185, 77–85. doi: 10.1007/BF00213603

Bannatyne, B. A., Edgley, S. A., Hammar, I., Jankowska, E., and Maxwell, D. J. (2003). Networks of inhibitory and excitatory commissural interneurons mediating crossed reticulospinal actions. *Eur. J. Neurosci.* 18, 2273–2284. doi: 10.1046/j.1460-9568.2003.02973.x

Basbaum, A. I., Clanton, C. H., and Fields, H. L. (1978). Three bulbospinal pathways from the rostral medulla of the cat: an autoradiographic study of pain modulating systems. *J. Comp. Neurol.* 178, 209–224. doi: 10.1002/cne.901780203

Basbaum, A. I., and Fields, H. L. (1979). The origin of descending pathways in the dorsolateral funiculus of the spinal cord of the cat and rat: further studies on the anatomy of pain modulation. *J. Comp. Neurol.* 187, 513–531. doi: 10.1002/cne.901870304

Bowker, R. M., Westlund, K. N., and Coulter, J. D. (1981). Origins of serotonergic projections to the spinal cord in rat: an immunocytochemical-retrograde transport study. *Brain Res.* 226, 187–199. doi: 10.1016/0006-8993(81)91092-1

Brodal, A., Walberg, F., and Blackstad, T. (1950). Termination of spinal afferents to inferior olive in cat. *J. Neurophysiol.* 13, 431–454.

Cavada, C., Huisman, A. M., and Kuypers, H. G. (1984). Retrograde double labeling of neurons: the combined use of horseradish peroxidase and diamidino yellow dihydrochloride (DY X 2HCl) compared with true blue and DY X 2HCl in rat descending brainstem pathways. *Brain Res.* 308, 123–136. doi: 10.1016/0006-8993(84)90923-5

Chen, S., and Aston-Jones, G. (1995). Evidence that cholera toxin B subunit (CTb) can be avidly taken up and transported by fibers of passage. *Brain Res.* 674, 107–111. doi: 10.1016/0006-8993(95)00020-Q

Coulon, P., Bras, H., and Vinay, L. (2011). Characterization of last-order premotor interneurons by transneuronal tracing with rabies virus in the neonatal mouse spinal cord. *J. Comp. Neurol.* 519, 3470–3487. doi: 10.1002/cne.22717

Drew, T., Prentice, S., and Schepens, B. (2004). Cortical and brainstem control of locomotion. *Prog. Brain Res.* 143, 251–261. doi: 10.1016/S0079-6123(03)43025-2

Du Beau, A., Shakya, S. S., Bannatyne, B. A., Jalicy, S. M., Linnen, S., and Maxwell, D. J. (2012). Neurotransmitter phenotypes of descending systems in the rat lumbar spinal cord. *Neuroscience* 227, 67–79. doi: 10.1016/j.neuroscience.2012.09.037

Ericson, H., and Blomqvist, A. (1988). Tracing of neuronal connections with cholera toxin subunit B: light and electron microscopic immunohistochemistry using monoclonal antibodies. *J. Neurosci. Methods* 24, 225–235. doi: 10.1016/0165-0270(88)90167-7

Floeter, M. K., Sholomenko, G. N., Gossard, J. P., and Burke, R. E. (1993). Disynaptic excitation from the medial longitudinal fasciculus to lumbosacral motoneurons: modulation by repetitive activation, descending pathways, and locomotion. *Exp. Brain Res.* 92, 407–419. doi: 10.1007/BF00229029

Galea, M. P., Hammar, I., Nilsson, E., and Jankowska, E. (2010). Bilateral postsynaptic actions of pyramidal tract and reticulospinal neurons on feline erector spinae motoneurons. *J. Neurosci.* 30, 858–869. doi: 10.1523/JNEUROSCI.4859-09.2010

Gossard, J. P., Floeter, M. K., Degtyarenko, A. M., Simon, E. S., and Burke, R. E. (1996). Disynaptic vestibulospinal and reticulospinal excitation in cat lumbosacral motoneurons: modulation during fictive locomotion. *Exp. Brain Res.* 109, 277–288. doi: 10.1007/BF00231787

Grillner, S., Cangiano, L., Hu, G., Thompson, R., Hill, R., and Wallen, P. (2000). The intrinsic function of a motor system–from ion channels to networks and behavior. *Brain Res.* 886, 224–236. doi: 10.1016/S0006-8993(00)03088-2

Grillner, S., and Lund, S. (1968). The origin of a descending pathway with monosynaptic action on flexor motoneurones. *Acta Physiol. Scand.* 74, 274–284. doi: 10.1111/j.1748-1716.1968.tb04236.x

Hancock, M. B., and Fougerousse, C. L. (1976). Spinal projections from the nucleus locus coeruleus and nucleus subcoeruleus in the cat and monkey as demonstrated by the retrograde transport of horseradish peroxidase. *Brain Res. Bull.* 1, 229–234. doi: 10.1016/0361-9230(76)90072-1

Hardy, S. G., Horecky, J. G., and Presley, K. G. (1998). Projections of the caudal ventrolateral medulla to the thoracic spinal cord in the rat. *Anat. Rec.* 250, 95–102. doi: 10.1002/(SICI)1097-0185(199801)250:1<95::AID-AR9>3.0.CO;2-D

Hasue, R. H., and Shammah-Lagnado, S. J. (2002). Origin of the dopaminergic innervation of the central extended amygdala and accumbens shell: a combined retrograde tracing and immunohistochemical study in the rat. *J. Comp. Neurol.* 454, 15–33. doi: 10.1002/cne.10420

Holstege, J. C. (1996). The ventro-medial medullary projections to spinal motoneurons: ultrastructure, transmitters and functional aspects. *Prog. Brain Res.* 107, 159–181. doi: 10.1016/S0079-6123(08)61864-6

Hossaini, M., Goos, J. A., Kohli, S. K., and Holstege, J. C. (2012). Distribution of glycine/GABA neurons in the ventromedial medulla with descending spinal projections and evidence for an ascending glycine/GABA projection. *PLoS ONE* 7:e35293. doi: 10.1371/journal.pone.0035293

Huisman, A. M., Kuypers, H. G., and Verburgh, C. A. (1981). Quantitative differences in collateralization of the descending spinal pathways from red nucleus and other brain stem cell groups in rat as demonstrated with the multiple fluorescent retrograde tracer technique. *Brain Res.* 209, 271–286. doi: 10.1016/0006-8993(81)90153-0

Jankowska, E. (2008). Spinal interneuronal networks in the cat: elementary components. *Brain Res. Rev.* 57, 46–55. doi: 10.1016/j.brainresrev.2007.06.022

Jankowska, E., Hammar, I., Slawinska, U., Maleszak, K., and Edgley, S. A. (2003). Neuronal basis of crossed actions from the reticular formation on feline hindlimb motoneurons. *J. Neurosci.* 23, 1867–1878. doi: 10.1046/j.1460-9568.2003.02973

Jankowska, E., Maxwell, D. J., and Bannatyne, B. A. (2007). On coupling and decoupling of spinal interneuronal networks. *Arch. Ital. Biol.* 145, 235–250.

Jones, B. E., and Yang T. Z. (1985). The efferent projections from the reticular formation and the locus coeruleus studied by anterograde and retrograde axonal transport in the rat. *J. Comp. Neurol.* 242, 56–92. doi: 10.1002/cne.902420105

Jordan, L. M., Liu, J., Hedlund, P. B., Akay, T., and Pearson, K. G. (2008). Descending command systems for the initiation of locomotion in mammals. *Brain Res. Rev.* 57, 183–191. doi: 10.1016/j.brainresrev.2007.07.019

Jovanovic, K., Pastor, A. M., and O'Donovan, M. J. (2010). The use of PRV-Bartha to define premotor inputs to lumbar motoneurons in the neonatal spinal cord of the mouse. *PLoS ONE* 5:e11743. doi: 10.1371/journal.pone.0011743

Kneisley, L. W., Biber, M. P., and LaVail, J. H. (1978). A study of the origin of brain stem projections to monkey spinal cord using the retrograde transport method. *Exp. Neurol.* 60, 116–139. doi: 10.1016/0014-4886(78)90172-3

Kuypers, H. G., and Maisky, V. A. (1975). Retrograde axonal transport of horseradish peroxidase from spinal cord to brain stem cell groups in the cat. *Neurosci. Lett.* 1, 9–14. doi: 10.1016/0304-3940(75)90004-X

Kuypers, H. G., and Maisky, V. A. (1977). Funicular trajectories of descending brain stem pathways in cat. *Brain Res.* 136, 159–165. doi: 10.1016/0006-8993(77)90141-X

Leong, S. K., Shieh, J. Y., and Wong, W. C. (1984). Localizing spinal-cord-projecting neurons in adult albino rats. *J. Comp. Neurol.* 228, 1–17. doi: 10.1002/cne.902280103

Loewy, A. D., and Burton, H. (1978). Nuclei of the solitary tract: efferent projections to the lower brain stem and spinal cord of the cat. *J. Comp. Neurol.* 181, 421–449. doi: 10.1002/cne.901810211

Martin, G. F., Humbertson, A. O. Jr., Laxson, L. C., Panneton, W. M., and Tschismadia, I. (1979). Spinal projections from the mesencephalic and pontine reticular formation in the North American Opossum: a study using axonal transport techniques. *J. Comp. Neurol.* 187, 373–399. doi: 10.1002/cne.901870208

Martin, G. F., Vertes, R. P., and Waltzer, R. (1985). Spinal projections of the gigantocellular reticular formation in the rat. Evidence for projections from different areas to laminae I and II and lamina IX. *Exp. Brain Res.* 58, 154–162. doi: 10.1007/BF00238963

Matsuyama, K., Kobayashi, Y., Takakusaki, K., Mori, S., and Kimura, H. (1993). Termination mode and branching patterns of reticuloreticular and reticulospinal fibers of the nucleus reticularis pontis oralis in the cat: an anterograde PHA-L tracing study. *Neurosci. Res.* 17, 9–21. doi: 10.1016/0168-0102(93)90024-K

Matsuyama, K., Mori, F., Kuze, B., and Mori, S. (1999). Morphology of single pontine reticulospinal axons in the lumbar enlargement of the cat: a study using the anterograde tracer PHA-L. *J. Comp. Neurol.* 410, 413–430.

Matsuyama, K., Mori, F., Nakajima, K., Drew, T., Aoki, M., and Mori, S. (2004). Locomotor role of the corticoreticular-reticulospinal-spinal interneuronal system. *Prog. Brain Res.* 143, 239–249. doi: 10.1016/S0079-6123(03)43024-0

Mitani, A., Ito, K., Mitani, Y., and McCarley, R. W. (1988). Descending projections from the gigantocellular tegmental field in the cat: cells of origin and their brainstem and spinal cord trajectories. *J. Comp. Neurol.* 268, 546–566. doi: 10.1002/cne.902680406

Nyberg-Hansen, R. (1965). Sites and mode of termination of reticulo-spinal fibers in the cat. An experimental study with silver impregnation methods. *J. Comp. Neurol.* 124, 71–99. doi: 10.1002/cne.901240107

Paxinos, G., and Watson, C. (2005). *The Rat Brain in Stereotaxic Coordinates. 5th Edn*. London; New York: Elsevier Academic Press.

Peterson, B. W., Maunz, R. A., Pitts, N. G., and Mackel, R. G. (1975). Patterns of projection and braching of reticulospinal neurons. *Exp. Brain Res.* 23, 333–351. doi: 10.1007/BF00238019

Reed, W. R., Shum-Siu, A., and Magnuson, D. S. (2008). Reticulospinal pathways in the ventrolateral funiculus with terminations in the cervical and lumbar enlargements of the adult rat spinal cord. *Neuroscience* 151, 505–517. doi: 10.1016/j.neuroscience.2007.10.025

Riddle, C. N., Edgley, S. A., and Baker, S. N. (2009). Direct and indirect connections with upper limb motoneurons from the primate reticulospinal tract. *J. Neurosci.* 29, 4993–4999. doi: 10.1523/JNEUROSCI.3720-08.2009

Rye, D. B., Lee, H. J., Saper, C. B., and Wainer, B. H. (1988). Medullary and spinal efferents of the pedunculopontine tegmental nucleus and adjacent mesopontine tegmentum in the rat. *J. Comp. Neurol.* 269, 315–341. doi: 10.1002/cne.902690302

Tavares, I., and Lima, D. (2002). The caudal ventrolateral medulla as an important inhibitory modulator of pain transmission in the spinal cord. *J. Pain* 3, 337–346. doi: 10.1054/jpai.2002.127775

Terashima, T., Inoue, K., Inoue, Y., Mikoshiba, K., and Tsukada, Y. (1984). Observations on the brainstem-spinal descending systems of normal and reeler mutant mice by the retrograde HRP method. *J. Comp. Neurol.* 225, 95–104. doi: 10.1002/cne.902250110

Torvik, A., and Brodal, A. (1957). The origin of reticulospinal fibers in the cat; an experimental study. *Anat. Rec.* 128, 113–137. doi: 10.1002/ar.1091280110

Tripodi, M., Stepien, A. E., and Arber, S. (2011). Motor antagonism exposed by spatial segregation and timing of neurogenesis. *Nature* 479, 61–66. doi: 10.1038/nature10538

VanderHorst, V. G., and Ulfhake, B. (2006). The organization of the brainstem and spinal cord of the mouse: relationships between monoaminergic, cholinergic, and spinal projection systems. *J. Chem. Neuroanat.* 31, 2–36. doi: 10.1016/j.jchemneu.2005.08.003

Voneida, T. J. (1967). The effect of pyramidal lesions on the performance of a conditioned avoidance response in cats. *Exp. Neurol.* 19, 483–493. doi: 10.1016/0014-4886(67)90168-9

Zemlan, F. P., Behbehani, M. M., and Beckstead, R. M. (1984). Ascending and descending projections from nucleus reticularis magnocellularis and nucleus reticularis gigantocellularis: an autoradiographic and horseradish peroxidase study in the rat. *Brain Res.* 292, 207–220. doi: 10.1016/0006-8993(84)90757-1

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Purines released from astrocytes inhibit excitatory synaptic transmission in the ventral horn of the spinal cord

Eva Meier Carlsen and Jean-François Perrier*

Laboratory of Neuronal Signaling, Department of Neuroscience and Pharmacology, University of Copenhagen, Copenhagen, Denmark

Edited by:
Brian R. Noga, University of Miami, USA

Reviewed by:
David Parker, Cambridge University, UK
Klaus Ballanyi, University of Alberta, Canada

***Correspondence:**
Jean-François Perrier, Laboratory of Neuronal Signaling, Department of Neuroscience and Pharmacology, University of Copenhagen, Blegdamsvej 3, 2200 Copenhagen N, Denmark
e-mail: perrier@sund.ku.dk

Spinal neuronal networks are essential for motor function. They are involved in the integration of sensory inputs and the generation of rhythmic motor outputs. They continuously adapt their activity to the internal state of the organism and to the environment. This plasticity can be provided by different neuromodulators. These substances are usually thought of being released by dedicated neurons. However, in other networks from the central nervous system synaptic transmission is also modulated by transmitters released from astrocytes. The star-shaped glial cell responds to neurotransmitters by releasing gliotransmitters, which in turn modulate synaptic transmission. Here we investigated if astrocytes present in the ventral horn of the spinal cord modulate synaptic transmission. We evoked synaptic inputs in ventral horn neurons recorded in a slice preparation from the spinal cord of neonatal mice. Neurons responded to electrical stimulation by monosynaptic EPSCs (excitatory monosynaptic postsynaptic currents). We used mice expressing the enhanced green fluorescent protein under the promoter of the glial fibrillary acidic protein to identify astrocytes. Chelating calcium with BAPTA in a single neighboring astrocyte increased the amplitude of synaptic currents. In contrast, when we selectively stimulated astrocytes by activating PAR-1 receptors with the peptide TFLLR, the amplitude of EPSCs evoked by a paired stimulation protocol was reduced. The paired-pulse ratio was increased, suggesting an inhibition occurring at the presynaptic side of synapses. In the presence of blockers for extracellular ectonucleotidases, TFLLR did not induce presynaptic inhibition. Puffing adenosine reproduced the effect of TFLLR and blocking adenosine A_1 receptors with 8-Cyclopentyl-1,3-dipropylxanthine prevented it. Altogether our results show that ventral horn astrocytes are responsible for a tonic and a phasic inhibition of excitatory synaptic transmission by releasing ATP, which gets converted into adenosine that binds to inhibitory presynaptic A_1 receptors.

Keywords: astrocyte, ATP, adenosine, purine, spinal cord, synapse, motor control

INTRODUCTION

Neuronal networks located in the ventral horns of the spinal cord are essential for motor control (Kjaerulff and Kiehn, 1996; Prut and Perlmutter, 2003; Lanuza et al., 2004; Dai et al., 2005; Brocard et al., 2010; Mui et al., 2012). Their architecture provides the necessary stability for generating stereotyped movements. In order to adapt to internal and external changes, the organism adjusts neuronal activity by different cellular mechanisms providing high degrees of flexibility on time scales ranging from milliseconds to hours. The activity of spinal networks is regulated by descending pathways releasing neuromodulators such as serotonin, noradrenaline, glutamate or GABA (Arvidsson et al., 1990; Jacobs and Azmitia, 1992; Ono and Fukuda, 1995; Schmidt and Jordan, 2000; Conn et al., 2005; Farrant and Nusser, 2005; Perrier and Delgado-Lezama, 2005; Cotel et al., 2013; Perrier et al., 2013). Flexibility is also ensured by intrinsic modulatory systems releasing transmitters such as endocannabinoids (Ameri, 1999; El Manira and Kyriakatos, 2010) or purines (Dale and Gilday, 1996). The modulation provided by purines is of particular interest because adenosine triphosphate (ATP) and its metabolic product adenosine exert opposite effects on neurons. In the spinal cord of embryonic *Xenopus*, ATP promotes swimming by inhibiting voltage gated potassium conductances (Dale and Gilday, 1996; Brown and Dale, 2002). Similarly, in the brainstem, ATP activates neurons from the retrotrapezoid nucleus, involved in breathing (Gourine et al., 2010). Once in the extracellular space, ATP is converted by ectonucleotidases into adenosine diphosphate (ADP), adenosine monophosphate (AMP), and adenosine (Dunwiddie et al., 1997). The binding of adenosine to metabotropic A_1 receptors lowers the excitability of spinal neurons belonging to motor circuits by inhibiting Ca^{2+} currents (Dale and Gilday, 1996; Brown and Dale, 2000). In agreement, (Taccola et al., 2012) and (Brockhaus and Ballanyi, 2000) showed that A_1 receptor activation depresses bicuculline-evoked seizure-like bursting in newborn rat spinal cords. In other systems such as the hippocampus or the cerebellum, the activation of presynaptic A_1 receptors decreases neurotransmitter release by inhibiting Ca^{2+} channels (Hollins and Stone, 1980; Wu and Saggau, 1994; Zhang et al., 2003).

Sources of ATP in the nervous system are multiple. ATP is co-released from neurons with neurotransmitters such as acetylcholine, noradrenaline, or GABA (Silinsky and Redman,

1996; Jo and Schlichter, 1999; Burnstock, 2007). In addition, ATP is secreted from astrocytes (Guthrie et al., 1999; Fields and Burnstock, 2006; Gourine et al., 2010; Christensen et al., 2013; Lalo et al., 2014). In the spinal cord, some of the modulatory actions induced by ATP are blocked by the glial metabolic poison fluoroacetate (Witts et al., 2012). It was therefore suggested that spinal glial cells also release ATP. However, a direct demonstration that ATP released from spinal astrocytes modulates synaptic transmission is still lacking.

In this study, we investigated if purines released from spinal astrocytes have any effect on synaptic transmission between neurons from the ventral horn. We found that astrocytes release ATP, which after being converted to adenosine produces both tonic and phasic inhibition of excitatory synaptic transmission by decreasing the probability of neurotransmitter release.

MATERIALS AND METHODS

Experiments were performed on neonatal (P4–P22) wild type (C7BJL6; Taconic) and transgenic mice expressing enhanced green fluorescent protein (E-GFP) under the promoter of glial fibrillary acidic protein [GFAP; line Tgn(hgFAPEGEP)] GFEC 335 (**Figures 1A,B**). Transgenic mice were kindly provided by Professor Frank Kirchhoff. The astrocytes of these mice were identified by their fluorescence (Nolte et al., 2001). The surgical procedures complied with Danish legislation. Mice were killed by decapitation.

SLICE PREPARATION

The lumbar enlargement of the spinal cord was removed and placed in cold artificial cerebrospinal fluid containing NaCl 125 mM, KCl 2.5 mM, $NaHCO_3$ 26 mM, $CaCl_2$ 2 mM, $MgCl_2$ 1 mM, NaH_2PO_4 1.25 mM, Glucose 25 mM. Ringer's solution was continuously carbogenated by gassing with 95% O_2 plus 5% CO_2. 300 μm thick slices were obtained with a vibratome (MicroM slicer HM 650V; Microm International GmbH, Germany) equipped with cooling unit CU65 set at 2°C. Slices were then positioned in a recording chamber and continuously perfused with Ringer's solution at room temperature.

PATCH CLAMP RECORDING

Visual guided patch clamp recording was done with a Multiclamp 700B amplifier (Molecular Devices, USA). Neurons were visualized by means of a BW51WI microscope (Olympus, Japan) equipped with an oblique illumination condenser. Astrocytes were identified by means of fluorescence illumination obtained with a monochromator (Polychrome V; Till Photonics, Germany) tuned at 488 nm. The pipette solution (in mM): 122 K-gluconate, 2.5 $MgCl_2$, 0.0003 $CaCl_2$, 5.6 Mg-gluconate, 5 K-HEPES, 5 H-HEPES, 5 Na_2ATP, 1 EGTA, 2.5 biocytine, 0.01 Alexa 488 hydrazide, sodium salt (Life Technologies, USA), and KOH to adjust the pH to 7.4. Calcium-clamp pipette solution contained (in mM): 40 K-gluconate, 30 K_4-BAPTA, 50 sucrose, 2.5 $MgCl_2$, 0.0003 $CaCl_2$, 5.6 Mg-gluconate, 5 K-HEPES, 5 H-HEPES, 5 Na_2ATP, 1 EGTA, 5 biocytine, 0.068 Alexa 568 hydrazide sodium salt (Life Technologies, USA) and the necessary amount of KOH to adjust the pH to 7.4. Electrodes had a resistance ranging from 4 to 8 MΩ. Recordings were sampled at 10 kHz with a 16-bit analog-to-digital converter (DIGIDATA 1440; Molecular Devices, USA) and displayed by means of Clampex 10.2 software (Molecular Devices, USA).

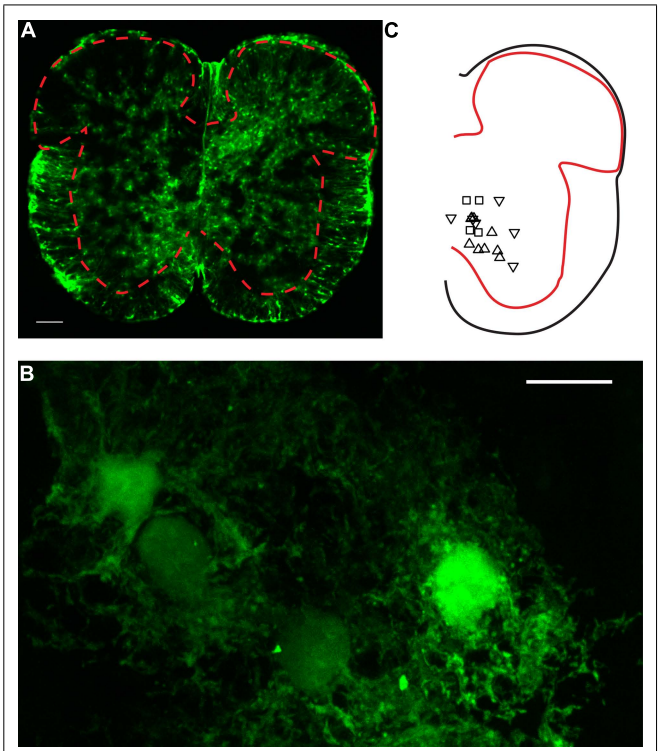

FIGURE 1 | Confocal microscopy of GFAP-EGFP astrocytes in spinal cord slices. (A) Image of a lumbar spinal cord slice from a neonatal mouse (P7) scanned with confocal microscopy. The red dashed line indicates the limits between gray and white matter. Note the presence of multiple protoplasmic fluorescent astrocytes in the gray matter. Scale bar: 100 μm. **(B)** High magnification picture (×63) of ventral horn E-GFP positive astrocytes. Twenty-four consecutives parallel confocal plans were superimposed. Scale bar: 10 μm. **(C)** Relative positions of a fraction of the neurons recorded for the study. Upward triangles indicate responses paired pulse stimulation protocol with a PPR > 1. Downward triangles correspond to a PPR < 1. Squares: PPR≈1.

ELECTRICAL STIMULATION

Local electric stimulation was performed with a bipolar concentric electrode (TM33CCNON; World Precision Instruments, Sarasota, FL, USA) connected to an isolation unit (Isolator 11, Axon Instruments, USA) triggered by an external signal. The electrode was positioned in areas devoid of cell bodies, in the vicinity of neurons recorded from. Different positions were tried until a reliable response was induced by stimulation. Responses to paired pulse stimulations (duration from 10 to 40 μs; amplitude from 0.1 to 1 mA; interval from 10 to 30 ms) were recorded in neurons in voltage-clamp mode. They were considered as putative excitatory monosynaptic postsynaptic currents (EPSCs) when all the following criteria were satisfied: (1) fixed latency and variable amplitude; (2) decrease of amplitude upon depolarization, as one would expect for EPSCs in contrast with IPSCs; (3) absence of response when reverting the polarity of stimulation to exclude possible artifacts caused by direct electrical

stimulation. For a fraction of neurons, we also tested responses to stimulation at high frequencies (10, 20, and 50 Hz). The neurons that did not follow these frequencies were discarded from the sample.

INPUT RESISTANCE

The input resistance of the neurons recorded from was calculated as the ratio of voltage to current measured during small depolarizing pulses generated in voltage-clamp mode.

FOCAL APPLICATION OF DRUGS

Electrodes made from borosilicate capillaries (tip diameter ranging from 1.5–2 μm; BF150-86-7.5, Sutter Instrument, USA) were either filled with TFLLR-NH_2 (10 μM; Tocris Bioscience, UK), ATP (1 mM; Sigma–Aldrich, St. Louis, MO, USA) or adenosine (1 mM; Sigma–Aldrich, St. Louis, MO, USA). Drugs were puff applied at 14–35 Pa by a homemade time-controlled pressure device.

DRUGS

The following drugs were used: BAPTA (30 mM; Invitrogen, Carlsbad, CA, USA), 6-*N*,*N*-Diethyl-D-β,γ-dibromomethylene ATP trisodium salt (ARL 67156 trisodium salt, 50 μM; Tocris Bioscience, UK), 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX, 5 μM; Tocris Bioscience, UK).

CONFOCAL MICROSCOPY

Confocal microscopy images were obtained at the core facility for integrated microscopy (CFIM) of the Faculty of Health and Medical Sciences of the University of Copenhagen. Pictures were taken with a LSM 700 confocal microscope (Zeiss, Germany) equipped with Plan-Neofluar, X5 (N.A. 0.15) and Plan-Apochromat X63 (N.A. 1.4) objectives. E-GFP positive cells were excited with a 488 nm diode laser (10 mW).

DATA ANALYSIS

Statistical analyses were performed offline by means of Origin software (version 8.6; MicroCal Inc., USA). The normality distribution of each sample was tested with Shapiro–Wilk test. Non-parametric tests were used for small samples ($n < 10$) and when Gaussian distribution could not be approximated. Data are represented as mean ± standard deviation of the mean.

RESULTS

ASTROCYTES PRODUCE TONIC PRESYNAPTIC INHIBITION IN THE VENTRAL HORN

We recorded ventral horn neurons (**Figure 1C**) from the spinal cord of neonatal mice with the whole-cell patch-clamp technique in voltage-clamp mode. Most of the neurons recorded from had a high input resistance (>500 MΩ) and were located in the medial part of the ventral horn. They were likely interneurons. In few instances putative motoneurons located in the lateral part of the ventral horn were also recorded. We did not investigate their identity further. Since all these neurons responded similarly to the different tests of our study, they were pooled in a single sample. We evoked pairs of monosynaptic EPSCs by stimulating a nearby region with a bipolar electrode (**Figures 2A,B**; see Materials and Methods). The second EPSC was bigger, similar or smaller than the first. Consequently, the paired pulse ratio (PPR), calculated as the relative value of the second and the first EPSCs was either below or above 1 (**Figure 1C**). To investigate if and how astrocytes modulate synaptic transmission, we used a second pipette filled with patch solution enriched with a high concentration of the Ca^{2+} chelator BAPTA (30 mM; see Materials and Methods). We approached it close to a neighboring fluorescent astrocyte (**Figures 1A,B**). Recording the astrocyte in cell-attached mode did not affect the evoked EPSCs (**Figure 2D**). However, few minutes (3–21) after breaking-in to whole-cell configuration, the amplitude of the first EPSC was significantly increased (**Figures 2B–D**; $n = 6$; significant increase for five of six cells considered individually; $6.8*10^{-13} < p < 0.033$; two sample *t*-test and two sample Kolmogorov–Smirnov; significant increase for the mean values of all cells pooled together; 105 ± 142 pA to 135 ± 179 pA, $p = 0.03$; Wilcoxon signed-rank test). This result suggests that astrocytes exert a tonic inhibition on ventral horn neurons by releasing a gliotransmitter through a Ca^{2+} dependent mechanism. To determine if the inhibition occurred pre- or postsynaptically, we considered the response induced by the second stimulation. On average, chelating Ca^{2+} in the astrocyte had no significant effect on the second EPSC (**Figure 2E**; 95 ± 63 pA to 97 ± 68 pA; $n = 6$; $p = 0.69$; Wilcoxon signed-rank test). Consequently the PPR was decreased in all the cells tested (**Figures 2F–H**; significant decrease for all the cells pooled together; 1.4 ± 0.5 to 1.0 ± 0.3, $p = 0.03$ Wilcoxon signed-rank test). The input resistance of the recorded neurons was not changed after chelating Ca^{2+} in astrocytes (514 ± 298 MΩ in control; 507 ± 284 MΩ after Ca^{2+} chelating, $n = 5$; $p = 0.3$; Wilcoxon signed-rank test). Altogether these results suggest that astrocytes produce a tonic inhibition in the ventral horn by releasing a gliotransmitter that inhibits excitatory transmission at the presynaptic level.

SPINAL ASTROCYTES GENERATE PHASIC PRESYNAPTIC INHIBITION

Next, we investigated if a phasic activation of astrocytes also produced an inhibition of synaptic transmission. For this purpose, we used a pipette filled with the peptide TFLLR, an agonist for the G-protein coupled PAR-1 receptor that is preferentially expressed in astrocytes (Lee et al., 2007; Shigetomi et al., 2008). TFLLR induces a Ca^{2+} increase in astrocytes (Lee et al., 2007; Shigetomi et al., 2008; Wang et al., 2013). When we puffed TFLLR between the stimulation and recording electrodes (**Figure 3A**), the amplitude of the first EPSC evoked by a paired stimulation protocol was significantly decreased (**Figures 3B–D**; $n = 25$; significant decrease for 20/25 cells tested individually; $3.3*10^{-6} < p < 0.05$; paired sample *t*-test, Kolmorogov–Smirnov test and two sample *t*-test; significant decrease for all the cells pooled together; $p = 1.9*10^{-7}$, paired sample *t*-test). The second EPSC was not significantly affected by TFLLR (**Figure 3E**; $n = 25$; $p = 0.06$; paired *t*-test). Consequently the PPR was increased (**Figures 3F–H**; $n = 25$; significant increase for all cells taken together; $p = 1.7*10^{-5}$, paired *t*-test). The effect of TFLLR was correlated with the PPR observed under control conditions: the inhibition was stronger for synapses with a high release probability (PPR < 1; significant correlation; $p = 0.04$; $R = -0.4$; Pearson correlation test; $n = 25$). Since neither the input resistance of the postsynaptic

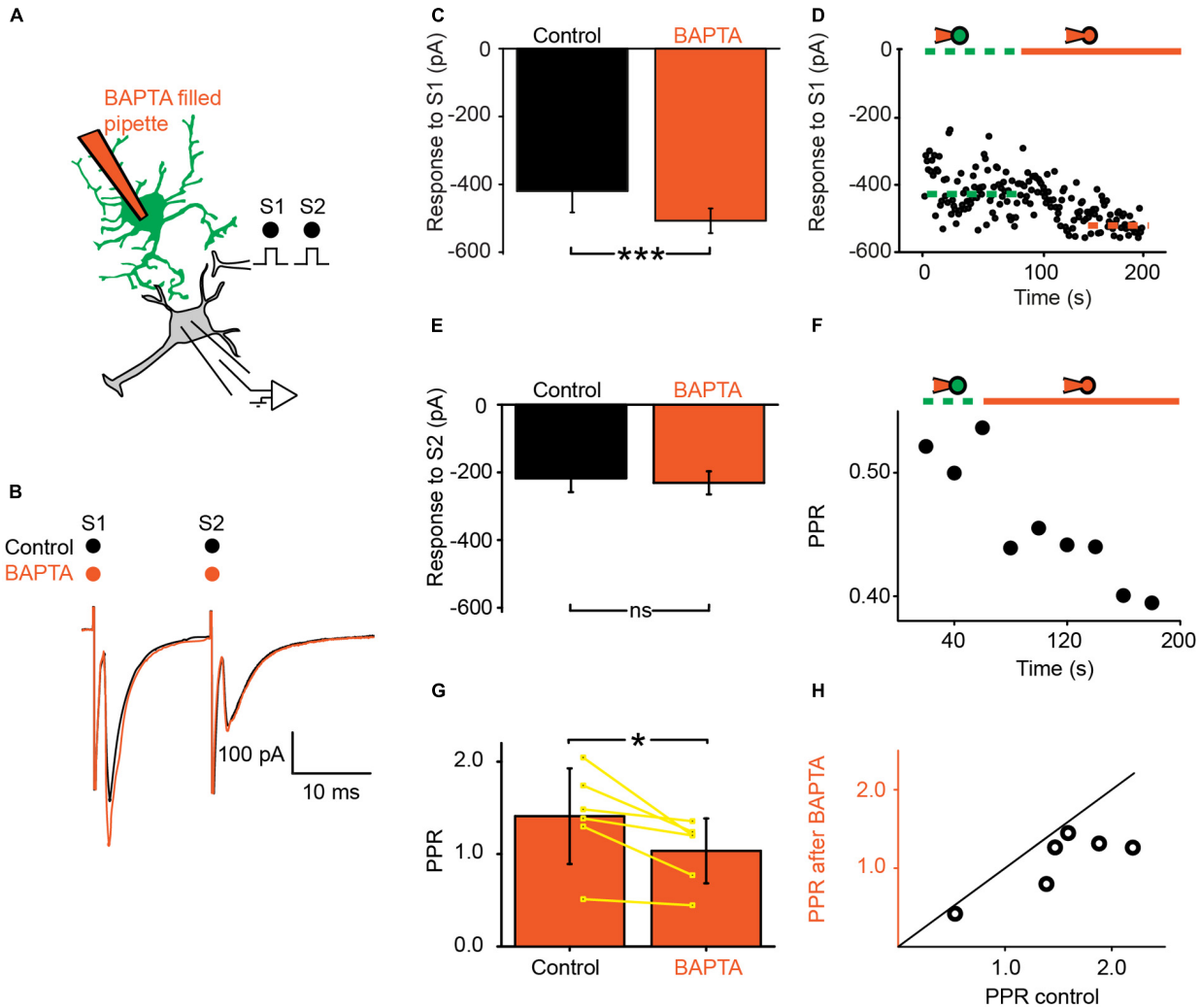

FIGURE 2 | Ventral horn astrocytes produce a tonic presynaptic inhibition of synaptic transmission. (A) Schema of the principle of the preparation. Ventral horn neuron recorded in whole-cell mode. Synaptic stimulation produced by a bipolar electrode (S1, S2). Whole-cell recording of an astrocyte with 30 mM BAPTA. **(B)** Response of a ventral horn neuron to two stimulations in control conditions (black) and after chelating Ca^{2+} in one astrocyte (red). The amplitude of the first EPSC was increased. **(C)** Mean amplitude of the first EPSC in control condition and after chelating Ca^{2+} in one astrocyte. The amplitude was significantly increased. $^{***}p < 0.001$. **(D)** Amplitude of the first EPSC when the astrocyte was recorded in cell-attached configuration (doted green line) and after breaking-in in whole-cell configuration (red line). Note the gradual increase in amplitude. The dot lines correspond to the mean amplitudes in both conditions. **(E)** Amplitude of the second EPSC in control condition and after chelating Ca^{2+} in one astrocyte. **(F)** Time course of the changes occurring for the paired pulse ratio (PPR) calculated as the amplitude of the second response divided by the first for the neuron recorded in (B). Recording the astrocyte in whole-cell mode (red line) decreased the PPR. **(G)** Mean PPR for all the cells recorded before and after chelating Ca^{2+} in one astrocyte. The yellow lines correspond to individual pairs of values. The PPR was significantly decreased. $^{*}p < 0.05$. **(H)** PPR after Ca^{2+} chelation in one astrocyte as a function of PPR in control conditions. If BAPTA had no effect, the points would be distributed around the line of equality (black). All the points are below the line of equality.

neuron, nor the holding current were affected by TFLLR (control input resistance: 730 ± 531 MΩ; after TFLLR: 741 ± 572 MΩ, $n = 23$; $p = 0.36$, Wilcoxon signed-rank test; control holding current: −63 ± 69 pA; after TFLLR: −67 ± 75 pA, $n = 25$; $p = 0.11$, Wilcoxon signed-rank test), our results suggest that activation of astrocytes triggers the release of a substance that produces presynaptic inhibition of excitatory synaptic transmission in the ventral horn of the spinal cord. To identify the gliotransmitter involved, we performed a range of pharmacological tests.

DUAL MODULATION OF SYNAPTIC TRANSMISSION BY ATP

Since purines are major intrinsic modulators in the spinal cord (Dale and Gilday, 1996) that can be released by astrocytes (Pascual et al., 2005; Fields and Burnstock, 2006; Panatier et al., 2011), we tested the effect of ATP on synaptic transmission (**Figure 4A**). A 10 s Puff of ATP (1 mM) between the stimulation and recording electrodes reduced the amplitude of evoked pairs EPSCs (**Figure 4B**; $n = 6$). A careful inspection of the recordings revealed that the effects induced by ATP evolved with time. During the puff itself, the amplitude of the first EPSC was reduced of 18% (**Figure 4C**;

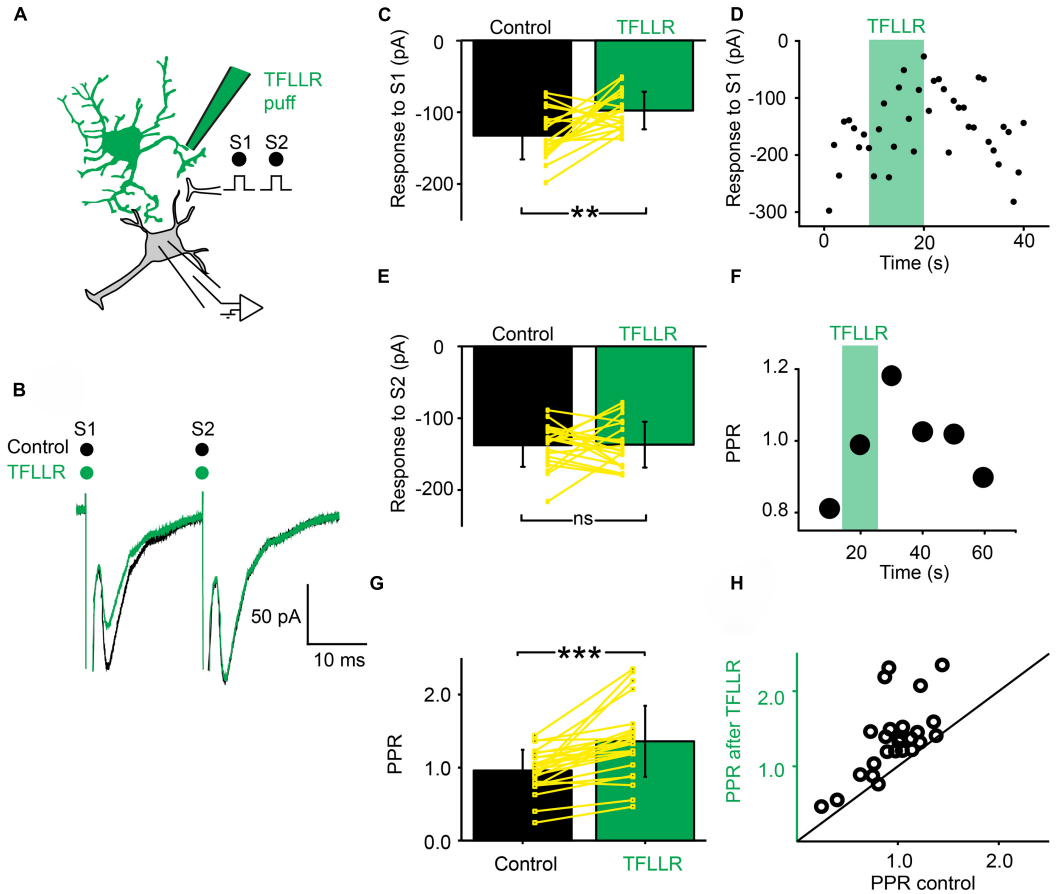

FIGURE 3 | Activation of astrocytes with TFLLR produces presynaptic inhibition of synaptic transmission. (A) Schema of the principle of the preparation. Ventral horn neuron recorded in whole-cell mode. Synaptic stimulation produced by a bipolar electrode (S1, S2). Puff application of TFLLR. **(B)** Response of a ventral horn neuron to two stimulations in control conditions (black) and after puffing TFLLR between the stimulation and recording electrode (green). The amplitude of the first EPSC was decreased. **(C)** Mean amplitude of the first EPSC in control condition and after puffing TFLLR. The amplitude was significantly decreased. $^{**}p < 0.01$. **(D)** Amplitude of the first EPSC before, during (green bar) and after puffing TFLLR. Note the decrease in amplitude during the puff. **(E)** Amplitude of the second EPSC in control conditions and after puffing TFLLR. **(F)** Time course of the changes occurring for the PPR calculated for the neuron recorded in (B). TFLLR increased the PPR. **(G)** Mean PPR for all the cells recorded before and after puffing TFLLR. The yellow lines correspond to individual values. The PPR was significantly increased. $^{***}p < 0.001$. **(H)** PPR after TFLLR in one astrocyte as a function of PPR in control conditions. Most points are above the line of equality.

from 131 ± 72 pA to 108 ± 37 pA; non-significant reduction, $p = 0.22$, Wilcoxon signed-rank test) while the second EPSC was decreased of 30% (**Figure 4D**; from 132 ± 71 pA to 93 ± 45 pA; significant reduction for all the cells tested together; $n = 6$; $p = 0.03$; Wilcoxon signed-rank test). Consequently, The PPR was significantly reduced (**Figures 4E,F**; from 1.08 ± 0.5 in control conditions to 0.85 ± 0.3 during ATP; $p = 0.03$, Wilcoxon signed-rank test). The effects initially induced by ATP were concomitant with a decrease in input resistance of postsynaptic neurons (from 480 ± 318 MΩ to 442 ± 295 MΩ; $p = 0.03$, Wilcoxon signed-rank test), suggesting a mixture of pre- and postsynaptic effects. However, 1 min after the puff, the effects induced by ATP were different. The response to the first chock was more decreased (**Figures 4B,C**; from 131 ± 72 pA to 81 ± 35 pA; $n = 6$; significant decrease for 4/6 cells, $0.0015 < p < 0.019$, two sample t-test; significant decrease for all cells pooled together, $p = 0.03$, Wilcoxon signed-rank test), while the response to the second chock was less affected (**Figure 4D**; from 132 ± 71 pA to 109 ± 59 pA, significant decrease for all cells pooled together, $p = 0.03$, Wilcoxon signed-rank test). Thus, after an initial decrease, ATP induced an increase of the PPR (**Figures 4E,F**; from 1.08 ± 0.5 to 1.35 ± 0.5, $p = 0.03$, Wilcoxon signed-rank test). Since 1 min after the puff, the input resistance of the recorded neuron was not significantly different from control conditions (480 ± 317 MΩ in control; 500 ± 363 MΩ 1 min after ATP; $p = 0.6$, Wilcoxon signed-rank test), our results suggest that ATP induced presynaptic inhibition of transmitter release.

ADENOSINE INDUCES PRESYNAPTIC INHIBITION

The latency of the effects induced by ATP suggests that presynaptic inhibition is not induced by ATP itself, but by one of its metabolic products. Since ATP gets converted into adenosine by extracellular ectonucleotidases (Fields and Burnstock, 2006), we tested the effect of a puff of adenosine (1 mM), using the

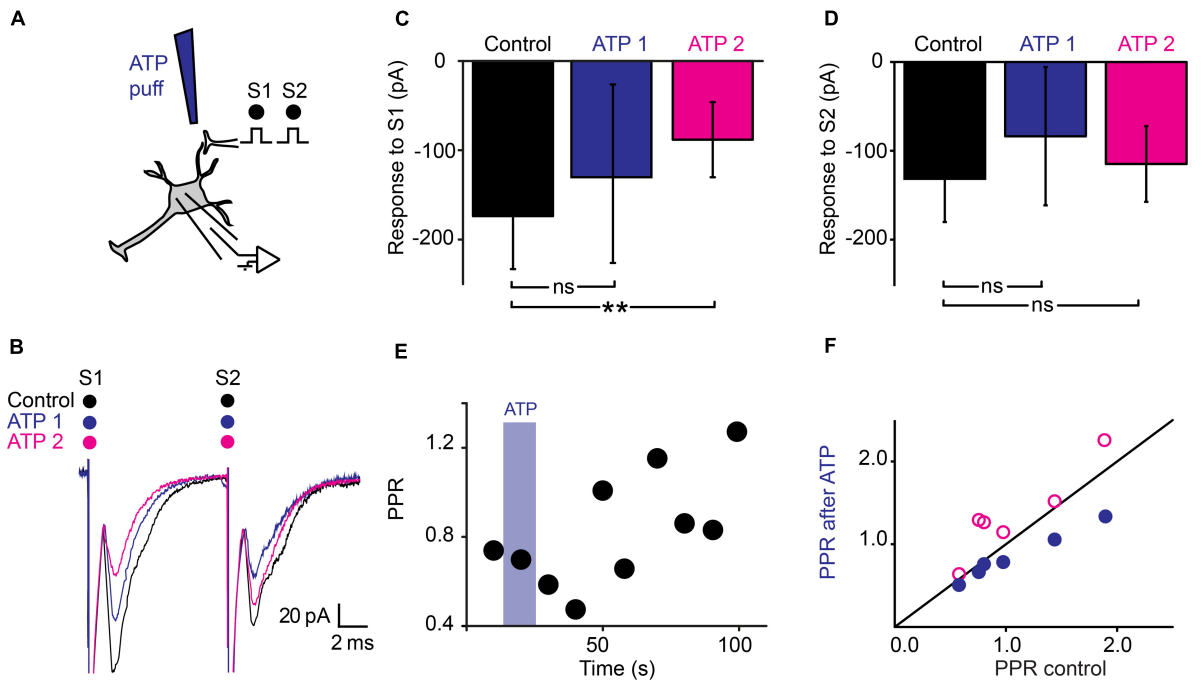

FIGURE 4 | Dual effects of ATP on synaptic transmission. (A) Schema of the principle of the preparation. Ventral horn neuron recorded in whole-cell mode. Synaptic stimulation produced by a bipolar electrode (S1, S2). Puff application of ATP. **(B)** Response of a ventral horn neuron to two stimulations in control conditions (black), during (ATP 1) and 1 min after puffing ATP (ATP 2) between the stimulation and recording electrode. The amplitude of the first EPSC got more decreased with time while the amplitude of the second EPSC got less decreased. **(C)** Amplitude of the first EPSC in control condition, during a puff of ATP (ATP 1) and 1 min after (ATP 2). After 1 min, the amplitude was significantly decreased. $^{**}p < 0.01$. **(D)** Amplitude of the second EPSC in control conditions, during a puff of ATP (ATP 1) and 1 min after (ATP 2). The difference is not significant for this particular example **(E)** Time course of the changes in PPR induced by ATP for the cell recorded in (B). Note the initial decrease followed by an increase. **(F)** PPR calculated during (blue filled circles), and 1 min after puffing ATP (purple open circles) as a function of PPR in control conditions. After 1 min, most points are above the line of equality. Significant increase.

same protocol (**Figure 5A**). We found that adenosine induced a strong inhibition of the first EPSC (**Figure 5C**; $n = 6$; from 133 ± 134 pA to 64 ± 52 pA; significant decrease for 5/6 cells; $5.9^{*}10^{-17} < p < 0.002$; two sample *t*-test and two sample Kolmogorov–Smirnov; significant decrease for all cells pooled together, $p = 0.03$, Wilcoxon signed rank test) and a weaker inhibition of the second EPSC (**Figure 5D**; $n = 6$; from 141 ± 170 pA to 85 ± 76 pA; significant decrease for 4/6 cells; $1.2^{*}10^{-12} < p < 0.008$, two sample *t*-test and two sample Kolmogorov–Smirnov; non-significant decrease for all cells pooled together, $p = 0.16$, Wilcoxon signed rank test). Here also, the PPR was significantly increased (**Figure 5E**; from 1.0 ± 0.3 to 1.4 ± 0.4; $p = 0.03$, Wilcoxon signed-rank test). In contrast with ATP, the effects of adenosine were homogenous and occurred without significant change in input resistance (1007 ± 794 MΩ in control; 949 ± 680 MΩ after adenosine; $p = 0.8$; Wilcoxon signed-rank test). Altogether, these results suggest that adenosine inhibits EPSCs via a presynaptic mechanism.

THE SELECTIVE BLOCKADE OF ECTONUCLEOTIDASES PREVENTS THE EFFECTS OF TFLLR

We tested further the possible involvement of ATP in presynaptic inhibition by means of the very selective ectonucleotidase inhibitor ARL 67156 (Levesque et al., 2007). We selected neurons for which TFLLR induced presynaptic inhibition (**Figures 6A,B**). After addition of ARL 67156 (50 μM) to the extracellular medium, the first EPSC was not affected anymore by a puff of TFLLR (**Figure 6E**; $n = 6$; EPSC in normal Ringer: −76.2 ± 30 pA; after TFLLR: −52.0 ± 31.6 pA; significant decrease for 4/6 cells, $3.3^{*}10^{-6} < p < 0.047$, two sample *t*-test and two sample Kolmogorov–Smirnov; significant decrease for all cells pooled together, $p = 0.03$, Wilcoxon signed-rank test; EPSC in ARL 67156: 70.6 ± 44.3 pA; after TFLLR: 73.2 ± 48.9 pA; no significant decrease for cells considered individually $0.11 < p < 0.81$, two sample *t*-test and Kolmogorov–Smirnov; no significant decrease for all cells pooled together, $p = 0.7$, Wilcoxon signed-rank test). Moreover, the PPR was not significantly affected (**Figures 6D,F**; $n = 6$: PPR in normal Ringer: 1.0 ± 0.2; TFLLR: 1.7 ± 0.4; $p = 0.03$ Wilcoxon signed-rank test; PPR in ARL 67156: 1.2 ± 0.4; TFLLR: 1.0 ± 0.2; $p = 0.2$ Wilcoxon signed-rank test). We therefore conclude that presynaptic inhibition is not mediated by ATP, but rather by one of its metabolic products.

TFLLR INDUCES PRESYNAPTIC INHIBITION BY ACTIVATING ADENOSINE A_1 RECEPTORS

Since adenosine induced presynaptic inhibition (**Figure 5**), we tested if blocking adenosine A_1 receptors prevented the induction

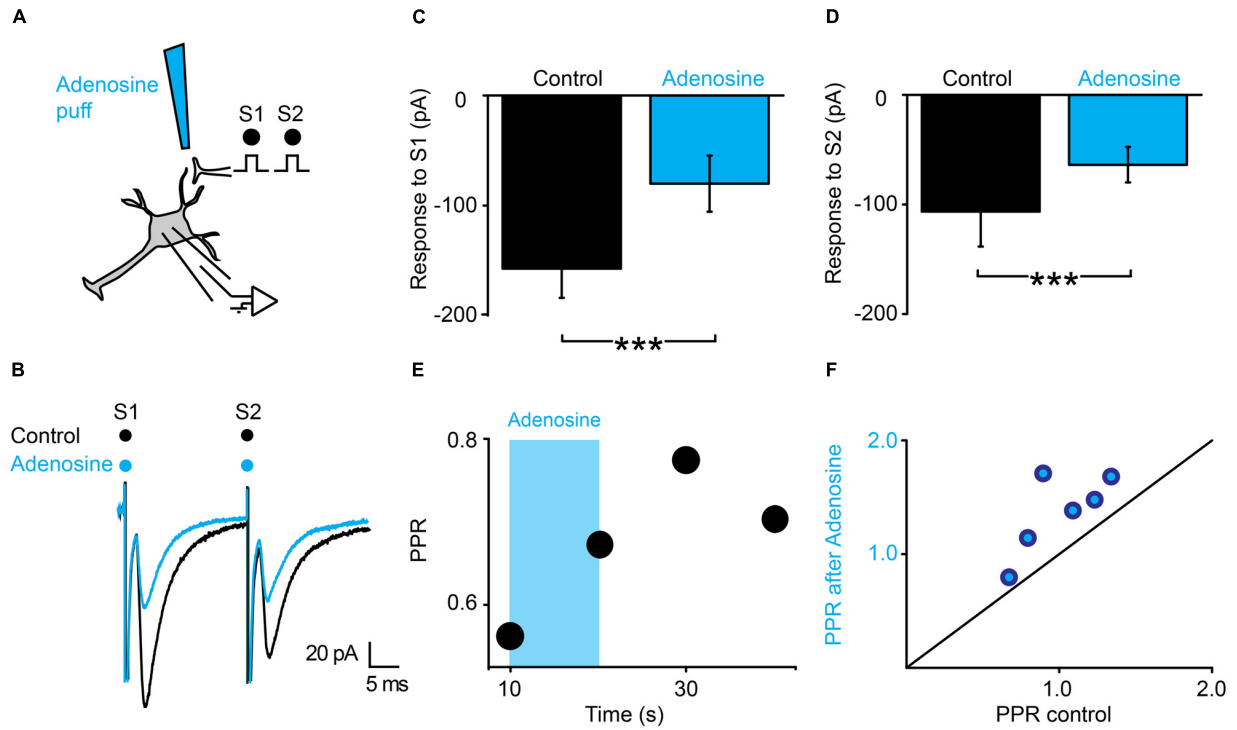

FIGURE 5 | Adenosine generates presynaptic inhibition. (A) Schema of the principle of the preparation. Ventral horn neuron recorded in whole-cell mode. Synaptic stimulation produced by a bipolar electrode (S1, S2). Puff application of adenosine. **(B)** Response of a ventral horn neuron to two stimulations in control conditions (black) and after puffing adenosine between the stimulation and recording electrode (blue). The amplitude of both EPSCs was decreased. **(C)** Amplitude of the first EPSC in control condition and after puffing adenosine. Significant decrease. $***p < 0.001$. **(D)** Amplitude of the second EPSC in control condition and after puffing adenosine. Significant decrease. $***p < 0.001$. **(E)** Time course of the changes in PPR induced by adenosine (blue bar) for the cell recorded in (B). **(F)** PPR after puffing adenosine as a function of PPR in control conditions. All points are above the line of equality. Adenosine significantly increased the PPR.

of presynaptic inhibition by TFLLR (**Figure 7A**). We found that in the presence of the selective adenosine A_1 receptor antagonist DPCPX (5 μM), puffing TFLLR did not affect the amplitude of evoked EPSCs anymore (**Figures 7B,E**; $n = 6$; effect of TFLLR in control conditions: from 153 ± 63 pA to 111 ± 47; significant decrease for 6/6 cells, $0.0017 < p < 0.043$, two sample t-test and two sample Kolmogorov–Smirnov; significant decrease for all cells pooled together, $p = 0.03$, Wilcoxon signed-rank test; effect of TFLLR in DPCPX: from 135 ± 63 pA to 131 ± 58 pA; no significant decrease for cells considered individually: $0.18 < p < 0.71$, two sample t-test and Kolmogorov–Smirnov; no significant decrease for all cells pooled together; $p = 0.3$, Wilcoxon signed-rank test). The PPR, which was significantly increased in control conditions (**Figures 7C,D**), was not altered anymore (**Figures 7D,F**; $n = 6$; increase of PPR in control conditions: from 1.1 ± 0.3 to 1.4 ± 0.5; $p = 0.03$, Wilcoxon signed-rank test; no change of PPR in DPCPX: from 1.2 ± 0.4 to 1.1 ± 0.4; $p = 0.3$, Wilcoxon signed-rank test). Our results demonstrate that the metabolic product of ATP that induces presynaptic inhibition of excitatory synaptic transmission is likely to be adenosine acting on A_1 receptors.

DISCUSSION

Our study suggests that astrocytes located in the ventral horn of the spinal cord induce both tonic and phasic presynaptic inhibition of excitatory synaptic transmission. Our results indicate that extracellular ectonucleotidases convert ATP released by astrocytes into adenosine, which binds to A_1 inhibitory receptors located on the presynaptic side of excitatory synapses (**Figure 8**).

THE MODULATION INDUCED BY ASTROCYTES IS BOTH TONIC AND PHASIC

Chelation of Ca^{2+} in one astrocyte induced a decrease of the PPR suggesting a removal of presynaptic inhibition (**Figure 2**). The most likely mechanism is an increase of the residual Ca^{2+} present in presynaptic terminals since both the release probability (p) and the pool of available quanta (n) are Ca^{2+} dependent (Zucker and Regehr, 2002). Even though the conductance measured in postsynaptic neurons was unaffected by the BAPTA treatment, we cannot rule out postsynaptic mechanisms (for example if there are two or more conductances affected in opposite ways).

This result suggests that astrocytes continuously release purines that produce presynaptic inhibition that might contribute to lower the activity of the spinal motor network. Tonic release of vesicular ATP from astrocytes was previously reported in the hippocampus (Pascual et al., 2005). Released ATP gets converted into adenosine by extracellular ectonucleotidases. This mechanism explains the persistent synaptic suppression mediated by adenosine (Pascual

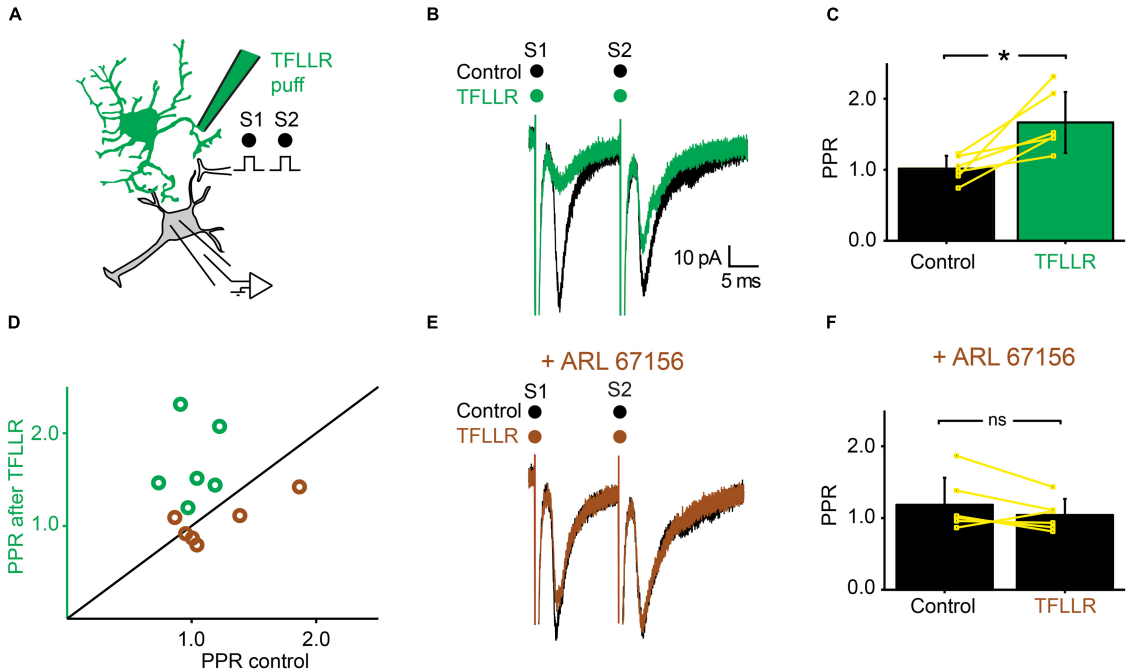

FIGURE 6 | Inhibiting extracellular ectonucleotidases blocks the effects of TFLLR. (A) Schema of the principle of the preparation. Ventral horn neuron recorded in whole-cell mode. Synaptic stimulation produced by a bipolar electrode (S1, S2). Puff application of TFLLR. **(B)** Response of a ventral horn neuron to two stimulations in control conditions (black) and after puffing TFLLR between the stimulation and recording electrode (green). The amplitude of the first EPSC was decreased. **(C)** Mean values of the PPR before and after puffing TFLLR. $*p < 0.05$. **(D)** PPR after puffing TFLLR as a function of PPR before puffing TFLLR. In control conditions, the PPR was significantly increased (green open circles). In the presence of ARL 67156, the PPR was not significantly increased anymore (brown open circles). **(E)** Same neuron as in **(B)**, recorded after addition of ARL 67156 to the bath. Puffing TFLLR did not affect the amplitude of the EPSC anymore (brown trace). **(F)** Mean values of the PPR before and after puffing TFLLR in the presence of ARL 67156.

et al., 2005). The central nervous system uses different strategies to decrease its excitability, the most common being a general increase of membrane conductance provided by an ambient concentration of GABA (Farrant and Nusser, 2005; Bautista et al., 2010).

Puffing TFLLR produced a transient inhibition of excitatory synaptic transmission (**Figure 3**). This suggests that the level of presynaptic inhibition provided by astrocytes is not only tonic but can also be regulated by the activity of the network. TFLLR is an exogenous molecule and does not activate astrocytes under physiological conditions. We did not investigate the nature of endogenous neurotransmitters responsible for the activation of astrocytes. The most likely scenario is that glutamate released from excitatory synapses binds to receptors located in the membrane of neighboring astrocytes that would in turn release ATP. Such a mechanism has been described in slices from the hippocampus where glutamate binding to metabotropic glutamate subtype 5 receptors triggers the release of ATP from astrocytes (Zhang et al., 2003; Panatier et al., 2011). Another possibility could be a change in pH. Brain hypoxia and ischemia lead to acidosis (Tombaugh and Sapolsky, 1993), which induces the release of ATP from astrocytes in the brainstem (Gourine et al., 2010). This process is essential for adjusting respiratory responses. These possibilities will be tested during future experiments.

The interpretation of our results relies on the hypothesis that the number of axons recruited by the electrical stimulation remained constant during each experiment. Puffing drug with pressure could in principle induce a mechanical artifact resulting in a change of the excitability of stimulated fibers. However, if it was the case, the number of recruited axons would either increase or decrease in a random manner. In contrast, puffing TFLLR induced either no effect or a reliable decrease of synaptic transmission. In addition, when blocking ectonucleotidases or A_1 receptors abolished the effect of TFLLR puff (see **Figures 6** and **7**), which rules out the possibility of a substantial change in the number of recruited axons.

IDENTITY OF THE PURINES AND OF THE RECEPTORS RESPONSIBLE FOR PRESYNAPTIC INHIBITION

Our results demonstrate that presynaptic inhibition is triggered by ATP, which, after enzymatic hydrolysis to adenosine binds to presynaptic receptors. Several arguments support this interpretation. First, presynaptic inhibition induced by TFLLR was both reproduced by ATP and adenosine (**Figures 4** and **5**). However, in contrast to adenosine, the inhibition produced by ATP was complex since, during the puff application, it was characterized by a decrease in PPR, which is not compatible with presynaptic inhibition. After 1 min, the inhibition produced by ATP was characterized by an increase in PPR, like the effect

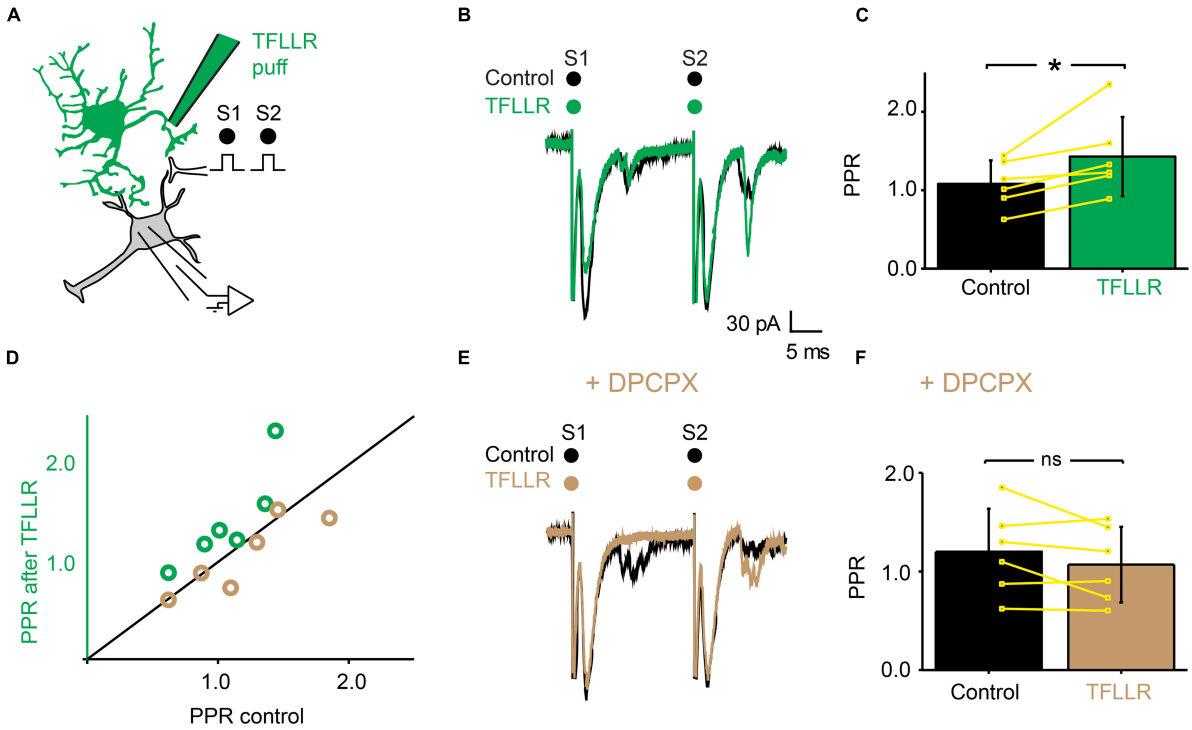

FIGURE 7 | The effects of TFLLR are blocked by DPCPX. (A) Schema of the principle of the preparation. Ventral horn neuron recorded in whole-cell mode. Synaptic stimulation produced by a bipolar electrode (S1, S2). Puff application of TFLLR. **(B)** Response of a ventral horn neuron to two stimulations in control conditions (black) and after puffing TFLLR between the stimulation and recording electrode (green). The amplitude of the first EPSC was decreased. **(C)** Mean values of the PPR before and after puffing TFLLR. $*p < 0.05$. **(D)** PPR after puffing TFLLR as a function of PPR before puffing TFLLR. In control conditions, TFLLR induced an increase of the PPR (green circles). In the presence of DPCPX, TFLLR did not change the PPR (beige circles). **(E)** Same neuron as in (B), recorded after addition of DPCPX to the bath. Puffing TFLLR did not affect the amplitude of the EPSP anymore (beige trace). **(F)** Mean values of the PPR before and after puffing TFLLR in the presence of DPCPX.

induced by adenosine. This suggests that adenosine rather than ATP is responsible for presynaptic inhibition. This interpretation is supported by the fact that the conversion of ATP to adenosine occurs within a range of 200 ms (Dunwiddie et al., 1997). Second, blocking the hydrolysis of ATP to adenosine by inhibiting extracellular ectonucleotidases with ARL 67156 suppressed the presynaptic inhibition triggered by TFLLR (**Figure 6**). This confirms that ATP itself does not induce presynaptic inhibition and suggests instead that a metabolic product of ATP such as ADP, AMP, or adenosine is involved (Fields and Burnstock, 2006). Third, blocking adenosine A_1 receptors with DPCPX suppressed the effects of TFLLR (**Figure 7**). This demonstrates that the purine responsible for presynaptic inhibition is actually adenosine.

Adenosine inhibits synaptic release of transmitter in other systems such as the dentate gyrus (Dolphin and Archer, 1983) and the CA1 region of the hippocampus (Corradetti et al., 1984; Wu and Saggau, 1994), the basal forebrain (Hawryluk et al., 2012), and the cerebral cortex (Hollins and Stone, 1980). In most cases, presynaptic adenosine A_1 receptors decrease transmitter release by inhibiting a Ca^{2+} current (Hollins and Stone, 1980; Wu and Saggau, 1994; Dale and Gilday, 1996; Brown and Dale, 2000; Zhang et al., 2003; Fields and Burnstock, 2006). In contrast, adenosine A_{2A} receptors facilitate synaptic transmission (Panatier et al., 2011). In agreement, we found that A_1 receptors mediate presynaptic inhibition in the ventral horn of the spinal cord (**Figure 7**).

FUNCTIONAL RELEVANCE

Regulating the excitability motor networks is essential for adjusting the motor command. Hyperexcitability of motor networks leads to involuntary movements and spasms. Tonic presynaptic inhibition of excitatory transmission may provide a general decrease of the activity of highly active networks and act as a homeostatic mechanism. In other regions of the central nervous system such as the hippocampus, adenosine released from astrocytes has neuroprotective effects and the activation of presynaptic A_1 receptors acts as en endogenous anticonvulsant (Boison, 2012). In the spinal cord, artificial spinal rhythmic activities induced by cocktails of pharmacological agents are modulated by adenosine. The activation of A_1 receptors depresses bicuculline-evoked seizures in newborn rats (Brockhaus and Ballanyi, 2000; Taccola et al., 2012). This suggests that adenosine also has anticonvulsant effects in the spinal cord. Indeed, in the spinal cord of young rats, acute hypoxia triggers the release of adenosine, which in turn depresses monosynaptic reflexes (Otsuguro et al., 2011). Such a neuroprotective role of adenosine is well documented in patients suffering from temporal lobe epilepsy. Seizures trigger a massive release of adenosine,

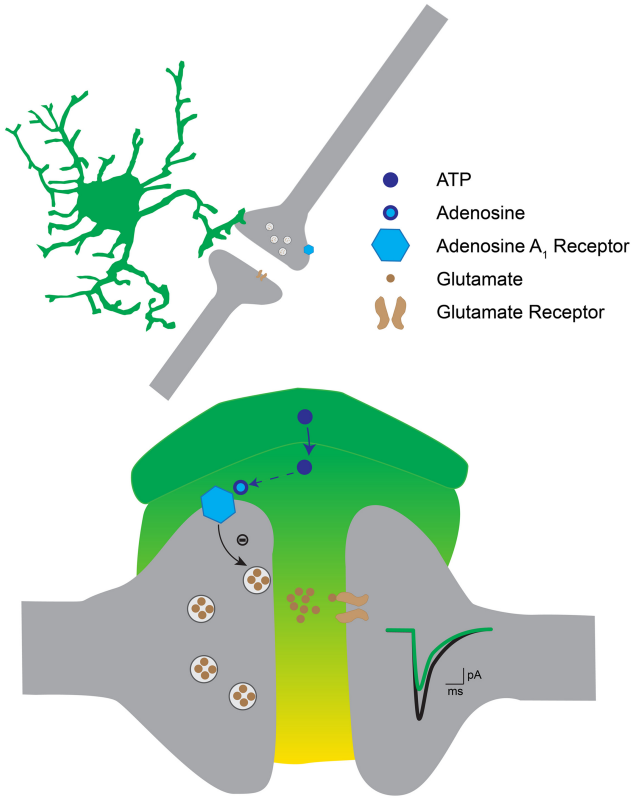

FIGURE 8 | Hypothetical mechanism for astrocytic induced presynaptic inhibition in the ventral horn. The activation of astrocytes induces the release of ATP, which gets converted into adenosine by extracellular ectonucleotidases. Adenosine binds to presynaptic A_1 receptors on excitatory terminals. This in turn decreases the probability of transmitter release. As a result excitatory postsynaptic currents are decreased.

which in turn activate A_1 receptors and thereby terminate the activity (Boison, 2012).

Adenosine might also be involved in the tuning of locomotor activity. Antagonists for adenosine receptors from the Methylxanthine family (such as caffeine or theophylline) increase locomotor activity (Snyder et al., 1981). During fictive locomotion induced in neonatal mice by a mixture of serotonin, NMDA and dopamine, bath application of adenosine slows down rhythmic activity by around 30% (Witts et al., 2012). To figure out the actual physiological role of purines during locomotion in adult mammals, one will have to measure their concentrations. The recent development of probes allowing the detection of ATP and adenosine in real time (Frenguelli et al., 2007) may allow figuring out if the concentration of purines changes tonically or rhythmically during movement. Interestingly, ATP increases the excitability of spinal locomotor networks (Dale and Gilday, 1996). Since the hydrolysis of ATP to adenosine occurs one range of magnitude faster than the locomotor cycle (Dunwiddie et al., 1997), a possible scenario could be that astrocytes release ATP cyclically, in phase with the rhythm. The fast degradation of ATP into adenosine would then induce a rhythmic inhibition of excitatory transmission. However, high-speed locomotion could be too fast for rhythmic mediated inhibition. We will test these hypotheses during future experiments.

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REFERENCES

Ameri, A. (1999). The effects of cannabinoids on the brain. *Prog. Neurobiol.* 58, 315–348. doi: 10.1016/S0301-0082(98)00087-2

Arvidsson, U., Cullheim, S., Ulfhake, B., Bennett, G. W., Fone, K. C., Cuello, A. C., et al. (1990). 5-Hydroxytryptamine, substance P, and thyrotropin-releasing hormone in the adult cat spinal cord segment L7: immunohistochemical and chemical studies. *Synapse* 6, 237–270. doi: 10.1002/syn.890060305

Bautista, W., Aguilar, J., Loeza-Alcocer, J. E., and Delgado-Lezama, R. (2010). Pre- and postsynaptic modulation of monosynaptic reflex by GABAA receptors on turtle spinal cord. *J. Physiol.* 588, 2621–2631. doi: 10.1113/jphysiol.2010.188979

Boison, D. (2012). Adenosine dysfunction in epilepsy. *Glia* 60, 1234–1243. doi: 10.1002/glia.22285

Brocard, F., Tazerart, S., and Vinay, L. (2010). Do pacemakers drive the central pattern generator for locomotion in mammals? *Neuroscientist* 16, 139–155. doi: 10.1177/1073858409346339

Brockhaus, J., and Ballanyi, K. (2000). Anticonvulsant A(1) receptor-mediated adenosine action on neuronal networks in the brainstem-spinal cord of newborn rats. *Neuroscience* 96, 359–371. doi: 10.1016/S0306-4522(99)00544-8

Brown, P., and Dale, N. (2000). Adenosine A1 receptors modulate high voltage-activated Ca2+ currents and motor pattern generation in the xenopus embryo. *J. Physiol.* 525(Pt 3), 655–667. doi: 10.1111/j.1469-7793.2000.00655.x

Brown, P., and Dale, N. (2002). Modulation of K(+) currents in Xenopus spinal neurons by p2y receptors: a role for ATP and ADP in motor pattern generation. *J. Physiol.* 540, 843–850. doi: 10.1113/jphysiol.2001.013192

Burnstock, G. (2007). Physiology and pathophysiology of purinergic neurotransmission. *Physiol. Rev.* 87, 659–797. doi: 10.1152/physrev.00043.2006

Christensen, R. K., Petersen, A. V., and Perrier, J. F. (2013). How do glial cells contribute to motor control? *Curr. Pharm. Des.* 19, 4385–4399. doi: 10.2174/13816128113199990384

Conn, P. J., Battaglia, G., Marino, M. J., and Nicoletti, F. (2005). Metabotropic glutamate receptors in the basal ganglia motor circuit. *Nat. Rev. Neurosci.* 6, 787–798. doi: 10.1038/nrn1763

Corradetti, R., Lo Conte, G., Moroni, F., Passani, M. B., and Pepeu, G. (1984). Adenosine decreases aspartate and glutamate release from rat hippocampal slices. *Eur. J. Pharmacol.* 104, 19–26. doi: 10.1016/0014-2999(84)90364-9

Cotel, F., Exley, R., Cragg, S. J., and Perrier, J. F. (2013). Serotonin spillover onto the axon initial segment of motoneurons induces central fatigue by inhibiting action potential initiation. *Proc. Natl. Acad. Sci. U.S.A.* 110, 4774–4779. doi: 10.1073/pnas.1216150110

Dai, X., Noga, B. R., Douglas, J. R., and Jordan, L. M. (2005). Localization of spinal neurons activated during locomotion using the c-fos immunohistochemical method. *J. Neurophysiol.* 93, 3442–3452. doi: 10.1152/Jn.00578.2004

Dale, N., and Gilday, D. (1996). Regulation of rhythmic movements by purinergic neurotransmitters in frog embryos. *Nature* 383, 259–263. doi: 10.1038/383259a0

Dolphin, A. C., and Archer, E. R. (1983). An adenosine agonist inhibits and a cyclic AMP analogue enhances the release of glutamate but not GABA from slices of rat dentate gyrus. *Neurosci. Lett.* 43, 49–54. doi: 10.1016/0304-3940(83)90127-1

Dunwiddie, T. V., Diao, L., and Proctor, W. R. (1997). Adenine nucleotides undergo rapid, quantitative conversion to adenosine in the extracellular space in rat hippocampus. *J. Neurosci.* 17, 7673–7682.

El Manira, A., and Kyriakatos, A. (2010). The role of endocannabinoid signaling in motor control. *Physiology (Bethesda)* 25, 230–238. doi: 10.11520/physiol.00007.2010

Farrant, M., and Nusser, Z. (2005). Variations on an inhibitory theme: phasic and tonic activation of GABA(A) receptors. *Nat. Rev. Neurosci.* 6, 215–229. doi: 10.1038/nrn1625

Fields, R. D., and Burnstock, G. (2006). Purinergic signalling in neuron-glia interactions. *Nat. Rev. Neurosci.* 7, 423–436. doi: 10.1038/nrn1928

Frenguelli, B. G., Wigmore, G., Llaudet, E., and Dale, N. (2007). Temporal and mechanistic dissociation of ATP and adenosine release during ischaemia in the mammalian hippocampus. *J. Neurochem.* 101, 1400–1413. doi: 10.1111/j.1471-4159.2007.04425.x

Gourine, A. V., Kasymov, V., Marina, N., Tang, F., Figueiredo, M. F., Lane, S., et al. (2010). Astrocytes control breathing through pH-dependent release of ATP. *Science* 329, 571–575. doi: 10.1126/science.1190721

Guthrie, P. B., Knappenberger, J., Segal, M., Bennett, M. V. L., Charles, A. C., and Kater, S. B. (1999). ATP released from astrocytes mediates glial calcium waves. *J. Neurosci.* 19, 520–528.

Hawryluk, J. M., Ferrari, L. L., Keating, S. A., and Arrigoni, E. (2012). Adenosine inhibits glutamatergic input to basal forebrain cholinergic neurons. *J. Neurophysiol.* 107, 2769–2781. doi: 10.1152/jn.00528.2011

Hollins, C., and Stone, T. W. (1980). Adenosine inhibition of gamma-aminobutyric acid release from slices of rat cerebral cortex. *Br. J. Pharmacol.* 69, 107–112. doi: 10.1111/j.1476-5381.1980.tb10888.x

Jacobs, B. L., and Azmitia, E. C. (1992). Structure and function of the brain serotonin system. *Physiol. Rev.* 72, 165–229.

Jo, Y. H., and Schlichter, R. (1999). Synaptic corelease of ATP and GABA in cultured spinal neurons. *Nat. Neurosci.* 2, 241–245. doi: 10.1038/6344

Kjaerulff, O., and Kiehn, O. (1996). Distribution of networks generating and coordinating locomotor activity in the neonatal rat spinal cord in vitro: a lesion study. *J. Neurosci.* 16, 5777–5794.

Lalo, U., Palygin, O., Rasooli-Nejad, S., Andrew, J., Haydon, P. G., and Pankratov, Y. (2014). Exocytosis of ATP from astrocytes modulates phasic and tonic inhibition in the neocortex. *PLoS Biol.* 12:e1001747. doi: 10.1371/journal.pbio.1001747

Lanuza, G. M., Gosgnach, S., Pierani, A., Jessell, T. M., and Goulding, M. (2004). Genetic identification of spinal interneurons that coordinate left-right locomotor activity necessary for walking movements. *Neuron* 42, 375–386. doi: 10.1016/S0896-6273(04)00249-1

Lee, C. J., Mannaioni, G., Yuan, H., Woo, D. H., Gingrich, M. B., and Traynelis, S. F. (2007). Astrocytic control of synaptic NMDA receptors. *J. Physiol.* 581, 1057–1081. doi: 10.1113/jphysiol.2007.130377

Levesque, S. A., Lavoie, E. G., Lecka, J., Bigonnesse, F., and Sevigny, J. (2007). Specificity of the ecto-ATPase inhibitor ARL 67156 on human and mouse ectonucleotidases. *Br. J. Pharmacol.* 152, 141–150. doi: 10.1038/sj.bjp.0707361

Mui, J. W., Willis, K. L., Hao, Z. Z., and Berkowitz, A. (2012). Distributions of active spinal cord neurons during swimming and scratching motor patterns. *J. Comp. Physiol. A Neuroethol. Sens. Neural. Behav. Physiol.* 198, 877–889. doi: 10.1007/s00359-012-0758-6

Nolte, C., Matyash, M., Pivneva, T., Schipke, C. G., Ohlemeyer, C., Hanisch, U. K., et al. (2001). GFAP promoter-controlled EGFP-expressing transgenic mice: a tool to visualize astrocytes and astrogliosis in living brain tissue. *Glia* 33, 72–86. doi: 10.1002/1098-1136(20010101)33:1<72::AID-GLIA1007>3.0.CO;2-A

Ono, H., and Fukuda, H. (1995). Pharmacology of descending noradrenergic systems in relation to motor function. *Pharmacol. Ther.* 68, 105–112. doi: 10.1016/0163-7258(95)02001-2

Otsuguro, K., Wada, M., and Ito, S. (2011). Differential contributions of adenosine to hypoxia-evoked depressions of three neuronal pathways in isolated spinal cord of neonatal rats. *Br. J. Pharmacol.* 164, 132–144. doi: 10.1111/j.1476-5381.2011.01333.x

Panatier, A., Vallee, J., Haber, M., Murai, K. K., Lacaille, J. C., and Robitaille, R. (2011). Astrocytes are endogenous regulators of basal transmission at central synapses. *Cell* 146, 785–798. doi: 10.1016/j.cell.2011.07.022

Pascual, O., Casper, K. B., Kubera, C., Zhang, J., Revilla-Sanchez, R., Sul, J. Y., et al. (2005). Astrocytic purinergic signaling coordinates synaptic networks. *Science* 310, 113–116. doi: 10.1126/science.1116916

Perrier, J. F., and Delgado-Lezama, R. (2005). Synaptic release of serotonin induced by stimulation of the raphe nucleus promotes plateau potentials in spinal motoneurons of the adult turtle. *J. Neurosci.* 25, 7993–7999. doi: 10.1523/JNEUROSCI.1957-05.2005

Perrier, J. F., Rasmussen, H. B., Christensen, R. K., and Petersen, A. V. (2013). Modulation of the intrinsic properties of motoneurons by serotonin. *Curr. Pharm. Des.* 19, 4371–4384. doi: 10.2174/1381612811 3199990341

Prut, Y., and Perlmutter, S. I. (2003). Firing properties of spinal interneurons during voluntary movement. I. State-dependent regularity of firing. *J. Neurosci.* 23, 9600–9610.

Schmidt, B. J., and Jordan, L. M. (2000). The role of serotonin in reflex modulation and locomotor rhythm production in the mammalian spinal cord. *Brain Res. Bull.* 53, 689–710. doi: 10.1016/S0361-9230(00)00402-0

Shigetomi, E., Bowser, D. N., Sofroniew, M. V., and Khakh, B. S. (2008). Two forms of astrocyte calcium excitability have distinct effects on NMDA receptor-mediated slow inward currents in pyramidal neurons. *J. Neurosci.* 28, 6659–6663. doi: 10.1523/JNEUROSCI.1717-08.2008

Silinsky, E. M., and Redman, R. S. (1996). Synchronous release of ATP and neurotransmitter within milliseconds of a motor nerve impulse in the frog. *J. Physiol.* 492(Pt 3), 815–822.

Snyder, S. H., Katims, J. J., Annau, Z., Bruns, R. F., and Daly, J. W. (1981). Adenosine receptors and behavioral actions of methylxanthines. *Proc. Natl. Acad. Sci. U.S.A.* 78, 3260–3264. doi: 10.1073/pnas.78.5.3260

Taccola, G., Olivieri, D., D'Angelo, G., Blackburn, P., Secchia, L., and Ballanyi, K. (2012). A(1) adenosine receptor modulation of chemically and electrically evoked lumbar locomotor network activity in isolated newborn rat spinal cords. *Neuroscience* 222, 191–204. doi: 10.1016/j.neuroscience.2012.07.030

Tombaugh, G. C., and Sapolsky, R. M. (1993). Evolving concepts about the role of acidosis in ischemic neuropathology. *J. Neurochem.* 61, 793–803. doi: 10.1111/j.1471-4159.1993.tb03589.x

Wang, F., Smith, N. A., Xu, Q., Goldman, S., Peng, W., Huang, J. H., et al. (2013). Photolysis of caged Ca2+ but not receptor-mediated Ca^{2+} signaling triggers astrocytic glutamate release. *J. Neurosci.* 33, 17404–17412. doi: 10.1523/JNEUROSCI.2178-13.2013

Witts, E. C., Panetta, K. M., and Miles, G. B. (2012). Glial-derived adenosine modulates spinal motor networks in mice. *J. Neurophysiol.* 107, 1925–1934. doi: 10.1152/jn.00513.2011

Wu, L. G., and Saggau, P. (1994). Adenosine inhibits evoked synaptic transmission primarily by reducing presynaptic calcium influx in area CA1 of hippocampus. *Neuron* 12, 1139–1148. doi: 10.1016/0896-6273(94)90321-2

Zhang, J. M., Wang, H. K., Ye, C. Q., Ge, W., Chen, Y., Jiang, Z. L., et al. (2003). ATP released by astrocytes mediates glutamatergic activity-dependent heterosynaptic suppression. *Neuron* 40, 971–982. doi: 10.1016/S0896-6273(03)00717-7

Zucker, R. S., and Regehr, W. G. (2002). Short-term synaptic plasticity. *Annu. Rev. Physiol.* 64, 355–405. doi: 10.1146/annurev.physiol.64.092501.114547

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Neurotrophins and spinal circuit function

Vanessa S. Boyce [†] ***and Lorne M. Mendell*** [*]

Department of Neurobiology and Behavior, Stony Brook University, Stony Brook, NY, USA

Edited by:
Shawn Hochman, Emory University, USA

Reviewed by:
James W. Grau, Texas A&M University, USA
Sandra Garraway, Emory University School of Medicine, USA

****Correspondence:***
Lorne M. Mendell, Department of Neurobiology and Behavior, Stony Brook University, Stony Brook, 100 Nicolls Road, NY 11794-5230, USA
e-mail: lorne.mendell@stonybrook.edu

[†]Present address:
Vanessa S. Boyce, 22 Highwoods Ct., Saint James, NY 11780, USA

Work early in the last century emphasized the stereotyped activity of spinal circuits based on studies of reflexes. However, the last several decades have focused on the plasticity of these spinal circuits. These considerations began with studies of the effects of monoamines on descending and reflex circuits. In recent years new classes of compounds called growth factors that are found in peripheral nerves and the spinal cord have been shown to affect circuit behavior in the spinal cord. In this review we will focus on the effects of neurotrophins, particularly nerve growth factor (NGF), brain derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), on spinal circuits. We also discuss evidence that these molecules can modify functions including nociceptive behavior, motor reflexes and stepping behavior. Since these substances and their receptors are normally present in the spinal cord, they could potentially be useful in improving function in disease states and after injury. Here we review recent findings relevant to these translational issues.

Keywords: neurotrophin, spinal cord injury, motor neuron, stretch reflex, pain, *c-Fos*, locomotion, nociceptor

INTRODUCTION

Classical studies of spinal cord function have centered on well-defined neural pathways that were considered to mediate stereotyped automatic functions such as stretch and flexion reflexes, and to distribute input from sensory fibers and from descending fibers to their appropriate targets. In the past several decades it has become apparent that these projections are modifiable and that this plays an important role in the physiology and pathology of functions mediated by the spinal cord. Thus a complete understanding the nature of spinal function as well as spinal dysfunction requires knowledge of these processes. In addition, such plasticity can serve potentially as a basis for reversing pathological changes after injury or disease. Initial studies of this type centered on the effects of monoamines on spinal circuits because of the well-known projections of serotonergic and noradrenergic projections to the spinal cord from the raphe and locus coeruleus, respectively (Jankowska et al., 2000). More recently, a different class of molecule has been shown capable of altering spinal circuit function. These are the neurotrophins, the most studied of which are nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3). These molecules interact with neurons that express the appropriate tropomyosin related kinase (trk) receptor- trkA (NGF), trkB (BDNF), and trkC (NT-3) as well as the p75 receptor common to all trk- expressing neurons. The receptor biology and intracellular signaling associated with trk receptors is reviewed elsewhere (Chao and Hempstead, 1995; Huang and Reichardt, 2001).

NEUROTROPHIN EFFECTS ON SENSORY NEURONS AND THEIR SPINAL PROJECTIONS

NGF

NGF was the first neurotrophin to be well characterized after discovery of its ability to promote the growth of neurites when applied to explanted dorsal root ganglia (reviewed in Cowan, 2001). Later, it was identified as a survival factor during the period of programmed cell death for small sensory neurons expressing the trkA receptor (reviewed in Mendell, 1995; Mendell et al., 1999b; Huang and Reichardt, 2001). In the immediate postnatal period, NGF continues to act as a survival factor, and subjecting the animal to an anti NGF antibody leads to failure of high threshold mechanoreceptors to survive (Lewin et al., 1992). Beginning about 2 days after birth, withdrawing NGF has little effect on nociceptor survival. However, during this same postnatal period, these cells begin to show sensitization of the response to noxious heat or capsaicin after application of NGF (Zhu et al., 2004). This switch, occurring in the immediate postnatal period, is associated with upregulation of ERK1/2 and PI3K/P110 in dorsal root ganglion (DRG) cells, and does not appear to be due to changes in expression of trkA or TRPV1 (Zhu and Oxford, 2011).

The sensitization elicited by NGF in adult nociceptive neurons in the DRG results from an increase in the current produced by TRPV1 receptor activation (Shu and Mendell, 1999a,b; Galoyan et al., 2003). This increase is not due to enhanced sensitivity of the TRPV1 receptors, but rather from recruitment of additional TRPV1 receptors from the interior of the cell (Zhang et al., 2005; Stein et al., 2006). The ability of NGF to sensitize the

response of nociceptors to noxious heat stimuli has important behavioral significance, namely that exposure of adult rats to systemic NGF results in robust mechanical and thermal hyperalgesia. Thermal hyperalgesia has a strong peripheral component while mechanical hyperalgesia induced by exposure to NGF is mediated largely centrally (Lewin et al., 1994). The central effects elicited from peripheral exposure to NGF are mediated by another neurotrophin, BDNF, whose expression in the DRG is enhanced several hours after administration of NGF (reviewed in Pezet and McMahon, 2006). The synaptic action of BDNF is discussed in more detail below.

NT-3

NT-3 plays a role similar to NGF in the survival of sensory afferents, but in this case it is muscle spindle (group Ia) afferent fibers expressing trkC (reviewed in Huang and Reichardt, 2001). NT-3 also promotes the elaboration of their terminal projections to motor neurons during late stages of development (Chen et al., 2003), and in addition potentiates the strength of the group Ia synaptic projection to motor neurons. In intact afferents this has been studied by delivering the NT-3 systemically or intramuscularly (Arvanian et al., 2003; Petruska et al., 2010) and it is difficult to determine the precise mode of action. For peripherally axotomized afferent fibers the NT-3 was delivered via a cuff on the cut nerve (Mendell et al., 1999). Because the effects were limited to the treated nerve, it seems likely that this was due to a direct effect of the NT-3 on the terminals of the fibers that were exposed to the NT-3, but possible mechanisms such as sprouting or facilitation of glutamate release could not be distinguished.

The monosynaptic projections from spindle afferent fibers to motor neurons also exhibit potentiation acutely when exposed to NT-3 in the isolated spinal cord (Arvanov et al., 2000). The acute effect is developmentally regulated because the postsynaptic (motor neuron) NMDA receptors required for the action of NT-3 become inactive about 10–14 days after birth due to Mg block (Arvanian and Mendell, 2001a; Arvanian et al., 2004). NT-3 also enhances the mechanical sensitivity of the neuroma of spindle afferents that have been axotomized and capped with a Gore-Tex sleeve (Munson et al., 1999).

BDNF

The effect of the trkB agonist BDNF on sensory neurons is more complex. Although BDNF also serves as a survival factor for sensory neurons during the period of programmed cell death, the correspondence to a particular phenotype as has been observed with NGF (nociceptors) and NT-3 (proprioceptors) is not clearcut. Furthermore, another neurotrophin, NT-4, also binds with the trkB receptor and can substitute for BDNF in some, but not in all cases, to assure the survival of sensory neuron populations (e.g., Erickson et al., 1996). BDNF, like NGF and NT-3, is known to regulate the sensitivity of low threshold sensory receptors, in this case the slowly adapting Type I mechanoreceptor (Carroll et al., 1998); NT-4 regulates the sensitivity of a different low threshold mechanoreceptor, the D- Hair (Hilaire et al., 2012). Thermal nociceptors are also reported to be sensitized by NT4 (Rueff and Mendell, 1996) and by BDNF (Shu et al., 1999; Shu and Mendell, 1999b).

An additional property of BDNF is that its expression in nociceptor somata is upregulated by exposure to NGF in the periphery as part of the response to peripheral inflammation (Pezet and McMahon, 2006). Enhanced expression of BDNF in the DRG results in increased levels of BDNF in the superficial dorsal horn (Lever et al., 2001). This is known to rapidly enhance the synaptic action of nociceptive afferents on cells of lamina II via a mechanism involving NMDA receptors (Garraway et al., 2003). It is interesting that NMDA receptors in these cells are not subject to Mg block in the adult unlike those in motor neurons (see above), presumably because their subunit composition differs (Arvanian et al., 2004).

BDNF also acutely strengthens the monosynaptic reflex pathway in the isolated spinal cord of neonates. However, this reverses into long lasting inhibition of transmission several minutes after BDNF exposure (Arvanian and Mendell, 2001b). The inhibitory effect is thought to be due to a presynaptic effect of BDNF in contrast to the potentiation, which has a postsynaptic mechanism, as is the case for NT-3 (reviewed in Mendell and Arvanian, 2002). In agreement with these long lasting postnatal changes, exposure of the prenatal rat to BDNF by i.p. injection of the mother reduces the monosynaptic EPSP measured postnatally, whereas prenatal exposure to NT-3 increases it (Seebach et al., 1999).

SUMMARY

In summary, there are several common features linking the effects of the different neurotrophins on sensory neurons. All sensory neurons express trk receptors whose identity determines which neurotrophin will influence their function. Neurotrophins all serve as survival factors during the period of programmed cell death. They all affect the response of receptors to natural stimulation, and finally they all are able to modify the synaptic effects of the affected sensory neurons. In the latter case the mechanisms are quite different. NGF elicits its effect by upregulating another neurotrophin, BDNF, which elicits its effect in the spinal cord by acting on NMDA receptors in cells of the superficial dorsal horn. The effect of peripherally delivered NT-3 is not established, but probably involves a presynaptic change in the affected fibers. However, studies of the acute action of NT-3 have also revealed the possibility of a postsynaptic NMDA receptor- mediated change as discussed for BDNF.

NEUROTROPHIN EFFECTS ON CIRCUITS IN THE INJURED SPINAL CORD

Since spinal neurons differ in their relationship with the inputs and outputs, we evaluate the spinal effects of neurotrophins by investigating their action on functional circuits rather than on individual neurons or neuron classes. In some cases the circuit is sensory and is driven by specific sensory receptors. An example is a circuit that processes nociceptive input. Another circuit is the pattern generator which delivers an output causing stepping. A third circuit is the sensorimotor circuit responsible for the stretch reflex that converts muscle stretch into muscle contraction.

Although NGF has played a prominent role in investigating the role of neurotrophins on sensory fibers, most studies of neurotrophin action on spinal circuit function have been limited

to BDNF and NT-3. TrkB and trkC receptors are expressed at a higher level than trkA receptors within the spinal cord (Liebl et al., 2001) thus raising the expectation that BDNF and NT-3 would be more effective in affecting spinal circuit function. Here we discuss the effect of BDNF and NT-3 on three circuits based on their effects on three behaviors: stepping, nociception, and the stretch reflex.

STEPPING

Application of neurotrophins to the injured spinal cord at the time of thoracic transection promotes stepping behavior in both cats (Boyce et al., 2007) and rats (Jakeman et al., 1998; Blits et al., 2003; Boyce et al., 2012b). Boyce et al. (2012b) reported that rats with intraspinal administration of AAV viruses engineered to express BDNF to a site just caudal to the T10 transection site could carry out plantar stepping with their hind limbs on a treadmill. Remarkably, these rats could also carry out overground stepping across a stationary surface requiring the provision of balance support only (i.e., without treadmill assistance). Training was not required for BDNF to exert any of these effects which developed over the first 2–4 weeks after transection and exposure to the AAV/BDNF.

Further evidence for BDNF- activation of neurons involved in stepping was a finding of elevated expression of *c-Fos* in cells of the intermediate zone in the upper lumbar spinal cord of these rats. Relatively little increase of *c-Fos* expression was observed in the ventral horn. There was a rostrocaudal gradient of *c-Fos* expression after administration at T10 such that it was greater at L2 than at L4/L5. Expression of *c-Fos* was not associated with enhanced neural activity *per se* because the rats had been anesthetized for an electrophysiological experiment for several hours prior to sacrifice. It is more likely that its expression was a direct effect of BDNF on these cells, as has been observed previously in dissociated embryonic hippocampal cells (Paul et al., 2001). However, *c-Fos* expression provided an indication that BDNF influenced L2 interneurons. Since L2 intermediate zone interneurons are likely crucial participants in the central pattern generator (CPG; reviewed in Goulding, 2009), it seems likely that this increase in *c-Fos* expression reflects the action of interneurons whose activity is responsible for the stepping behavior elicited by BDNF, i.e., *c-Fos* is a biomarker for stepping, at least under these conditions. The mechanism of BDNF action on this circuits remains to be determined, i.e., whether it is a generalized increase in excitability, activation of a specific subgroup of cells in this circuit or of cells driving it.

These findings in the transected cord suggest that the exogenous BDNF acts on trkB receptors to elevate activity in neurons of the locomotor circuit. This effect may be diminished to some extent by the presence of truncated trkB receptors whose expression is elevated in the spinal cord after contusion (Liebl et al., 2001). These authors suggest that the truncated receptors with a non-signaling intracellular component act to sequester BDNF thereby reducing its effect. More recent data have shown that deleting the truncated form of trkB receptor improves locomotor recovery after spinal contusion injury even without provision of exogenous BDNF (Wu et al., 2013).

NOCICEPTION

Elevation of BDNF levels also results in increased sensitivity to a noxious heat stimulation as would be expected by sensitizing or activating cells in the superficial dorsal horn (Garraway et al., 2003). Cells in this region express elevated *c-Fos* indicating a response to BDNF as reported for cells in the intermediate zone. These findings raise the question as to whether activating nociceptive circuits is a requirement for BDNF to elicit overground stepping, i.e., stepping is a component of the response to noxious stimulation. There are no direct studies of this issue, but a recent report (Garraway et al., 2011) indicates that the relationship between BDNF, stepping and nociception is at the very least quite complex.

In experiments comparing the effects of NT-3 and BDNF on stepping under comparable conditions, NT-3 promoted only treadmill-assisted stepping with the further requirement, not needed for BDNF, for perineal stimulation accomplished by squeezing the base of the tail (Boyce et al., 2012b). Furthermore, NT-3 did not sensitize the response to nociceptive stimulation. Consistent with both of these findings was the finding of no increase in *c-Fos* expression in the spinal cord, either in the intermediate zone or in the superficial dorsal horn. It is not known whether this is due to differences in signaling elicited by trkB and trkC activation or whether there are few trkC receptors on cells eliciting the nociceptive and stepping behavior.

Another model demonstrating the pro-nociceptive role of BDNF comes from the response to peripheral nerve injury. Under these conditions BDNF has been shown to be released from microglia in the superficial dorsal horn that have been sensitized by the purinergic transmitter ATP released from the central terminals of damaged nociceptors (Tsuda et al., 2003). The activated microglia release BDNF that in turn reduces the level of the Cl^- transporter KCC2 in neurons of the dorsal horn. This diminishes the inhibitory action of GABA, and in extreme cases converts it to an excitatory action (Coull et al., 2005). Thus neurotrophins can be released from non-neuronal cells, a mechanism that has been known for many years in the periphery where Schwann Cells associated with damaged peripheral fibers release neurotrophins whose identity is associated with the fiber type (reviewed in Lehmann and Höke, 2010).

MOTONEURONS AND THE MONOSYNAPTIC REFLEX

BDNF causes increased excitability of motor neurons in the injured cord as evidenced by a reduced rheobase (Gonzalez and Collins, 1997; Boyce et al., 2012b) despite the lack of significantly altered *c-Fos* expression in the ventral horn. Of interest in this context is the finding that BDNF delivered in a single dose 24 h after transection has been reported to increase expression of the KCC2 chloride transporter in motor neurons of transected preparations (Boulenguez et al., 2010). This would oppose any intrinsic increase in excitability of motor neurons by enhancing the magnitude of inhibition due to establishment of a more negative chloride equilibrium potential. However, chronically transected preparations treated with continuous BDNF administration via engineered AAV viruses exhibit reduction in expression of the KCC2 transporter (see also preliminary data in Boyce et al., 2012a; Ziemlinska et al., 2014). This is an important area that

requires further study, particularly in view of the recent finding indicating elevated levels of GABA and GABA- synthetic enzymes caudal to spinal transection (Ziemlinska et al., 2014). A combined reduction in motor neuron rheobase and a reduction in synaptic inhibition of motor neurons would be expected to promote spasticity as has been reported after BDNF administration (Boyce et al., 2012b; Fouad et al., 2013; Ziemlinska et al., 2014).

The effect of NT-3 on motor neuron excitability is opposite to that of BDNF, i.e., a reduced excitability (increased rheobase; see also Petruska et al., 2010; Boyce et al., 2012b). However, the decrease in intrinsic motor neuron excitability after NT-3 treatment is opposed by an increase in monosynaptic EPSP amplitude and a decrease in the after hyperpolarization (AHP) following the spike, each of which should enhance the strength of excitatory response directly (elevated EPSP) or indirectly (diminished AHP). Unlike NT-3, BDNF has no effect on the strength of synaptic projections to motor neurons in these preparations although as discussed above it enhances motor neuron excitability and can reduce synaptic inhibition via reduced KCC2 at least under some conditions.

SELECTIVITY OF NEUROTROPHIN EXPRESSION

Figure 1 shows that *summing* the separate effects of BDNF and NT-3 on the electrophysiology of motor neurons and their spindle synaptic input gives a result that is in qualitative agreement with the effects of motor training in spinal transected rats (Petruska et al., 2007) where the levels of BDNF and NT-3 are both increased (Ying et al., 2005; Côté et al., 2011; de Leon et al., 2011). This is consistent with the idea that training exerts its effects via changes in neurotrophin expression (Gómez-Pinilla et al., 2001). The increase in both BDNF and NT-3 after motor training differs from what is observed after increase in activity limited to proprioceptive pathways where NT-3 levels are increased but BDNF levels are not (Gajewska-Wozniak et al., 2013). This indicates that locomotor training involves more than altering the stretch reflex pathway despite its enhancement after step training (Petruska et al., 2007), and the beneficial effects of increasing it by operant conditioning (Thompson et al., 2013). These beneficial effects of stretch reflex enhancement may be due to improved weight support which facilitates stepping elicited by independent activation of the CPG.

The pathway- selective increase in neurotrophin levels is also observed for BDNF in that inflammatory pain results in upregulation of BDNF in trkA- expressing nociceptive neurons in the DRG (Pezet and McMahon, 2006) with subsequent release into the superficial dorsal horn of the spinal cord (Lever et al., 2001). Thus activity- driven elevation in neurotrophin levels is circuit-dependent and acts to potentiate the central effect of the activated fibers.

	MN rheobase	MN AHP	Monosynaptic EPSP
↑NT-3	↑	↓	↑
↑BDNF	↓	↔	↔
Training ↑NT-3 and ↑BDNF	↔	↓	↑

FIGURE 1 | Comparison of effects of NT-3, BDNF and step training on electrophysiological properties of motor neurons. Note that training gives results that are qualitatively equivalent to the sum of the separate effects of NT-3 (data row 1) and BDNF (data row 2). The data are derived from Petruska et al. (2007), Petruska et al. (2010) and Boyce et al. (2012b).

NEUROTROPHINS AND REPAIR OF SPINAL CIRCUITS

Investigating a possible role for neurotrophins in the treatment of spinal injuries was prompted by earlier results in the developing spinal cord suggesting that these agents could encourage growth of axons. Thus there are many studies in which bridges of Schwann cells, olfactory ensheathing glia or other substrates were treated with neurotrophins to enhance elongation of cut axons (reviewed in Boyce et al., 2014; Boyce and Mendell, 2014). Additional studies in which neurotrophins were applied either to the severed proximal end of descending fibers in the vicinity of a spinal injury or to the cell body in the brainstem or cerebral cortex revealed their ability to promote the growth of axons descending from more rostral centers with some specificity. For example, BDNF stimulates growth of vestibulospinal fibers whereas NT-3 enhances corticospinal fiber growth although the specificity of action may not be absolute (see reviews in Boyce et al., 2014; Boyce and Mendell, 2014). As expected from the specificity of neurotrophin receptor expression on sensory axons (see Section Neurotrophin Effects on Sensory Neurons and their Spinal Projections), NGF enhances the ability of nociceptive afferent fibers to grow past a crush injury in the dorsal root and terminate in the appropriate area of the superficial dorsal horn whereas after a similar injury NT-3 stimulates growth of proprioceptors into the ventral horn (see review by Smith et al., 2012). At present, there is little information concerning direct effects of neurotrophins on the activity of spinal neurons recorded *in vivo*, in part because any observed activity changes might be an indirect action, e.g., due to activation of sensory fibers. Nonetheless, direct effects on spinal neuron activity would be expected because there are reports of trk receptors on these neurons (Liebl et al., 2001). Furthermore, some of the synaptic effects of exogenously applied neurotrophins in the isolated spinal cord have been attributed to trk receptors on the postsynaptic spinal neurons (Arvanov et al., 2000; Garraway et al., 2003).

Long term intrathecal treatment with neurotrophins can also promote the development of novel functional pathways in the spinal cord, specifically the formation of functional detours around zones of injury both in neonatal (Arvanian et al., 2006) and adult rats (García-Alías et al., 2011; Schnell et al., 2011). In adults the neurotrophin works best if supplemented with agents that overcome the inhibitory effect of the tissue on axonal growth, e.g., anti Nogo or chondroitinase (reviewed in Fawcett et al., 2012), and provision of NMDA subunit NR2D to bring these receptors back to their juvenile state (Arvanian et al., 2004). In these experiments, neurotrophins were introduced intrathecally using either engineered fibroblasts or viruses. The fibroblasts or the cells infected by the AAV viral vectors released the neurotrophin which interacted with the trk or p75- (reviewed in Nicol and Vasko, 2007) expressing cells resident in the CNS. Some success has also been reported in altering connections to motoneurons retrogradely by administering viruses expressing a

neurotrophin to muscle (Fortun et al., 2009; Petruska et al., 2010). The peripheral administration approach might have important translational advantages.

Many investigators are now making efforts to repair the injured spinal cord using cell transplants including stem cells. In evaluating the results of these procedures it is important to keep in mind that these cells constitutively release neurotrophins (Lu et al., 2003), which could affect the function of spinal neurons and circuits in addition potentially promoting axonal elongation.

MALADAPTIVE EFFECTS OF NEUROTROPHINS

Although neurotrophins have numerous potentially useful effects on the injured spinal cord, there are many potential drawbacks. Improving stepping might be negated by increased pain or spasticity both of which have been reported (Boyce et al., 2012b; Fouad et al., 2013; see review by Weishaupt et al., 2012). This may be the result of the multiplicity of actions of neurotrophins, particularly BDNF which elicits activity- based plasticity as well as activating microglia leading to pain. One might question why the same molecule has developed adaptive and maladaptive roles. In evaluating this issue it is important to remember that the different mechanisms are being studied in isolation in preparations that are often so severely injured (e.g., peripheral nerve injury, spinal cord injury) that any evolutionary significance is doubtful.

It remains to be seen whether adjusting the dosage of BDNF will help to balance the positive and negative effects of this treatment so that it might be used translationally. Alternatively, an approach allowing BDNF release to be turned on and off using a regulatable viral system might be possible. Finally, a discrete infusion of BDNF into motor pools via an indwelling catheter might improve the balance between the positive and negative effects of this agent. In support of this approach, Mantilla et al. (2013) recently demonstrated enhanced respiration leading to increased survival of cervically hemisected rats after chronic infusion of BDNF into the phrenic motor neuron pool.

CONCLUSION

It appears, then, that neurotrophins, particularly BDNF, can have numerous actions that affect circuit function in the spinal cord (**Figure 2**). It follows that any attempt to use neurotrophins to promote repair will require targeting them to specific classes of cells, e.g., the CPG but not the nociceptive circuits in the superficial dorsal horn. So far this has not been possible using engineered cells, viral vectors or osmotic minipumps. Alternatively, it may be possible to devise cell based treatments which are easily turned off to control side effects. At present, what is clear is that neurotrophins are powerful molecules that could be very useful in repairing the injured cord. It will be necessary to recognize that they have significant effects on functional properties of cells and circuits as well as on axon elongation and to take this into account when devising protocols for their use.

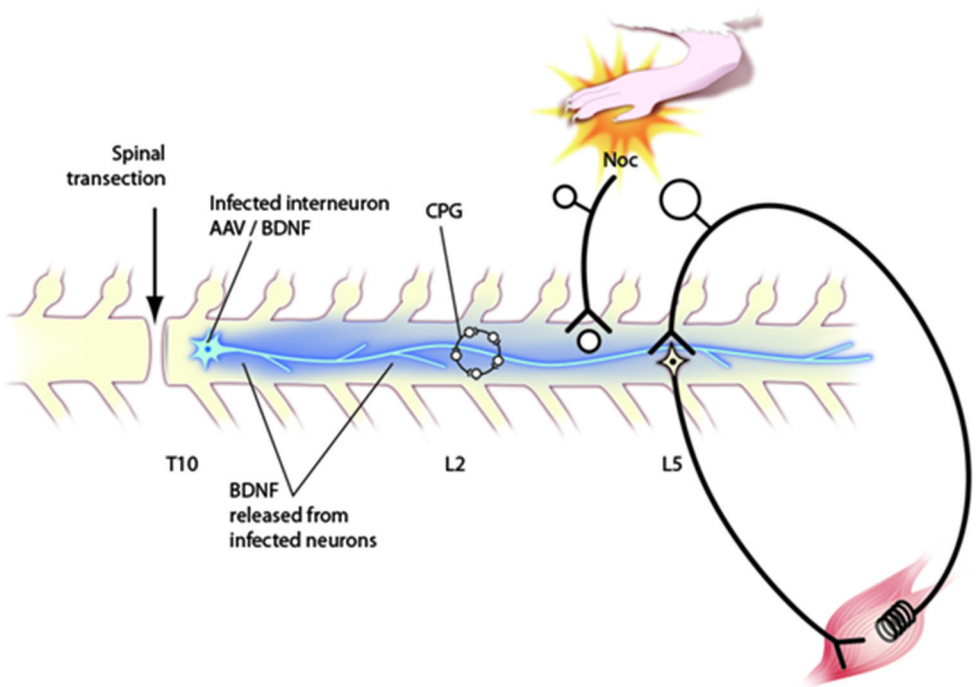

FIGURE 2 | Schematic diagram of circuitry affected by neurotrophins after application via engineered AAV viruses to the transected spinal cord. Behavioral, electrophysiological and immunocytochemical data indicate that such injections affect the CPG in upper lumbar segments, nociceptive processing in the dorsal horn and the stretch reflex pathway. Further discussion of these effects in the text.

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REFERENCES

Arvanian, V. L., Bowers, W. J., Anderson, A. J., Horner, P. J., Federoff, H. J., and Mendell, L. M. (2006). Combined delivery of neurotrophin-3 and NMDA receptors 2D subunit strengthens synaptic transmission in contused and staggered double hemisected spinal cord of neonatal rat. *Exp. Neurol.* 197, 347–352. doi: 10.1016/j.expneurol.2005.10.008

Arvanian, V. L., Bowers, W. J., Petruska, J. C., Manuzon, H., Narrow, W. C., Motin, V., et al. (2004). Viral delivery of NR2D subunits reduces Mg^{2+} block of NMDA receptor and restores NT-3-induced potentiation of AMPA/kainate responses in maturing rat motor neurons. *J. Neurophysiol.* 92, 2394–2404. doi: 10.1152/jn.00278.2004

Arvanian, V. L., Horner, P. J., Gage, F. H., and Mendell, L. M. (2003). Chronic neurotrophin-3 strengthens synaptic connections to motor neurons in the neonatal rat. *J. Neurosci.* 23, 8706–8712.

Arvanian, V. L., and Mendell, L. M. (2001a). Removal of NMDA receptor Mg^{2+} block extends the action of neurotrophin-3 on synaptic transmission in neonatal rat motor neurons. *J. Neurophysiol.* 86, 123–129.

Arvanian, V. L., and Mendell, L. M. (2001b). Acute modulation of synaptic transmission to motor neurons by BDNF in the neonatal rat spinal cord. *Eur. J. Neurosci.* 14, 1800–1808. doi: 10.1046/j.0953-816x.2001.01811.x

Arvanov, V. L., Seebach, B. S., and Mendell, L. M. (2000). NT-3 evokes an LTP- like facilitation of AMPA/Kainate- mediated synaptic transmission in the neonatal rat spinal cord. *J. Neurophysiol.* 84, 752–758.

Blits, B., Oudega, M., Boer, G. J., Bartlett Bunge, M., and Verhaagen, J. (2003). Adeno-associated viral vector-mediated neurotrophin gene transfer in the injured adult rat spinal cord improves hind-limb function. *Neuroscience* 118, 271–281. doi: 10.1016/s0306-4522(02)00970-3

Boulenguez, P., Liabeuf, S., Bos, R., Bras, H., Jean-Xavier, C., Brocard, C., et al. (2010). Down-regulation of the potassium-chloride cotransporter KCC2 contributes to spasticity after spinal cord injury. *Nat. Med.* 16, 302–307. doi: 10.1038/nm.2107

Boyce, V. S., Klees, L. M., Kim, B., and Mendell, L. M. (2012a). BDNF induced motor recovery: toward an understanding of its mechanism of action. 2012 Neuroscience Meeting Planner, Society for Neuroscience New Orleans, LA.

Boyce, V. S., Levine, J. M., and Mendell, L. M. (2014). "Neurotrophin repair of spinal cord damage," in *Textbook of Neural Repair and Rehabilitation* (Vol. 1), eds M. E. Selzer, S. Clarke, L. G. Cohen, G. Kwakkel and R. H. Miller, 2nd Edn. (Cambridge, UK: Cambridge University Press), 400–413.

Boyce, V. S., and Mendell, L. M. (2014). Neurotrophic factors in spinal cord injury. *Handb. Exp. Pharmacol.* 220, 443–460. doi: 10.1007/978-3-642-45106-5_16

Boyce, V. S., Park, J., Gage, F. H., and Mendell, L. M. (2012b). Differential effects of BDNF and NT-3 on hindlimb function in paraplegic rats. *Eur. J. Neurosci.* 35, 221–232. doi: 10.1111/j.1460-9568.2011.07950.x

Boyce, V. S., Tumolo, M., Fischer, I., Murray, M., and Lemay, M. A. (2007). Neurotrophic factors promote and enhance locomotor recovery in untrained spinalized cats. *J. Neurophysiol.* 98, 1988–1996. doi: 10.1152/jn.00861.2007

Carroll, P., Lewin, G. R., Koltzenburg, M., Toyka, K. V., and Thoenen, H. (1998). A role for BDNF in mechanosensation. *Nat. Neurosci.* 1, 42–46.

Chao, M. V., and Hempstead, B. L. (1995). p75 and Trk: a two-receptor system. *Trends Neurosci.* 18, 321–326. doi: 10.1016/0166-2236(95)93922-k

Chen, H. H., Hippenmeyer, S., Arber, S., and Frank, E. (2003). Development of the monosynaptic stretch reflex circuit. *Curr. Opin. Neurobiol.* 13, 96–102. doi: 10.1016/s0959-4388(03)00006-0

Côté, M. P., Azzam, G. A., Lemay, M. A., Zhukareva, V., and Houle, J. D. (2011). Activity-dependent increase in neurotrophic factors is associated with an enhanced modulation of spinal reflexes after spinal cord injury. *J. Neurotrauma* 28, 299–309. doi: 10.1089/neu.2010.1594

Coull, J. A., Beggs, S., Boudreau, D., Boivin, D., Tsuda, M., Inoue, K., et al. (2005). BDNF from microglia causes the shift in neuronal anion gradient underlying neuropathic pain. *Nature* 438, 1017–1021. doi: 10.1038/nature04223

Cowan, W. M. (2001). Viktor Hamburger and Rita Levi-Montalcini: the path to the discovery of nerve growth factor. *Ann. Rev. Neurosci.* 24, 551–600. doi: 10.1146/annurev.neuro.24.1.551

de Leon, R. D., See, P. A., and Chow, C. H. (2011). Differential effects of low versus high amounts of weight supported treadmill training in spinally transected rats. *J. Neurotrauma* 28, 1021–1033. doi: 10.1089/neu.2010.1699

Erickson, J. T., Conover, J. C., Borday, V., Champagnat, J., Barbacid, M., Yancopoulos, G., et al. (1996). Mice lacking brain-derived neurotrophic factor exhibit visceral sensory neuron losses distinct from mice lacking NT4 and display a severe developmental deficit in control of breathing. *J. Neurosci.* 16, 5361–5371.

Fawcett, J. W., Schwab, M. E., Montani, L., Brazda, N., and Müller, H. W. (2012). Defeating inhibition of regeneration by scar and myelin components. *Handb. Clin. Neurol.* 109, 503–522. doi: 10.1016/B978-0-444-52137-8.00031-0

Fortun, J., Puzis, R., Pearse, D. D., Gage, F. H., and Bunge, M. B. (2009). Muscle injection of AAV-NT-3 promotes anatomical reorganization of CST axons and improves behavioral outcome following SCI. *J. Neurotrauma* 26, 941–953. doi: 10.1089/neu.2008-0807

Fouad, K., Bennett, D. J., Vavrek, R., and Blesch, A. (2013). Long-term viral brain-derived neurotrophic factor delivery promotes spasticity in rats with a cervical spinal cord hemisection. *Front. Neurol.* 4:187. doi: 10.3389/fneur.2013.00187

Gajewska-Wozniak, O., Skup, M., Kasicki, S., Ziemlinska, E., and Czarkowska-Bauch, J. (2013). Enhancing proprioceptive input to motor neurons differentially affects expression of neurotrophin 3 and brain-derived neurotrophic factor in rat hoffmann-reflex circuitry. *PLoS One* 8:e65937. doi: 10.1371/journal.pone.0065937

Galoyan, S. M., Petruska, J., and Mendell, L. M. (2003). Mechanisms of sensitization of the response of single dorsal root ganglion cells from adult rat to noxious heat. *Eur. J. Neurosci.* 18, 535–541. doi: 10.1046/j.1460-9568.2003.02775.x

García-Alías, G., Petrosyan, H., Schnell, H., Horner, P. J., Bowers, W. J., Mendell, L. M., et al. (2011). Chondroitinase ABC combined with NT-3 secretion and NR2D expression promotes axonal plasticity and functional recovery in rats with lateral hemisection of the spinal cord. *J. Neurosci.* 31, 17788–17799. doi: 10.1523/JNEUROSCI.4308-11.2011

Garraway, S. M., Petruska, J. C., and Mendell, L. M. (2003). BDNF sensitizes the response of lamina II neurons to high threshold primary afferent inputs. *Eur. J. Neurosci.* 18, 2467–2476. doi: 10.1046/j.1460-9568.2003.02982.x

Garraway, S. M., Turtle, J. D., Huie, J. R., Lee, K. H., Hook, M. A., Woller, S. A., et al. (2011). Intermittent noxious stimulation following spinal cord contusion injury impairs locomotor recovery and reduces spinal brain-derived neurotrophic factor-tropomyosin-receptor kinase signaling in adult rats. *Neuroscience* 199, 86–102. doi: 10.1016/j.neuroscience.2011.10.007

Gómez-Pinilla, F., Ying, Z., Opazo, P., Roy, R. R., and Edgerton, V. R. (2001). Differential regulation by exercise of BDNF and NT-3 in rat spinal cord and skeletal muscle. *Eur. J. Neurosci.* 13, 1078–1084. doi: 10.1046/j.0953-816x.2001.01484.x

Gonzalez, M., and Collins, W. F. III. (1997). Modulation of motorneuron excitability by brain-derived neurotrophic factor. *J. Neurophysiol.* 77, 502–506.

Goulding, M. C. (2009). Circuits controlling vertebrate locomotion: moving in a new direction. *Nat. Rev. Neurosci.* 10, 507–518. doi: 10.1038/nrn2608

Hilaire, C., Lucas, O., Valmier, J., and Scamps, F. (2012). Neurotrophin-4 modulates the mechanotransducer Cav3.2 T-type calcium current in mice down-hair neurons. *Biochem. J.* 441, 463–471. doi: 10.1042/bj20111147

Huang, E. J., and Reichardt, L. F. (2001). Neurotrophins: roles in neuronal development and function. *Ann. Rev. Neurosci.* 24, 677–736. doi: 10.1146/annurev.neuro.24.1.677

Jakeman, L. B., Wei, P., Guan, Z., and Stokes, B. T. (1998). Brain-derived neurotrophic factor stimulates hindlimb stepping and sprouting of cholinergic fibers after spinal cord injury. *Exp. Neurol.* 154, 170–184. doi: 10.1006/exnr.1998.6924

Jankowska, E., Hammar, I., Chojnicka, B., and Hedén, C. H. (2000). Effects of monoamines on interneurons in four spinal reflex pathways from group I and/or group II muscle afferents. *Eur. J. Neurosci.* 12, 701–714. doi: 10.1046/j.1460-9568.2000.00955.x

Lehmann, H. C., and Höke, A. (2010). Schwann cells as a therapeutic target for peripheral neuropathies. *CNS Neurol. Disord. Drug Targets* 9, 801–806. doi: 10.2174/187152710793237412

Lever, I. J., Bradbury, E. J., Cunningham, J. R., Adelson, D. W., Jones, M. G., McMahon, S. B., et al. (2001). Brain- derived neurotrophic factor is released in the dorsal horn by distinctive patterns of afferent fiber stimulation. *J. Neurosci.* 21, 4469–4477.

Lewin, G. R., Ritter, A. M., and Mendell, L. M. (1992). On the role of Nerve Growth Factor in the development of myelinated nociceptors. *J. Neurosci.* 12, 1896–1905.

Lewin, G. R., Rueff, A., and Mendell, L. M. (1994). Peripheral and central mechanisms of NGF-induced hyperalgesia. *Eur. J. Neurosci.* 6, 1903–1912. doi: 10.1111/j.1460-9568.1994.tb00581.x

Liebl, D. J., Huang, W., Young, W., and Parada, L. F. (2001). Regulation of Trk receptors following contusion of the rat spinal cord. *Exp. Neurol.* 167, 15–26. doi: 10.1006/exnr.2000.7548

Lu, P., Jones, L. L., Snyder, E. Y., and Tuszynski, M. H. (2003). Neural stem cells constitutively secrete neurotrophic factors and promote extensive host axonal growth after spinal cord injury. *Exp. Neurol.* 181, 115–129. doi: 10.1016/s0014-4886(03)00037-2

Mantilla, C. B., Gransee, H. M., Zhan, W. Z., and Sieck, G. C. (2013). Motor neuron BDNF/TrkB signaling enhances functional recovery after cervical spinal cord injury. *Exp. Neurol.* 247, 101–109. doi: 10.1016/j.expneurol.2013.04.002

Mendell, L. M. (1995). Neurotrophic factors and the specification of neural function. *Neuroscientist* 1, 26–34. doi: 10.1177/107385849500100105

Mendell, L. M., Albers, K. M., and Davis, B. M. (1999b). Neurotrophins, nociceptors and pain. *Microsc. Res. Tech.* 45, 252–261. doi: 10.1002/(sici)1097-0029(19990515/01)45:4/5<252::aid-jemt9>3.0.co;2-n

Mendell, L. M., and Arvanian, V. L. (2002). Diversity of neurotrophin action in the postnatal spinal cord. *Brain Res. Brain Res. Rev.* 40, 230–239. doi: 10.1016/s0165-0173(02)00205-9

Mendell, L. M., Johnson, R. D., and Munson, J. B. (1999). Neurotrophin modulation of the monosynaptic reflex after peripheral nerve transection. *J. Neurosci.* 19, 3162–3170.

Munson, J. B., Johnson, R. D., and Mendell, L. M. (1999). Neurotrophin-3 and maintenance of muscle afferent function. *Prog. Brain Res.* 123, 157–163. doi: 10.1016/s0079-6123(08)62853-8

Nicol, G. D., and Vasko, M. R. (2007). Unraveling the story of NGF-mediated sensitization of nociceptive sensory neurons: ON or OFF the Trks? *Mol. Interv.* 7, 26–41. doi: 10.1124/mi.7.1.6

Paul, J., Gottmann, K., and Lessmann, V. (2001). NT-3 regulates BDNF-induced modulation of synaptic transmission in cultured hippocampal neurons. *Neuroreport* 12, 2635–2639. doi: 10.1097/00001756-200108280-00010

Petruska, J. C., and Mendell, L. M. (2009). "Nerve growth factor," in *Encyclopedia of Neuroscience* (Vol. 6), ed L. R. Squire (Oxford: Academic Press), 71–78.

Petruska, J. C., Ichiyama, R. M., Crown, E. D., Tansey, K. E., Roy, R. R., Edgerton, V. R., et al. (2007). Changes in motor neuron properties and synaptic inputs related to step training following spinal cord transection in rats. *J. Neurosci.* 27, 4460–4471. doi: 10.1523/jneurosci.2302-06.2007

Petruska, J. C., Kitay, B., Boyce, V. S., Kaspar, B. K., Pearse, D. D., Gage, F. H., et al. (2010). Intramuscular AAV delivery of NT-3 alters synaptic transmission to motor neurons in adult rats. *Eur. J. Neurosci.* 32, 997–1005. doi: 10.1111/j.1460-9568.2010.07392.x

Pezet, S., and McMahon, S. B. (2006). Neurotrophins: mediators and modulators of pain. *Annu. Rev. Neurosci.* 29, 507–538. doi: 10.1146/annurev.neuro.29.051605.112929

Rueff, A., and Mendell, L. M. (1996). Nerve growth factor and NT–5 induce increased thermal sensitivity of cutaneous nociceptors in vitro. *J. Neurophysiol.* 76, 3593–3596.

Schnell, L., Hunanyan, A., Bowers, W., Horner, P., Federoff, H., Gullo, M., et al. (2011). Combined delivery of Nogo-A antibody, neurotrophin-3 and NMDA-2D subunits establishes a functional "detour" in a hemisected spinal cord. *Eur. J. Neurosci.* 34, 1256–1267. doi: 10.1111/j.1460-9568.2011.07862.x

Seebach, B. S., Arvanov, V., and Mendell, L. M. (1999). Neurotrophin influence on the development of segmental reflexes in the rat. *J. Neurophysiol.* 81, 2398–2405.

Shu, X., and Mendell, L. M. (1999a). Nerve growth factor acutely sensitizes the response of adult rat sensory neurons to capsaicin. *Neurosci. Lett.* 274, 159–162. doi: 10.1016/s0304-3940(99)00701-6

Shu, X., and Mendell, L. M. (1999b). Neurotrophins and hyperalgesia. *Proc. Natl. Acad. Sci. U S A* 96(Suppl.), 7693–7696. doi: 10.1073/pnas.96.14.7693

Shu, X., Llinas, A., and Mendell, L. M. (1999). Effects of trkB and trkC neurotrophin receptor agonists on thermal nociception: a behavioural and electrophysiological study. *Pain* 80, 463–470. doi: 10.1016/s0304-3959(99)00042-1

Smith, G. M., Falone, A. E., and Frank, E. (2012). Sensory axon regeneration: rebuilding functional connections in the spinal cord. *Trends Neurosci.* 35, 156–163. doi: 10.1016/j.tins.2011.10.006

Stein, A. T., Ufret-Vincenty, C. A., Hua, L., Santana, L. F., and Gordon, S. E. (2006). Phosphoinositide 3-kinase binds to TRPV1 and mediates NGF-stimulated TRPV1 trafficking to the plasma membrane. *J. Gen. Physiol.* 128, 509–522. doi: 10.1085/jgp.200609576

Thompson, A. K., Pomerantz, F. R., and Wolpaw, J. R. (2013). Operant conditioning of a spinal reflex can improve locomotion after spinal cord injury in humans. *J. Neurosci.* 33, 2365–2375. doi: 10.1523/JNEUROSCI.3968-12.2013

Tsuda, M., Shigemoto-Mogami, Y., Koizumi, S., Mizokoshi, A., Kohsaka, S., Salter, M. W., et al. (2003). P2X4 receptors induced in spinal microglia gate tactile allodynia after nerve injury. *Nature* 424, 778–783. doi: 10.1038/nature01786

Weishaupt, N., Blesch, A., and Fouad, K. (2012). BDNF: the career of a multifaceted neurotrophin in spinal cord injury. *Exp. Neurol.* 238, 254–264. doi: 10.1016/j.expneurol.2012.09.001

Wu, J., Renn, C. L., Faden, A. I., and Dorsey, S. G. (2013). TrkB.T1 contributes to neuropathis pain after spinal cord injury through regulation of cell cycle pathways. *J. Neurosci.* 33, 12447–12463. doi: 10.1523/NEUROSCI.0486-13.2013

Ying, Z., Roy, R. R., Edgerton, V. R., and Gómez-Pinilla, F. (2005). Exercise restores levels of neurotrophins and synaptic plasticity following spinal cord injury. *Exp. Neurol.* 193, 411–419. doi: 10.1016/j.expneurol.2005.01.015

Zhang, X., Huang, J., and McNaughton, P. A. (2005). NGF rapidly increases membrane expression of TRPV1 heat-gated ion channels. *EMBO J.* 24, 4211–4223. doi: 10.1038/sj.emboj.7600893

Zhu, W., and Oxford, G. S. (2011). Differential gene expression of neonatal and adult DRG neurons correlates with the differential sensitization of TRPV1 responses to nerve growth factor. *Neurosci. Lett.* 500, 192–196. doi: 10.1016/j.neulet.2011.06.034

Zhu, W., Galoyan, S. M., Petruska, J. C., Oxford, G. S., and Mendell, L. M. (2004). A developmental switch in acute sensitization of small dorsal root ganglion (DRG) neurons to capsaicin or noxious heating by NGF. *J. Neurophysiol.* 92, 3148–3152. doi: 10.1152/jn.00356.2004

Ziemlinska, E., Kügler, S., Schachner, M., Wewiór, I., Czarkowska-Bauch, J., and Skup, M. (2014). Overexpression of BDNF increases excitability of the lumbar spinal network and leads to robust early locomotor recovery in completely spinalized rats. *PLoS One* 9:e88833. doi: 10.1371/journal.pone.0088833

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Dopamine D3 receptor dysfunction prevents anti-nociceptive effects of morphine in the spinal cord

Kori L. Brewer[1]*†, Christine A. Baran[1], Brian R. Whitfield[1], A. Marley Jensen[2] and Stefan Clemens[2†]

[1] Department of Emergency Medicine, Brody School of Medicine, East Carolina University, Greenville, NC, USA
[2] Department of Physiology, Brody School of Medicine, East Carolina University, Greenville, NC, USA

Edited by:
Brian R. Noga, University of Miami, USA

Reviewed by:
Deborah Baro, Georgia State University, USA
Florian Klinker, University Medical Centre, Göttingen, Germany

***Correspondence:**
Kori L. Brewer, Department of Emergency Medicine, Brody School of Medicine, East Carolina University, 600 Moye Blvd., Mail Stop #625, Greenville, NC 27834, USA
e-mail: brewerk@ecu.edu

† Co-senior authors.

Dopamine (DA) modulates spinal reflexes, including nociceptive reflexes, in part via the D3 receptor subtype. We have previously shown that mice lacking the functional D3 receptor (D3KO) exhibit decreased paw withdrawal latencies from painful thermal stimuli. Altering the DA system in the CNS, including D1 and D3 receptor systems, reduces the ability of opioids to provide analgesia. Here, we tested if the increased pain sensitivity in D3KO might result from a modified μ-opioid receptor (MOR) function at the spinal cord level. As D1 and D3 receptor subtypes have competing cellular effects and can form heterodimers, we tested if the changes in MOR function may be mediated in D3KO through the functionally intact D1 receptor system. We assessed thermal paw withdrawal latencies in D3KO and wild type (WT) mice before and after systemic treatment with morphine, determined MOR and phosphorylated MOR (p-MOR) protein expression levels in lumbar spinal cords, and tested the functional effects of DA and MOR receptor agonists in the isolated spinal cord. *In vivo*, a single morphine administration (2 mg/kg) increased withdrawal latencies in WT but not D3KO, and these differential effects were mimicked *in vitro*, where morphine modulated spinal reflex amplitudes (SRAs) in WT but not D3KO. Total MOR protein expression levels were similar between WT and D3KO, but the ratio of pMOR/total MOR was higher in D3KO. Blocking D3 receptors in the isolated WT cord precluded morphine's inhibitory effects observed under control conditions. Lastly, we observed an increase in D1 receptor protein expression in the lumbar spinal cord of D3KO. Our data suggest that the D3 receptor modulates the MOR system in the spinal cord, and that a dysfunction of the D3 receptor can induce a morphine-resistant state. We propose that the D3KO mouse may serve as a model to study the onset of morphine resistance at the spinal cord level, the primary processing site of the nociceptive pathway.

Keywords: dopamine d3 receptor, mu-opiod receptor, nociception, second messenger cross-talk, dopamine d1 recptor, spinal reflexes

INTRODUCTION

Opiate analgesics are the classical first line treatment for strong and persistent pain, but their effectiveness in long-term treatment is limited by the emergence of tolerance (Colpaert, 2002; Dupen et al., 2007; Bekhit, 2010; Joseph et al., 2010). This morphine-resistant condition is thought to arise over time and involve a dysfunction of μ-opioid receptor (MOR)- and dopamine (DA)-receptor mediated cAMP/PKA second messenger pathways in the brain (Suzuki et al., 2001; Schmidt et al., 2002; Fazli-Tabaei et al., 2006; Zhang et al., 2008, 2012; Le Marec et al., 2011; Enoksson et al., 2012). However, under this tenet the role of the spinal cord remains overlooked. The spinal cord is the first site for the processing for nociceptive information, and it houses both DA and MOR receptors in the dorsal horn (Abbadie et al., 2001; Levant and McCarson, 2001; Ray and Wadhwa, 2004; Zhu et al., 2007, 2008). DA regulates spinal cord circuits, including pain-associated responses (Garraway and Hochman, 2001; Clemens and Hochman, 2004; Yang et al., 2005; Keeler et al., 2012), and both D1 and D3 receptors are present in the dorsal horn (Levant and McCarson, 2001; Zhu et al., 2007). Further, the MOR is associated with inhibitory G proteins (G_i and G_o) and is present in the spinal cord (Mansour et al., 1994; Ji et al., 1995; Ray and Wadhwa, 1999, 2004; Abbadie et al., 2001, 2002; Zhang et al., 2006). Upon ligand binding to the receptor, signaling cascades are activated that inhibit cAMP production, resulting in the opening of K^+ channels or closing of Ca^{2+} channels (Connor and Christie, 1999; Connor et al., 1999; Williams et al., 2001). Its desensitization is under the control of G-protein-coupled receptor kinase (GRK)-mediated phosphorylation (Connor et al., 2004; Gainetdinov et al., 2004; Garzon et al., 2005), followed by ß-arrestin-mediated internalization (Bohn et al., 1999, 2000; Ohsawa et al., 2003; Connor et al., 2004). Blocking ß-arrestin expression improves morphine-mediated analgesia (Li et al., 2009), and recent data suggest that morphine-induced ß-arrestin complex formation primarily requires D1 receptors (Urs et al., 2011).

Interestingly, D1 receptors form heterodimers with D3 receptors (Surmeier et al., 1996; Fiorentini et al., 2008; Maggio et al., 2009; Missale et al., 2010; Cruz-Trujillo et al., 2013) and both

play a role in opioid tolerance (Lin et al., 1996; Cook et al., 2000; Richtand et al., 2000; Fazli-Tabaei et al., 2006). We recently found that animals lacking a functional D3 receptor (D3KO) exhibited facilitated pain-associated reflexes (Keeler et al., 2012), and we address here the role of the spinal D3 receptor on MOR functional states and morphine responsiveness.

We evaluated thermal pain paw withdrawal latencies in D3KO and wild type (WT) controls before and after administration of morphine, determined naïve MOR and phosphorylated MOR (p-MOR) protein expression levels in the lumbar spinal cord, and compared spinal reflex amplitudes (SRA) in isolated spinal cords from WT and D3KO *in vitro*, to assess the role of the D3 receptor in mediating morphine actions in isolated WT spinal cords.

We found that naïve D3KO were unresponsive to morphine administration, both *in vivo* and *in vitro*, and that this lack of responsiveness was associated with increased levels of spinal pMOR in D3KO. Moreover, while the highly-specific MOR agonist, DAMGO, was able to decrease SRAs in both WT and D3KO *in vitro*, this effect was significantly smaller in D3KO than WT. Finally, acute pharmacological block of the D3 receptor in the isolated WT cord was sufficient to induce a D3KO-like phenotype and prevent the modulatory actions of morphine on SRAs observed under control conditions.

MATERIALS AND METHODS

All experimental procedures complied with NIH guidelines for animal care and were approved by the East Carolina University Institutional Animal Care and Use Committee. Male dopamine D3 receptor knockout mice (D3KO; strain B6.129S4-*Drd3*^{tm1dac}*/J;* stock # 002958, Jackson Laboratory, Bar Harbor, ME) and their appropriate associated wild-type (WT) controls (C57BL/6) were used for *in vivo* behavioral testing and Western blot experiments (3–4 months), while neonatal pups of either sex were used for extracellular electrophysiology and pharmacology (postnatal days 7–14).

BEHAVIORAL ASSESSMENTS

Thermal withdrawal latencies were tested on 10 D3KO and 8 WT males using a Hargreaves apparatus. Prior to baseline testing, mice were acclimated to the apparatus by being placed in the apparatus for 2 h per day for a total of 5 days. Baseline thresholds were obtained over three test sessions, with each test session occurring at the same time of day, and each mouse being placed in the chamber to which it was acclimated. Each test session consisted of three trials (i.e., application of the heat stimulus at 56°C) separated by at least 5 min. The latencies obtained over the 3 trials were averaged to get the mean baseline latency for each test day. After baselines were established, a randomly chosen subset of mice (5 D3KO and 4 WT) was administered morphine sulfate (Sigma, 2 mg/kg, i.p.) and tested 30 min later as outlined above.

TISSUE COLLECTION AND WESTERN BLOT ANALYSIS

One week after behavioral testing, animals were deeply anesthetized with inhaled isoflurane (4–5%), decapitated, and spinal cords were dissected, frozen in liquid nitrogen and stored at -80°C. Proteins were homogenized in RIPA buffer containing protease and phosphatase inhibitors (Sigma-Aldrich, St. Lois, MO), lysates centrifuged, and the supernatant collected. Total protein was quantified using an EZQ Quantitation Kit (R33200; Invitrogen, Grand Island, NY). Equal concentrations of protein were separated using a SDS-PAGE (Criterion TGX Any kD, Bio-Rad, Canton, MA) and transferred onto a PVDF membrane (Immobolin-P, Millipore, Germany). To verify consistent protein transfer across the lanes, we measured total protein staining of the membrane with Coomassie Blue, and compared the protein staining with the β-actin expression in the corresponding lanes. We did not observe any significant difference in β-actin protein expression between WT and D3KO ($p = 0.54$ and 0.2, respectively). For MOR and pMOR protein expression assessments, membranes were blocked using 5% BSA overnight and probed with primary antibodies for the MOR (ab10275, Abcam, UK) at 1:1000 and p-MOR (bs3724R, BIOSS, UK) at 1:1000 overnight. This MOR antibody can detect bands at ∼50 kDa (Kerros et al., 2010) and has been verified in a different study by comparing it to the effects of another MOR antibody (Loyd et al., 2008). The pMOR antibody we used shows a strong band at ∼44 kDa (http://biossusa.com/store/bs-3724r.html), and control ELISA data provided by BIOSS show an increased binding of this antibody to pMOR over MOR (data not shown, BIOSS, personal communication). Membranes were washed and incubated in anti-mouse and anti-rabbit secondary antibodies (R&D Systems, Minneapolis, MN) respectively. Target proteins were visualized using ECL Plus detection reagent (80916; Invitrogen, Grand Island, NY) according to the manufacturer's recommendations, quantified using relative integrated density normalized against β-actin (ab8226, Abcam, UK) at 1:2000, and analyzed with ImageJ software (version 1.48S, NIH). Expression values are given as the ratio of MOR or pMOR to β-actin.

For D1 receptor protein assessments, membranes were blocked overnight using 5% BSA at 4°C and probed with primary antibodies for the D1 receptor (ab81296, Abcam, Cambridge, MA) and β-actin (ab8226, Abcam, UK) at 1:1000 overnight. This D1 receptor antibody can detect bands at both 50 and 75 kDa (Mizuta et al., 2013). Membranes were washed four times at 5 min in TBS-T and incubated for 30 min at room temperature using 5% BSA in anti-rabbit IR800 (35571, Thermo Scientific, Rockford, IL) and anti-mouse IR680 (926-68070, Li-Cor, Lincoln, NE) secondary antibodies at 1:30000. The membranes were then washed another three times for 5 min in TBS-T followed by two washes at 5 min in PBS. Target proteins were visualized using the Li-Cor detection system (Odyssey Clx, Li-Cor Biosciences, Lincoln, NE) and associated software (Image Studio, Li-Cor), and quantified with ImageJ. D1 receptor protein expression values were normalized to β-actin protein expression.

ELECTROPHYSIOLOGY AND PHARMACOLOGY

A total of 19 WT and 17 D3KO neonatal pups of either sex (postnatal days 7–14) were used for extracellular electrophysiological and pharmacological experiments. As reported earlier (Clemens and Hochman, 2004; Keeler et al., 2012), animals were deeply anesthetized with i.p. injection (50 μl/10 g) of a ketamine (90 mg/ml)/xylazine (10 mg/ml) mix, and after verification of deep anesthesia decapitated. Spinal cords were removed quickly, usually completed within 10 min, placed in a Sylgard-lined Petri

dish, the Dura mater desheathed and the cords hemisected. Throughout this process the preparations were submersed in oxygenated (95 O_2/5% CO_2) ice-cold high-sucrose artificial cerebrospinal fluid (containing in mM: 342 sucrose, 180.2 Glucose, 203.3 $MgCl_2$, 147.02 $CaCl_2$, 137.99 NaH_2PO_4, 84.01 $NaHCO_3$, 74.56 KCl, pH 7.4). After these initial dissection steps, the high-sucrose solution replaced with oxygenated artificial cerebrospinal fluid (ACSF; containing in mM: 128 NaCl, 1.9 KCl, 10 Glucose, 1.3 $MgSO_4$, 2.4 $CaCl_2$, 1.2 KH_2PO_4, 26 $NaHCO_3$, pH 7.4), acclimated to room temperature. Subsequently, small glass suction electrodes were carefully attached to identified dorsal and ventral lumbar roots (usually L2-L5).

After a recovery of phase of ∼30–60 min, reflex responses were elicited with a constant current stimulator (Iso-Stim 01D, NPI Electronics, Tamm, Germany, or a custom-built Linear Isolation Unit, Model MI 401, Department of Animal Physiology, University of Cologne) with pulses of 100–500 μA for 100–250 μs. Signals were recorded and amplified with a 4-channel differential AC amplifier (Model 1700, A-M Systems, Sequim, WA), digitized with a Digidata 1440A, and analyzed with pClamp v.10.2 software (Molecular Devices, Sunnyvale, CA). Single pulse stimulations were delivered every 60 s (WT) or 120–180 s (D3KO) to avoid habituation (data not shown). Reflex responses were recorded and analyzed by rectified integration of the recorded responses. SRAs were obtained before (control conditions) and during application of morphine sulfate (Sigma, 1 μM), the D3 receptor-preferring antagonist, nafadotride (Tocris, 20 μM), or the specific MOR agonist DAMGO (Tocris, 10 μM). For testing the interactions between the D3 and MOR in the WT spinal cord, we first bath-applied the D3-receptor preferring antagonist, nafadotride, to establish the effects of this drug, before adding morphine to the bath. Drugs were bath-applied and for 30–60 min, during which time the stimulus protocol was maintained. Following the drug application testing, drugs were carefully washed out (through ACSF exchange of 4–5 times the bath volume). For analytical purposes, we compared the last 10 consecutive SRAs in the control condition with the last 10 SRAs during the drug application.

STATISTICAL ANALYSIS

For behavioral experiments, baseline and post-morphine thermal thresholds were compared between WT and D3KO mice using a RM ANOVA (SigmaPlot, Systat, San Jose, CA) with $p < 0.05$ indicating significance. For determining differences in MOR and p-MOR protein expression levels between groups, we used using a paired *t*-test (JMP v.10, SAS, Inc., Cary NC) with $p < 0.05$ indicating significance. For electrophysiological experiments, SRAs under drug conditions were averaged and normalized to the mean of the pre-drug values, and statistical significance was determined with *t*-tests or ANOVA and subsequent *post-hoc* comparisons, as appropriate, and with α set <0.05 (SigmaPlot, Systat, San Jose, CA).

RESULTS

BEHAVIOR

Under baseline conditions, pain withdrawal latencies of WT were significantly higher than those of D3KO (WT: 6.24 ± 0.64, $n = 4$; D3KO: 4.84 ± 1.08 s, $p < 0.001$, $n = 5$, *t*-test, **Figure 1**, left panel), suggesting an increased excitability to thermal stimulation of the hindpaw in the D3KO animals. A single injection of morphine (2 mg/kg, i.p.) significantly increased thermal withdrawal latencies in WT from 6.24 ± 0.64 s to 11.01 ± 1.91 s ($p < 0.001$, **Figure 1**, right panel), whereas they remained unaltered in D3KO (4.84 ± 1.08 s, vs. 3.94 ± 0.95, $p = 0.902$, RM-ANOVA). Consequently, the difference in pain withdrawal latencies between morphine-treated WT and D3KO remained significant ($p < 0.001$, *t*-test). These data suggest that under naïve conditions D3KO are unresponsive to the system-wide application of morphine.

MOR PROTEIN EXPRESSION IN THE LUMBAR CORD

To test if changes in MOR levels or phosphorylation status might underlie the lack of the opioid effect observed in D3KO, we assessed MOR and pMOR protein expression levels in lumbar spinal cords of WT and D3KO (**Figure 2**). We probed lumbar spinal cords of 4 naïve (untreated) WT and 4 D3KO for MOR (**Figure 2A**) and pMOR protein expression (**Figure 2B**). In both experiments, we first probed for the respective receptor protein expression before stripping the blot and then probing for ß-actin. We found that, after normalization to ß-actin, WT and D3KO had similar levels of total MOR (WT: 1.08 ± 0.32 a.u.; D3KO: 1.146 ± 0.08 a.u., $p = 0.85$, $n = 4$; **Figure 2C**, left panels). In contrast, pMOR protein expression levels of pMOR in D3KO were significantly elevated over WT (WT: 0.127 ± 0.03 a.u., D3KO: 0.992 ± 0.13 a.u., $p < 0.001$; **Figure 2C**, $p < 0.01$, right panels). As overall MOR but not pMOR expression levels were similar, and both sample groups passed Normality (Shapiro-Wilk; $p = 0.441$ and $p = 0.509$, respectively) and Equal Variance Tests ($p = 0.085$ and $p = 0.505$, respectively), we next calculated the ratio of pMOR to MOR expression levels (**Figure 2D**). We found that in WT 36.7 ± 10.6% of total MOR was phosphorylated,

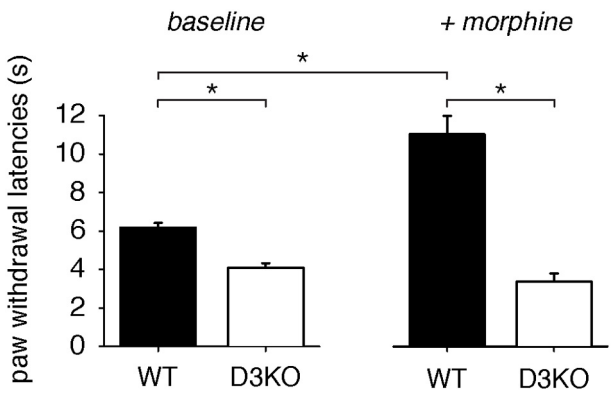

FIGURE 1 | Thermal pain withdrawal latencies at baseline and after a single i.p. application of 2 mg/kg morphine. Left panel: Baseline data for WT ($n = 8$) and D3KO ($n = 10$). Under control conditions, D3KO displayed a significantly lower time to withdrawal than WT. **Right panel:** D3KO ($n = 5$) continued to maintain a lower withdrawal time than WT ($n = 4$), but morphine significantly increased latencies in WT but not D3KO. ∗ indicates $p < 0.001$.

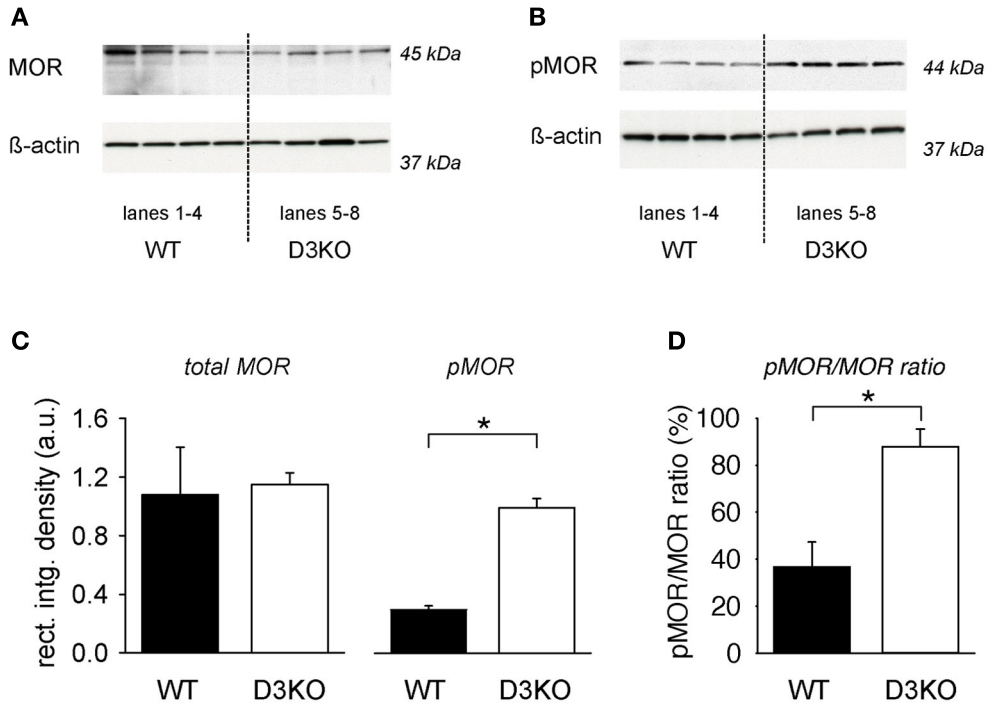

FIGURE 2 | Protein expression levels of total MOR and pMOR in lumbar spinal cord, normalized to β-actin. (A) Total MOR protein expression in WT and D3KO (top panels), and their respective ß-actin protein expressions (bottom panels). Lanes 1–4 represent lumbar spinal cords from four independent WT, while lanes 5–8 represent tissues from four independent D3KO. Total MOR protein expression levels were overall similar between WT and D3KO. Note that we first probed for MOR expression before stripping and re-probing for ß-actin. **(B)** pMOR protein expression levels in WT and D3KO (top lanes), and their respective ß-actin protein expression (bottom panels). Note that pMOR expression levels showed a stronger labeling in D3KO than WT. **(C)** Quantification of expression data: After normalization of MOR and pMOR data to their respective ß-actin expression in each lane, total MOR protein expression was similar between WT and D3KO ($^*p = 0.85$, $n = 4$ each). In contrast, pMOR protein expression was significantly increased in D3KO over WT ($p < 0.001$, $n = 4$ each). **(D)** Ratio of pMOR/MOR protein expression in the lumbar spinal cord of WT and D3KO. In WT, pMOR was low, whereas in D3KO a large majority of MORs was phosphorylated ($^*p = 0.008$, $n = 4$ each).

while in D3KO this rate 87.8 ± 7.6% ($p = 0.008$), suggesting a reduced availability of the MOR for ligand binding in D3KO.

EXTRACELLULAR ELECTROPHYSIOLOGY AND PHARMACOLOGY

To test the functional effects of morphine mediated responses at the spinal cord level alone, we next elicited and recorded SRAs in isolated spinal cords of WT and D3KO before and during exogenous application of morphine (**Figure 3**). Constant-current stimulation of dorsal lumbar roots via glass suction electrodes elicited within a few milliseconds a monosynaptic stretch reflex on the corresponding ventral roots (MSR, **Figure 3A1**) that generally lasted less than 5–6 ms. The signal traces in the MSR time window were rectified and integrated, and the resulting data points at the end of each sweep were used as a measure of SRA (**Figure 3A2**).

Bath-application of morphine (1 μM) induced in WT a gradual decrease in SRA amplitude (**Figure 3B1**), and led to a significant reduction in WT SRAs at the end of the recorded application interval, from 100.3 ± 0.2 to 75.2 ± 6.8% ($p = 0.004$, $n = 6$, **Figure 3B2**). In contrast, in D3KO, bath-application of 1 μM morphine had no significant modulatory effect on SRA (**Figures 3C1,C2**; control: 99.8 ± 0.2%; morphine: 93.2 ± 4.2%, $p = 0.052$, $n = 12$). These data mirror the effects observed in the thermal pain paradigm, and they suggest that the behavioral changes observed in the intact animals stem from changes in the D3 receptor system at the spinal cord level.

While preferentially targeting MORs, morphine activates ∂ and κ receptors as well. To test if a targeted activation of the MOR pathway alone can mimic the effects observed with morphine, we bath-applied the MOR-preferring agonist, DAMGO (10 μM), to isolated WT and D3KO spinal cord preparations and assessed its role in modulating SRAs (**Figure 4**). In WT, DAMGO decreased SRAs from 99.9 ± 0.1 to 66.6 ± 4.5% ($n = 15$, $p < 0.001$, **Figure 4A**), and it decreased them in D3KO from 100 ± 0.15 to 80.9 ± 3.4% ($n = 8$, **Figure 4B**). Importantly, the difference in DAMGO-mediated actions between WT and D3KO was significant ($p = 0.013$). These data suggest that in isolated spinal cords of D3KO, MORs are still functionally active and can be recruited by a highly specific agonist, albeit at a smaller magnitude than in WT.

The D3KO animal is a functional, global knockout, and consequently D3 receptor function is compromised in every tissue. To test if D3 receptor dysfunction in the spinal cord is sufficient to mimic the morphine effects observed in the

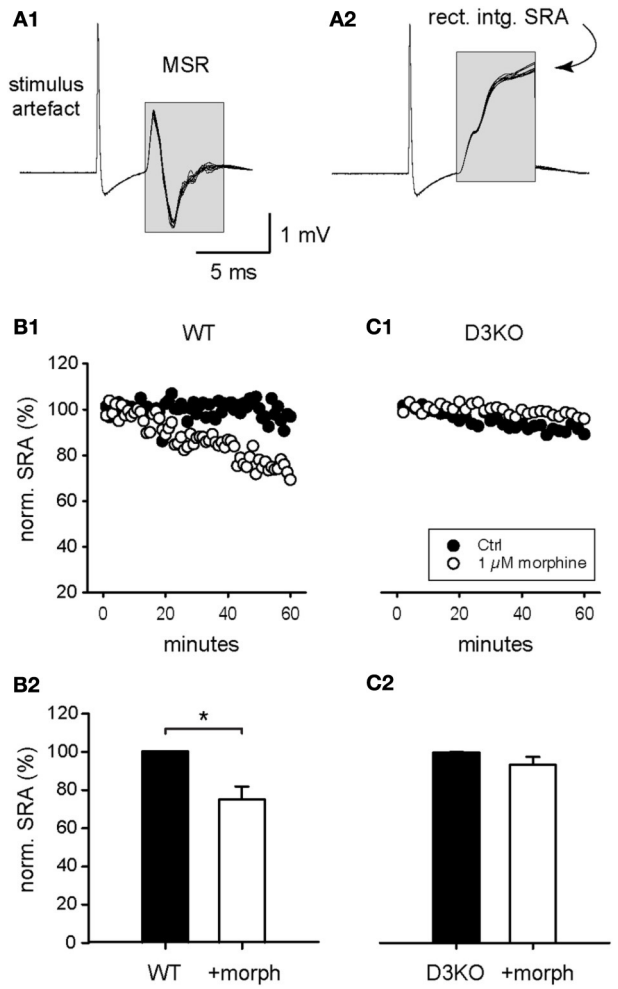

FIGURE 3 | Modulation of spinal reflex amplitudes (SRAs) by morphine. (A1) Example of a reflex response recorded from ventral roots (10 consecutive sweeps superimposed). Stimulation of a dorsal root (at stimulus artifact) induced a monosynaptic reflex response (MSR) in the corresponding ventral root. The boxed region identifies the epochs chosen to calculate the amplitude of the spinal reflex amplitude (SRA) represented in **(A2)**. **(A2)** The rectification and subsequent integration of the epochs identified in **(A1)** led to the signal values highlighted in the boxed region. The final maximal values of each epoch were used as a measurement of the SRA for each sweep, and used in the subsequent analysis steps represented in panels **(B,C)**, and **Figures 4**, **5**. **(B1)** Typical time course of control SRAs in WT before and during bath-application of 1 μM morphine (control: black symbols; morphine: white symbols). During morphine, reflex amplitudes gradually decreased in amplitude. **(B2)** Normalized SRAs in WT ($n = 6$). Morphine led to a significant reduction in SRA. $^*p = 0.004$. **(C1)** Typical time course of control SRAs in D3KO before and during bath-application of 1 μM morphine (control: black symbols; morphine: white symbols). **(C2)** Normalized SRAs in D3KO ($n = 12$). In these animals, morphine had no significant effect on SRA.

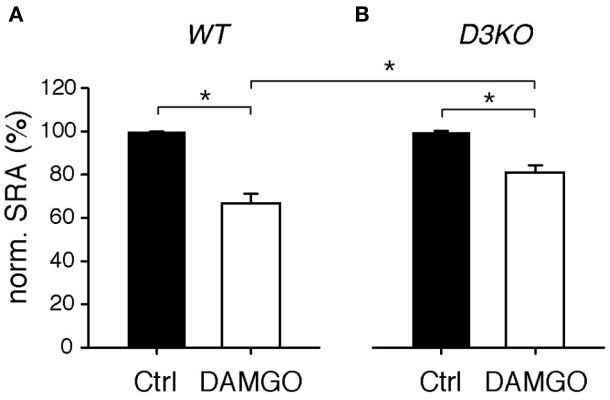

FIGURE 4 | Modulation of spinal reflex amplitudes (SRAs) by the MOR-preferring agonist, DAMGO. (A) In WT, DAMGO induced a significant decrease in SRA (to 66.6 ± 4.5% of control, $n = 15$). WT: $^*p < 0.001$. **(B)** In D3KO, DAMGO equally significant reduced SRA, albeit to lesser degree than in WT (to 80.9 ± 3.4%, $n = 8$). D3KO: $^*p < 0.001$. The difference between DAMGO-induced effects in WT and D3KO was significant ($p = 0.013$).

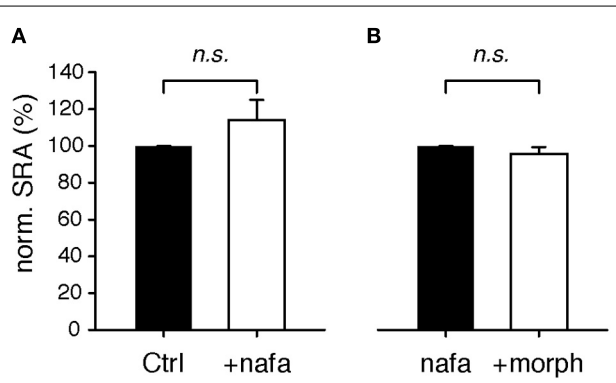

FIGURE 5 | Modulation of spinal reflex amplitudes (SRAs) by the D3-receptor preferring antagonist, nafadotride. (A) In the isolated WT spinal cord, bath-application of nafadotride led to slight, albeit insignificant increase in SRAs ($n = 8$). **(B)** In the presence of nafadotride, additional application of 1 μM morphine failed to exert any modulatory action on SRAs ($n = 5$).

D3KO both *in vivo* and *in vitro*, we tested the consequence of an acute block of the D3 receptor system in the isolated spinal cord prior to morphine exposure (**Figure 5**). Following baseline recordings, WT spinal cords were exposed to the D3 receptor-preferring antagonist, nafadotride (**Figure 5A**), before additionally bath-applying morphine (**Figure 5B**). Application of nafadotride alone (20 μM) led to a slight, yet insignificant increase in SRA from 99.7 ± 0.2 to 113.9 ± 11.1% ($p = 0.87$, $n = 8$, **Figure 5A**). Subsequent application of morphine, in the presence of nafadotride, and at the same dose that caused the significant inhibition of SRAs under control conditions (cf. **Figure 3**) failed to exert any significant modulatory action (ctrl: 99.8 ± 0.1 vs. 95.5 ± 3.9%, $p = 0.69$, $n = 5$, **Figure 5B**). These data are testimony that an induced acute block of spinal D3 receptors is sufficient to prevent morphine effects.

D1 RECEPTOR EXPRESSION

As the D3 receptor is closely associated with the D1 receptor through heterodimerization (Surmeier et al., 1996; Fiorentini et al., 2008; Maggio et al., 2009; Missale et al., 2010; Cruz-Trujillo

et al., 2013), we next tested if the dysfunction of the D3 receptor system leads to changes in the D1 receptor system (**Figure 6**). We found that in D3KO ($n = 5$), D1 receptor expression (49 kDa band) increased significantly from 99.99 ± 4.6% (in WT) to 170.6 ± 8.9% ($n = 5$, $p < 0.001$). These data support the notion that the increased excitability in the D3KO spinal cord could be, at least in part, driven by an increased expression in D1 receptor levels.

DISCUSSION

We show here that a dysfunction of the dopamine D3 receptor is associated with a morphine-resistant state *in vivo*, that this behavioral phenotype can be mimicked in the spinal cord *in vitro*, and that this altered morphine responsiveness is associated with an increase of pMOR but not total MOR expression in the lumbar spinal cord. In addition, we found that an acute block of D3 receptor function in the isolated spinal cord completely abolished the modulatory capabilities of morphine on SRAs, suggesting that the disruption of D3 receptor function *in vitro* is sufficient to induce a state of morphine resistance.

The D3 receptor is part of the D2-like family, and an evaluation of D1- and D2 receptor agonists, at a time when other DA receptors had not yet been described, reported that D2 activation produced antinociception, whereas D1 receptor activation induced mild hyperalgesia (Rooney and Sewell, 1989). More recently it was shown that co-administration of morphine with nafadotride could effectively suppress the morphine-induced behavioral sensitization observed in WT mice after acute morphine administration (Li et al., 2010), and it was suggested that D3 receptors regulate basal nociception (Li et al., 2012). Together, these data support our findings that the D3 receptor may have a critical role in regulating the morphine response in the spinal cord.

Loss of morphine analgesia is generally associated with a reduction in functional MOR receptors, either through down-regulation or desensitization of receptors (Williams et al., 2013). Such a down-regulation is characterized by a decrease in functional receptors present on the cell membrane due to degradation or decreased biosynthesis (Finn and Whistler, 2001). We found that D3KO and WT mice had similar levels of total MOR expression indicating that MOR receptor down-regulation *per se* is not the primary mechanism behind the lack of morphine responsiveness in D3KO mice *in vivo* (**Figure 2**). Our studies, however, were insensitive to receptor distribution between cellular compartments which would effect of the availability of functional MOR. Desensitization involves molecular changes at the receptor signaling level (Yu et al., 1997; Bohn et al., 2000), with receptor phosphorylation being the first step. MOR desensitization is under the control of GRK-mediated phosphorylation (Connor et al., 2004; Gainetdinov et al., 2004; Garzon et al., 2005), followed by ß-arrestin-mediated internalization (Bohn et al., 1999, 2000; Ohsawa et al., 2003; Connor et al., 2004). Our data show that, while total MOR protein levels were similar across groups, a greater proportion of those receptors were phosphorylated in D3KO compared to WT, suggesting that these receptors were desensitized and will not effectively signal, even in the presence of a ligand (**Figure 2**). While the MOR can be phosphorylated by both GPCR kinase (GRK) and non-GRK mechanisms, the antibody used for this experiment was designed to recognize phosphorylation of the Ser375 residue of the MOR, a site that is critical for GRK phosphorylation, arrestin recruitment and endocytosis (El Kouhen et al., 2001). This site has also been shown to be phosphorylated in states of morphine tolerance and with sustained release of β-endorphin, the endogenous ligand for MOR (Petraschka et al., 2007). Such alterations in binding efficiency of MOR to endogenous opioid ligands may provide one explanation for our findings why D3KO mice have lower baseline thermal pain thresholds than WT (Keeler et al., 2012), and they may also explain the lack of analgesia with exogenous morphine treatment. Studies assessing GRK activity levels and endogenous opioid levels in D3KO mice are needed to determine if the baseline phosphorylation of the MOR results from either increased GRK activity and/or continuous activation due to an increased availability of endogenous ligand.

A possible limitation of our immunohistochemical findings is that the commercially-generated antibodies used in our study were not, or only to a limited extent, validated in external peer review processes, and thus may not be optimized or only partially effective in detecting the proteins of interest (e.g., the 42 kDa band we observed exclusively in the D3KO spinal cord when probing for the D1 receptor, **Figure 6**). However, as the 49 kDa band (s. arrows in **Figure 6A**) corresponds to the predicted D1 receptor protein size, has together with a 75 kDa band also been observed at this size recently (Mizuta et al., 2013), and was both present and differentially expressed in WT and D3KO (**Figure 6B**), we are confident that future work will corroborate the molecular changes reported in this study. At the same time, given the high structural homology between D1 and D5 receptors, we can not exclude the possibility that such studies may identify an up-regulation of the D5 receptor instead of the D1 receptor as suggested by our findings. Yet, given the similar functional properties of D1 and D5 receptors, both of which primarily mediate excitatory actions, and the lack of evidence of D1/D5 receptor interactions, we have based our model on the existing literature that has identified D1/D3 interaction and heterodimers, which could act in synergistic fashion to control and mediate DA and morphine actions.

Our finding that the MOR agonist DAMGO can induce a modulatory response in both WT and D3KO could be the result of its higher specificity in activating the MOR than morphine (Minami et al., 1995; Onogi et al., 1995; Saidak et al., 2006), which also activate ∂-receptors (Walwyn et al., 2009), and/or its high affinity to the MOR (Pak et al., 1996). As we observed lower levels of non-p-MOR in D3KO than in WT (**Figure 2**), it is conceivable that DAMGO but not morphine may be able to activate this receptor population in D3KO, thus allowing the modulation of the SRAs *in vitro*.

Resistance to opioid treatment involves in part the DA system, including the D3 receptor system (Richtand et al., 2001; Sokoloff et al., 2001; Kosten et al., 2002; Vorel et al., 2002; Le Foll et al., 2007; Heidbreder, 2008; Hell, 2009; Li et al., 2012). Such tolerance can arise from a dysfunction of MOR and DA receptor mediated cAMP/PKA second messenger pathways in the brain (Zhao et al., 2007; Barraud et al., 2010). Both DA and MORs are located pre- and post-synaptically in spinal cord sensory

neurons (Xie et al., 1998; Levant and McCarson, 2001; Abbadie et al., 2002; Millan, 2002) and they control the activation of adenylyl-cyclase (AC). Generally, DA D1-like receptors (D1, D5) mediate excitatory actions by increasing AC activation and raising cAMP levels in the target neuron (Missale et al., 1998; Neve et al., 2004), whereas D2-like (D2, D3, and D4) and MOR pathways reduce AC activation and decrease cAMP levels (Yu et al., 1990; Jaber et al., 1996; Mamiya et al., 2001; Neve et al., 2004; Sheng et al., 2009). As D1 and D3 receptors often co-localize or form heterodimers (Karasinska et al., 2005; Fiorentini et al., 2008, 2010; Marcellino et al., 2008; Beaulieu and Gainetdinov, 2011) and oppositely regulate cAMP/PKA-mediated second messenger pathways, it is conceivable that the dysfunction of the D3 receptor might directly modify D1 receptor function. While we previously did not observe differences in D1 mRNA expression in the D3KO spinal cord (Zhu et al., 2008), probing for the affiliated protein expression revealed a significantly increased D1 receptor protein expression in D3KO (**Figure 6**), supporting the notion of a D3-mediated influence on D1 function. Additionally, the similarities in MOR- and D3-mediated second messenger pathways suggest that they may have synergistic effects on reducing pain transmission (Li et al., 2012; Saghaei et al., 2012), and the co-localization of D1 with D3 receptors suggest that interactions in their common cAMP/PKA signaling cascade might play a role in the emergence of the morphine resistance seen in D3KO (**Figure 7**).

In WT, a nociceptive stimulus induces the release of DA and endogenous opioids, including beta-endorphin, which preferentially bind the D3 and MOR, respectively, and initiate signaling cascades that inhibit cAMP production, resulting in the

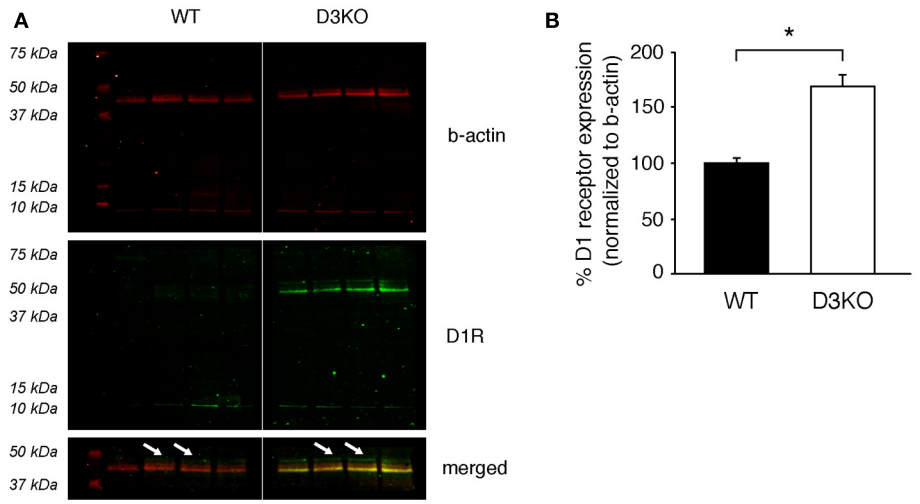

FIGURE 6 | Protein expression levels of the dopamine D1 receptor in lumbar spinal cord. (A) ß-actin (top panels, red), D1R (middle panels, green), and merged images of the 37–50 kDa band of interest (bottom panels) of the same blot. Note that ß-actin expression is similar in WT and D3KO. In contrast, the D1R antibody recognizes a strong signal at the 42 kDa band that is present only in D3KO, and a weaker signal at the predicted band size of 49 kDa that is differently expressed in WT and D3KO (arrows) and that was used for the subsequent analysis. **(B)** The quantification of the 49 kDa expression labeling, normalized to ß-actin expression, revealed a significant increase in D1 receptor protein expression levels in D3KO (*$p < 0.001$).

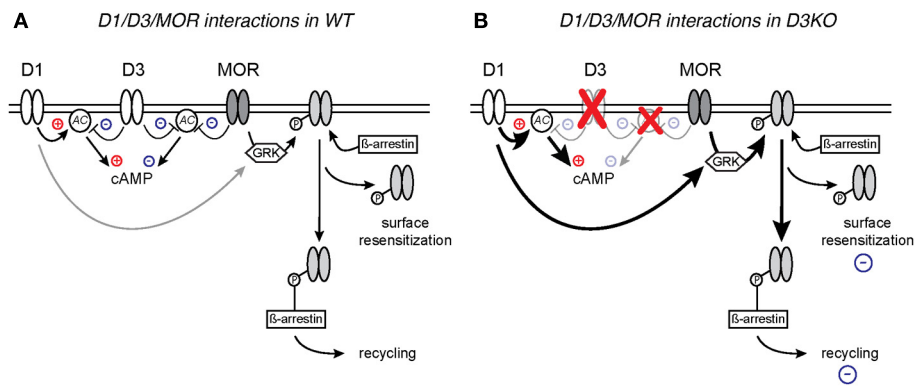

FIGURE 7 | Proposed model of D1 and D3 receptor interactions with MOR in WT (A) and D3KO (B). (A) Naïve WT with intact D1, D3, and MOR signaling pathways. **(B)** D3KO with compromised D3 function and altered second messenger cascades.

opening of K^+ channels or closing of Ca^{2+} channels (Connor and Christie, 1999; Connor et al., 1999; Williams et al., 2001) thus decreasing the transmission of the nociceptive signal. MOR binding is followed by GRK-mediated phosphorylation (Connor et al., 2004; Gainetdinov et al., 2004; Garzon et al., 2005), and ß-arrestin-mediated internalization (Bohn et al., 1999, 2000; Ohsawa et al., 2003; Connor et al., 2004) of the receptor, followed by recycling or de-phosphorylation and subsequent reinsertion into the membrane. More recent evidence also demonstrates that recovery from phosphorylation and desensitization can occur on the cell membrane, without the need for endocytosis of the receptor (Arttamangkul et al., 2006; Doll et al., 2011).

This intracellular overlap between the MOR and DA systems, along with evidence that D1, but not D2 receptors are involved in mediating the behavioral responses to morphine by acting to recruit GRK and ß-arrestin to the MOR (Urs et al., 2011) and that blocking ß-arrestin expression has been shown to enhance morphine-mediated analgesia (Li et al., 2009), has led to the model proposed in **Figure 7**. According to our model, activation of the D3 receptor leads in WT to a reduction of adenylate cyclase (AC) activity, which will result in a reduction of cAMP levels and decreased cAMP-mediated signaling, in a manner that is synergistic to the MOR in response to its ligand. However, under this scenario, D1 activation can also activate AC and compensate for a D3- or MOR-mediated reduction in cAMP levels, thus permitting a bi-directional modulation of cAMP-mediated pathways. As D1 and D3 receptors often co-localize or form heterodimers in the brain (Karasinska et al., 2005; Fiorentini et al., 2008, 2010; Marcellino et al., 2008; Beaulieu and Gainetdinov, 2011) and oppositely regulate cAMP/PKA-mediated second messenger pathways, we postulate that, in D3KO, the dysfunction of the D3 receptor prevents the D3-mediated block of AC, and leaves D1 receptor actions unopposed. Under such circumstances, cAMP pathways might be continuously up-regulated, and additional application of cAMP nucleotides might fail to further increase cellular excitability. Further, activation of the D1 receptor induces ß-arrestin signaling complex formation, in which β-arrestin acts as a scaffold for different kinases and phosphatases (Beaulieu et al., 2005; Urs et al., 2011) which in turn may lead to desensitization of receptors through G protein-independent signaling mechanisms (Pierce and Lefkowitz, 2001; Lefkowitz and Shenoy, 2005) and increased pMOR levels. We postulate that these changes to the second messenger systems, either individually or in concert, can create the overly excitable cellular state that is evidenced by decreased sensory thresholds at baseline, and the resistance to the analgesic effects of the opiates.

Taken together, our data reinforce the idea that changes in the ability of opioids to provide analgesia can arise from a dysfunctional D3 receptor, as demonstrated using spinally-mediated behaviors and reflex circuits, and that this unresponsiveness to morphine can be induced acutely in the isolated spinal cord by blocking the D3 receptor. Therefore, the D3KO mouse may be a powerful tool with which to study the alterations to the MOR second messenger-signaling cascade, to decipher the initial mechanisms that may underlie the waning of morphine effectiveness over time.

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REFERENCES

Abbadie, C., Lombard, M. C., Besson, J. M., Trafton, J. A., and Basbaum, A. I. (2002). Mu and delta opioid receptor-like immunoreactivity in the cervical spinal cord of the rat after dorsal rhizotomy or neonatal capsaicin: an analysis of pre- and postsynaptic receptor distributions. *Brain Res.* 930, 150–162. doi: 10.1016/S0006-8993(02)02242-4

Abbadie, C., Pasternak, G. W., and Aicher, S. A. (2001). Presynaptic localization of the carboxy-terminus epitopes of the μ opioid receptor splice variants MOR-1C and MOR-1D in the superficial laminae of the rat spinal cord. *Neuroscience* 106, 833–842. doi: 10.1016/S0306-4522(01)00317-7

Arttamangkul, S., Torrecilla, M., Kobayashi, K., Okano, H., and Williams, J. T. (2006). Separation of mu-opioid receptor desensitization and internalization: endogenous receptors in primary neuronal cultures. *J. Neurosci.* 26, 4118–4125. doi: 10.1523/JNEUROSCI.0303-06.2006

Barraud, Q., Obeid, I., Aubert, I., Barriere, G., Contamin, H., McGuire, S., et al. (2010). Neuroanatomical study of the A11 diencephalospinal pathway in the non-human primate. *PLoS ONE* 5:e13306. doi: 10.1371/journal.pone.0013306

Beaulieu, J. M., and Gainetdinov, R. R. (2011). The physiology, signaling, and pharmacology of dopamine receptors. *Pharmacol. Rev.* 63, 182–217. doi: 10.1124/pr.110.002642

Beaulieu, J. M., Sotnikova, T. D., Marion, S., Lefkowitz, R. J., Gainetdinov, R. R., and Caron, M. G. (2005). An Akt/beta-arrestin 2/PP2A signaling complex mediates dopaminergic neurotransmission and behavior. *Cell* 122, 261–273. doi: 10.1016/j.cell.2005.05.012

Bekhit, M. H. (2010). Opioid-induced hyperalgesia and tolerance. *Am. J. Ther.* 17, 498–510. doi: 10.1097/MJT.0b013e3181ed83a0

Bohn, L. M., Gainetdinov, R. R., Lin, F. T., Lefkowitz, R. J., and Caron, M. G. (2000). Mu-opioid receptor desensitization by beta-arrestin-2 determines morphine tolerance but not dependence. *Nature* 408, 720–723. doi: 10.1038/35047086

Bohn, L. M., Lefkowitz, R. J., Gainetdinov, R. R., Peppel, K., Caron, M. G., and Lin, F. T. (1999). Enhanced morphine analgesia in mice lacking beta-arrestin 2. *Science* 286, 2495–2498. doi: 10.1126/science.286.5449.2495

Clemens, S., and Hochman, S. (2004). Conversion of the modulatory actions of dopamine on spinal reflexes from depression to facilitation in D3 receptor knock-out mice. *J. Neurosci.* 24, 11337–11345. doi: 10.1523/JNEUROSCI.3698-04.2004

Colpaert, F. C. (2002). Mechanisms of opioid-induced pain and antinociceptive tolerance: signal transduction. *Pain* 95, 287–288. doi: 10.1016/S0304-3959(01)00445-6

Connor, M., and Christie, M. D. (1999). Opioid receptor signalling mechanisms. *Clin. Exp. Pharmacol. Physiol.* 26, 493–499. doi: 10.1046/j.1440-1681.1999.03049.x

Connor, M., Osborne, P. B., and Christie, M. J. (2004). Mu-opioid receptor desensitization: is morphine different? *Br. J. Pharmacol.* 143, 685–696. doi: 10.1038/sj.bjp.0705938

Connor, M., Schuller, A., Pintar, J. E., and Christie, M. J. (1999). Mu-opioid receptor modulation of calcium channel current in periaqueductal grey neurons from C57B16/J mice and mutant mice lacking MOR-1. *Br. J. Pharmacol.* 126, 1553–1558. doi: 10.1038/sj.bjp.0702457

Cook, C. D., Barrett, A. C., Syvanthong, C., and Picker, M. J. (2000). Modulatory effects of dopamine D3/2 agonists on kappa opioid-induced antinociception and diuresis in the rat. *Psychopharmacology (Berl.)* 152, 14–23. doi: 10.1007/s002130000519

Cruz-Trujillo, R., Avalos-Fuentes, A., Rangel-Barajas, C., Paz-Bermudez, F., Sierra, A., Escartin-Perez, E., et al. (2013). D3 dopamine receptors interact with dopamine D1 but not D4 receptors in the GABAergic terminals of the SNr of the rat. *Neuropharmacology* 67, 370–378. doi: 10.1016/j.neuropharm.2012.11.032

Doll, C., Konietzko, J., Poll, F., Koch, T., Hollt, V., and Schulz, S. (2011). Agonist-selective patterns of micro-opioid receptor phosphorylation revealed by phosphosite-specific antibodies. *Br. J. Pharmacol.* 164, 298–307. doi: 10.1111/j.1476-5381.2011.01382.x

Dupen, A., Shen, D., and Ersek, M. (2007). Mechanisms of opioid-induced tolerance and hyperalgesia. *Pain Manag. Nurs.* 8, 113–121. doi: 10.1016/j.pmn.2007.02.004

El Kouhen, R., Burd, A. L., Erickson-Herbrandson, L. J., Chang, C. Y., Law, P. Y., and Loh, H. H. (2001). Phosphorylation of Ser363, Thr370, and Ser375 residues within the carboxyl tail differentially regulates mu-opioid receptor internalization. *J. Biol. Chem.* 276, 12774–12780. doi: 10.1074/jbc.M009571200

Enoksson, T., Bertran-Gonzalez, J., and Christie, M. J. (2012). Nucleus accumbens D2- and D1-receptor expressing medium spiny neurons are selectively activated by morphine withdrawal and acute morphine, respectively. *Neuropharmacology* 62, 2463–2471. doi: 10.1016/j.neuropharm.2012.02.020

Fazli-Tabaei, S., Yahyavi, S. H., Nouri, M., Zartab, H., Javid, G., Loghavi, S., et al. (2006). Dopamine receptor mechanism(s) and antinociception and tolerance induced by swim stress in formalin test. *Behav. Pharmacol.* 17, 341–347. doi: 10.1097/01.fbp.0000224383.63744.69

Finn, A. K., and Whistler, J. L. (2001). Endocytosis of the mu opioid receptor reduces tolerance and a cellular hallmark of opiate withdrawal. *Neuron* 32, 829–839. doi: 10.1016/S0896-6273(01)00517-7

Fiorentini, C., Busi, C., Gorruso, E., Gotti, C., Spano, P., and Missale, C. (2008). Reciprocal regulation of dopamine D1 and D3 receptor function and trafficking by heterodimerization. *Mol. Pharmacol.* 74, 59–69. doi: 10.1124/mol.107.043885

Fiorentini, C., Busi, C., Spano, P., and Missale, C. (2010). Dimerization of dopamine D1 and D3 receptors in the regulation of striatal function. *Curr. Opin. Pharmacol.* 10, 87–92. doi: 10.1016/j.coph.2009.09.008

Gainetdinov, R. R., Premont, R. T., Bohn, L. M., Lefkowitz, R. J., and Caron, M. G. (2004). Desensitization of G protein-coupled receptors and neuronal functions. *Annu. Rev. Neurosci.* 27, 107–144. doi: 10.1146/annurev.neuro.27.070203.144206

Garraway, S. M., and Hochman, S. (2001). Modulatory actions of serotonin, norepinephrine, dopamine, and acetylcholine in spinal cord deep dorsal horn neurons. *J. Neurophysiol.* 86, 2183–2194.

Garzon, J., Rodriguez-Munoz, M., and Sanchez-Blazquez, P. (2005). Morphine alters the selective association between mu-opioid receptors and specific RGS proteins in mouse periaqueductal gray matter. *Neuropharmacology* 48, 853–868. doi: 10.1016/j.neuropharm.2005.01.004

Heidbreder, C. (2008). Selective antagonism at dopamine D3 receptors as a target for drug addiction pharmacotherapy: a review of preclinical evidence. *CNS Neurol. Disord. Drug Targets* 7, 410–421. doi: 10.2174/187152708786927822

Hell, J. W. (2009). Hooked on the D3 receptor: CaMKII's new addiction. *Neuron* 61, 335–336. doi: 10.1016/j.neuron.2009.01.025

Jaber, M., Robinson, S. W., Missale, C., and Caron, M. G. (1996). Dopamine receptors and brain function. *Neuropharmacology* 35, 1503–1519. doi: 10.1016/S0028-3908(96)00100-1

Ji, R. R., Zhang, Q., Law, P.-Y., Low, H. H., Elde, R., and Hökfelt, T. (1995). Expression of μ-, δ-, and κ-opioid receptor-like immunoreactivities in rat dorsal root ganglia after carrageenan-induced inflammation. *J. Neurosci.* 15, 8156–8166.

Joseph, E. K., Reichling, D. B., and Levine, J. D. (2010). Shared mechanisms for opioid tolerance and a transition to chronic pain. *J. Neurosci.* 30, 4660–4666. doi: 10.1523/JNEUROSCI.5530-09.2010

Karasinska, J. M., George, S. R., Cheng, R., and O'Dowd, B. F. (2005). Deletion of dopamine D1 and D3 receptors differentially affects spontaneous behaviour and cocaine-induced locomotor activity, reward and CREB phosphorylation. *Eur. J. Neurosci.* 22, 1741–1750. doi: 10.1111/j.1460-9568.2005.04353.x

Keeler, B. E., Baran, C. A., Brewer, K. L., and Clemens, S. (2012). Increased excitability of spinal pain reflexes and altered frequency-dependent modulation in the dopamine D3-receptor knockout mouse. *Exp. Neurol.* 238, 273–283. doi: 10.1016/j.expneurol.2012.09.002

Kerros, C., Brood, I., Sola, B., Jauzac, P., and Allouche, S. (2010). Reduction of cell proliferation and potentiation of Fas-induced apoptosis by the selective kappa-opioid receptor agonist U50 488 in the multiple myeloma LP-1 cells. *J. Neuroimmunol.* 220, 69–78. doi: 10.1016/j.jneuroim.2010.01.010

Kosten, T. R., George, T. P., and Kosten, T. A. (2002). The potential of dopamine agonists in drug addiction. *Expert Opin. Investig. Drugs* 11, 491–499. doi: 10.1517/13543784.11.4.491

Lefkowitz, R. J., and Shenoy, S. K. (2005). Transduction of receptor signals by beta-arrestins. *Science* 308, 512–517. doi: 10.1126/science.1109237

Le Foll, B., Goldberg, S. R., and Sokoloff, P. (2007). Dopamine D3 receptor ligands for the treatment of tobacco dependence. *Expert Opin. Investig. Drugs* 16, 45–57. doi: 10.1517/13543784.16.1.45

Le Marec, T., Marie-Claire, C., Noble, F., and Marie, N. (2011). Chronic and intermittent morphine treatment differently regulates opioid and dopamine systems: a role in locomotor sensitization. *Psychopharmacology (Berl.)* 216, 297–303. doi: 10.1007/s00213-011-2223-6

Levant, B., and McCarson, K. E. (2001). D(3) dopamine receptors in rat spinal cord: implications for sensory and motor function. *Neurosci. Lett.* 303, 9–12. doi: 10.1016/S0304-3940(01)01692-5

Li, T., Hou, Y., Cao, W., Yan, C. X., Chen, T., and Li, S. B. (2012). Role of dopamine D3 receptors in basal nociception regulation and in morphine-induced tolerance and withdrawal. *Brain Res.* 1433, 80–84. doi: 10.1016/j.brainres.2011.11.045

Li, T., Hou, Y., Yan, C. X., Chen, T., Zhao, Y., and Li, S. B. (2010). Dopamine D3 receptor knock-out mice display deficits in locomotor sensitization after chronic morphine administration. *Neurosci. Lett.* 485, 256–260. doi: 10.1016/j.neulet.2010.09.025

Li, Y., Liu, X., Liu, C., Kang, J., Yang, J., Pei, G., et al. (2009). Improvement of morphine-mediated analgesia by inhibition of beta-arrestin 2 expression in mice periaqueductal gray matter. *Int. J. Mol. Sci.* 10, 954–963. doi: 10.3390/ijms10030954

Lin, C. W., Bianchi, B. R., Miller, T. R., Stashko, M. A., Wang, S. S., Curzon, P., et al. (1996). Persistent activation of the dopamine D1 receptor contributes to prolonged receptor desensitization: studies with A-77636. *J. Pharmacol. Exp. Ther.* 276, 1022–1029.

Loyd, D. R., Wang, X., and Murphy, A. Z. (2008). Sex differences in micro-opioid receptor expression in the rat midbrain periaqueductal gray are essential for eliciting sex differences in morphine analgesia. *J. Neurosci.* 28, 14007–14017. doi: 10.1523/JNEUROSCI.4123-08.2008

Maggio, R., Aloisi, G., Silvano, E., Rossi, M., and Millan, M. J. (2009). Heterodimerization of dopamine receptors: new insights into functional and therapeutic significance. *Parkinsonism Relat. Disord.* 15(Suppl. 4), S2–S7. doi: 10.1016/S1353-8020(09)70826-0

Mamiya, T., Noda, Y., Ren, X., Hamdy, M., Furukawa, S., Kameyama, T., et al. (2001). Involvement of cyclic AMP systems in morphine physical dependence in mice: prevention of development of morphine dependence by rolipram, a phosphodiesterase 4 inhibitor. *Br. J. Pharmacol.* 132, 1111–1117. doi: 10.1038/sj.bjp.0703912

Mansour, A., Fox, C. A., Burke, S., Meng, F., Thompson, R. C., Akil, H., et al. (1994). Mu, delta, and kappa opioid receptor mRNA expression in the rat CNS: an *in situ* hybridization study. *J. Comp. Neurol.* 350, 412–438. doi: 10.1002/cne.903500307

Marcellino, D., Ferre, S., Casado, V., Cortes, A., Le Foll, B., Mazzola, C., et al. (2008). Identification of dopamine D1-D3 receptor heteromers. Indications for a role of synergistic D1-D3 receptor interactions in the striatum. *J. Biol. Chem.* 283, 26016–26025. doi: 10.1074/jbc.M710349200

Millan, M. J. (2002). Descending control of pain. *Prog. Neurobiol.* 66, 355–474. doi: 10.1016/S0301-0082(02)00009-6

Minami, M., Onogi, T., Nakagawa, T., Katao, Y., Aoki, Y., Katsumata, S., et al. (1995). DAMGO, a mu-opioid receptor selective ligand, distinguishes between mu-and kappa-opioid receptors at a different region from that for the distinction between mu- and delta-opioid receptors. *FEBS Lett.* 364, 23–27. doi: 10.1016/0014-5793(95)00340-F

Missale, C., Fiorentini, C., Collo, G., and Spano, P. (2010). The neurobiology of dopamine receptors: evolution from the dual concept to heterodimer complexes. *J. Recept. Signal Transduct. Res.* 30, 347–354. doi: 10.3109/10799893.2010.506192

Missale, C., Nash, S. R., Robinson, S. W., Jaber, M., and Caron, M. G. (1998). Dopamine receptors: from structure to function. *Physiol. Rev.* 78, 189–225.

Mizuta, K., Zhang, Y., Xu, D., Mizuta, F., D'Ovidio, F., Masaki, E., et al. (2013). The dopamine D1 receptor is expressed and facilitates relaxation in airway smooth muscle. *Respir. Res.* 14, 89. doi: 10.1186/1465-9921-14-89

Neve, K. A., Seamans, J. K., and Trantham-Davidson, H. (2004). Dopamine receptor signaling. *J. Recept. Signal Transduct. Res.* 24, 165–205. doi: 10.1081/RRS-200029981

Ohsawa, M., Mizoguchi, H., Narita, M., Nagase, H., Dun, N. J., and Tseng, L. F. (2003). Involvement of beta-arrestin-2 in modulation of the spinal

antinociception induced by mu-opioid receptor agonists in the mouse. *Neurosci. Lett.* 346, 13–16. doi: 10.1016/S0304-3940(03)00591-3

Onogi, T., Minami, M., Katao, Y., Nakagawa, T., Aoki, Y., Toya, T., et al. (1995). DAMGO, a mu-opioid receptor selective agonist, distinguishes between mu- and delta-opioid receptors around their first extracellular loops. *FEBS Lett.* 357, 93–97. doi: 10.1016/0014-5793(94)01341-W

Pak, Y., Kouvelas, A., Scheideler, M. A., Rasmussen, J., O'Dowd, B. F., and George, S. R. (1996). Agonist-induced functional desensitization of the mu-opioid receptor is mediated by loss of membrane receptors rather than uncoupling from G protein. *Mol. Pharmacol.* 50, 1214–1222.

Petraschka, M., Li, S., Gilbert, T. L., Westenbroek, R. E., Bruchas, M. R., Schreiber, S., et al. (2007). The absence of endogenous beta-endorphin selectively blocks phosphorylation and desensitization of mu opioid receptors following partial sciatic nerve ligation. *Neuroscience* 146, 1795–1807. doi: 10.1016/j.neuroscience.2007.03.029

Pierce, K. L., and Lefkowitz, R. J. (2001). Classical and new roles of beta-arrestins in the regulation of G-protein-coupled receptors. *Nat. Rev. Neurosci.* 2, 727–733. doi: 10.1038/35094577

Ray, S. B., and Wadhwa, S. (1999). Mu opioid receptors in developing human spinal cord. *J. Anat.* 195(Pt 1), 11–18. doi: 10.1046/j.1469-7580.1999.19510011.x

Ray, S. B., and Wadhwa, S. (2004). Expression of mu-opioid receptors in developing rat spinal cord: an autoradiographic study. *Indian J. Exp. Biol.* 42, 533–537.

Richtand, N. M., Goldsmith, R. J., Nolan, J. E., and Berger, S. P. (2001). The D3 dopamine receptor and substance dependence. *J. Addict. Dis.* 20, 19–32. doi: 10.1300/J069v20n03_03

Richtand, N. M., Logue, A. D., Welge, J. A., Perdiue, J., Tubbs, L. J., Spitzer, R. H., et al. (2000). The dopamine D3 receptor antagonist nafadotride inhibits development of locomotor sensitization to amphetamine. *Brain Res.* 867, 239–242. doi: 10.1016/S0006-8993(00)02247-2

Rooney, K. F., and Sewell, R. D. (1989). Evaluation of selective actions of dopamine D-1 and D-2 receptor agonists and antagonists on opioid antinociception. *Eur. J. Pharmacol.* 168, 329–336. doi: 10.1016/0014-2999(89)90794-2

Saghaei, E., Moini Zanjani, T., Sabetkasaei, M., and Naseri, K. (2012). Enhancement of antinociception by co-administrations of nefopam, morphine, and nimesulide in a rat model of neuropathic pain. *Korean J. Pain* 25, 7–15. doi: 10.3344/kjp.2012.25.1.7

Saidak, Z., Blake-Palmer, K., Hay, D. L., Northup, J. K., and Glass, M. (2006). Differential activation of G-proteins by mu-opioid receptor agonists. *Br. J. Pharmacol.* 147, 671–680. doi: 10.1038/sj.bjp.0706661

Schmidt, B. L., Tambeli, C. H., Barletta, J., Luo, L., Green, P., Levine, J. D., et al. (2002). Altered nucleus accumbens circuitry mediates pain-induced antinociception in morphine-tolerant rats. *J. Neurosci.* 22, 6773–6780.

Sheng, H. Y., Qu, C. L., Huo, F. Q., Du, J. Q., and Tang, J. S. (2009). D2-like but not D1-like dopamine receptors are involved in the ventrolateral orbital cortex-induced antinociception: a GABAergic modulation mechanism. *Exp. Neurol.* 215, 128–134. doi: 10.1016/j.expneurol.2008.09.018

Sokoloff, P., Le Foll, B., Perachon, S., Bordet, R., Ridray, S., and Schwartz, J. C. (2001). The dopamine D3 receptor and drug addiction. *Neurotox. Res.* 3, 433–441. doi: 10.1007/BF03033202

Surmeier, D. J., Song, W. J., and Yan, Z. (1996). Coordinated expression of dopamine receptors in neostriatal medium spiny neurons. *J. Neurosci.* 16, 6579–6591.

Suzuki, T., Kishimoto, Y., Ozaki, S., and Narita, M. (2001). Mechanism of opioid dependence and interaction between opioid receptors. *Eur. J. Pain* 5(Suppl. A), 63–65. doi: 10.1053/eujp.2001.0282

Urs, N. M., Daigle, T. L., and Caron, M. G. (2011). A dopamine D1 receptor-dependent beta-arrestin signaling complex potentially regulates morphine-induced psychomotor activation but not reward in mice. *Neuropsychopharmacology* 36, 551–558. doi: 10.1038/npp.2010.186

Vorel, S. R., Ashby, C. R. Jr., Paul, M., Liu, X., Hayes, R., Hagan, J. J., et al. (2002). Dopamine D3 receptor antagonism inhibits cocaine-seeking and cocaine-enhanced brain reward in rats. *J. Neurosci.* 22, 9595–9603.

Walwyn, W., John, S., Maga, M., Evans, C. J., and Hales, T. G. (2009). Delta receptors are required for full inhibitory coupling of mu-receptors to voltage-dependent Ca(2+) channels in dorsal root ganglion neurons. *Mol. Pharmacol.* 76, 134–143. doi: 10.1124/mol.109.055913

Williams, J. T., Christie, M. J., and Manzoni, O. (2001). Cellular and synaptic adaptations mediating opioid dependence. *Physiol. Rev.* 81, 299–343.

Williams, J. T., Ingram, S. L., Henderson, G., Chavkin, C., Von Zastrow, M., Schulz, S., et al. (2013). Regulation of mu-opioid receptors: desensitization, phosphorylation, internalization, and tolerance. *Pharmacol. Rev.* 65, 223–254. doi: 10.1124/pr.112.005942

Xie, G. X., Jones, K., Peroutka, S. J., and Palmer, P. P. (1998). Detection of mRNAs and alternatively spliced transcripts of dopamine receptors in rat peripheral sensory and sympathetic ganglia. *Brain Res.* 785, 129–135. doi: 10.1016/S0006-8993(97)01394-2

Yang, H. W., Zhou, L. J., Hu, N. W., Xin, W. J., and Liu, X. G. (2005). Activation of spinal d1/d5 receptors induces late-phase LTP of C-fiber-evoked field potentials in rat spinal dorsal horn. *J. Neurophysiol.* 94, 961–967. doi: 10.1152/jn.01324.2004

Yu, V. C., Eiger, S., Duan, D. S., Lameh, J., and Sadee, W. (1990). Regulation of cyclic AMP by the mu-opioid receptor in human neuroblastoma SH-SY5Y cells. *J. Neurochem.* 55, 1390–1396. doi: 10.1111/j.1471-4159.1990.tb03151.x

Yu, Y., Zhang, L., Yin, X., Sun, H., Uhl, G. R., and Wang, J. B. (1997). Mu opioid receptor phosphorylation, desensitization, and ligand efficacy. *J. Biol. Chem.* 272, 28869–28874. doi: 10.1074/jbc.272.46.28869

Zhang, D., Zhang, H., Jin, G. Z., Zhang, K., and Zhen, X. (2008). Single dose of morphine produced a prolonged effect on dopamine neuron activities. *Mol. Pain* 4, 57.

Zhang, Y., Pan, Y. X., Kolesnikov, Y., and Pasternak, G. W. (2006). Immunohistochemical labeling of the mu opioid receptor carboxy terminal splice variant mMOR-1B4 in the mouse central nervous system. *Brain Res.* 1099, 33–43. doi: 10.1016/j.brainres.2006.04.133

Zhang, Y., Zhang, F., Yang, C., Jin, H., Yang, Y., and Xu, M. (2012). Dopamine affects the change of pain-related electrical activity induced by morphine dependence. *Neurochem. Res.* 37, 977–982. doi: 10.1007/s11064-011-0690-0

Zhao, H., Zhu, W., Pan, T., Xie, W., Zhang, A., Ondo, W. G., et al. (2007). Spinal cord dopamine receptor expression and function in mice with 6-OHDA lesion of the A11 nucleus and dietary iron deprivation. *J. Neurosci. Res.* 85, 1065–1076. doi: 10.1002/jnr.21207

Zhu, H., Clemens, S., Sawchuk, M., and Hochman, S. (2007). Expression and distribution of all dopamine receptor subtypes (D(1)-D(5)) in the mouse lumbar spinal cord: a real-time polymerase chain reaction and non-autoradiographic *in situ* hybridization study. *Neuroscience* 149, 885–897. doi: 10.1016/j.neuroscience.2007.07.052

Zhu, H., Clemens, S., Sawchuk, M., and Hochman, S. (2008). Unaltered D1, D2, D4, and D5 dopamine receptor mRNA expression and distribution in the spinal cord of the D3 receptor knockout mouse. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 194, 957–962. doi: 10.1007/s00359-008-0368-5

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Dopamine: a parallel pathway for the modulation of spinal locomotor networks

Simon A. Sharples[1,2], Kathrin Koblinger[1,2], Jennifer M. Humphreys[1,2] and Patrick J. Whelan[1,2,3,4]*

[1] Hotchkiss Brain Institute, University of Calgary, Calgary, AB, Canada
[2] Department of Comparative Biology and Experimental Medicine, University of Calgary, Calgary, AB, Canada
[3] Department of Physiology and Pharmacology, University of Calgary, Calgary, AB, Canada
[4] Department of Clinical Neurosciences, University of Calgary, Calgary, AB, Canada

Edited by:
Brian R. Noga, University of Miami, USA

Reviewed by:
Ronald M. Harris-Warrick, Cornell University, USA
Pierre Guertin, Laval University, Canada

***Correspondence:**
Patrick J. Whelan, Hotchkiss Brain Institute, University of Calgary, HSC 2119, 3330 Hospital Drive NW, Calgary, AB T2N 1N4, Canada
e-mail: whelan@ucalgary.ca

The spinal cord contains networks of neurons that can produce locomotor patterns. To readily respond to environmental conditions, these networks must be flexible yet at the same time robust. Neuromodulators play a key role in contributing to network flexibility in a variety of invertebrate and vertebrate networks. For example, neuromodulators contribute to altering intrinsic properties and synaptic weights that, in extreme cases, can lead to neurons switching between networks. Here we focus on the role of dopamine in the control of stepping networks in the spinal cord. We first review the role of dopamine in modulating rhythmic activity in the stomatogastric ganglion (STG) and the leech, since work from these preparations provides a foundation to understand its role in vertebrate systems. We then move to a discussion of dopamine's role in modulation of swimming in aquatic species such as the larval xenopus, lamprey and zebrafish. The control of terrestrial walking in vertebrates by dopamine is less studied and we review current evidence in mammals with a focus on rodent species. We discuss data suggesting that the source of dopamine within the spinal cord is mainly from the A11 area of the diencephalon, and then turn to a discussion of dopamine's role in modulating walking patterns from both *in vivo* and *in vitro* preparations. Similar to the descending serotonergic system, the dopaminergic system may serve as a potential target to promote recovery of locomotor function following spinal cord injury (SCI); evidence suggests that dopaminergic agonists can promote recovery of function following SCI. We discuss pharmacogenetic and optogenetic approaches that could be deployed in SCI and their potential tractability. Throughout the review we draw parallels with both noradrenergic and serotonergic modulatory effects on spinal cord networks. In all likelihood, a complementary monoaminergic enhancement strategy should be deployed following SCI.

Keywords: dopamine, monoamines, central pattern generator, locomotion, spinal cord

Neuromodulators are the key ingredient allowing motor networks the flexibility to produce multiple patterns of output. When one considers a task such as stepping, a number of patterns need to be produced to run, walk, hop, go up and down inclines and walk in a circular pattern. While we focus on stepping in this review, neuromodulators contribute to the rhythmic operation of most, if not all, biological networks. A complete understanding of the role of neuromodulators in motor control must take into account the combinatorial effects of the complement of transmitters released both synaptically and extrasynaptically. This is an immense challenge for the field of motor control, especially when small invertebrate motor circuits are compared directly with their larger, less accessible and more complex mammalian cousins. However, in the last decade new genetic tools have become available that allow spinal circuits that compose a motor network to be identified (Goulding, 2009). In addition, the birth of optogenetics and pharmacogenetics has provided advanced tools for activating and inactivating neuromodulatory systems (Shapiro et al., 2012; Aston-Jones and Deisseroth, 2013). Even with these new tools it will still be a challenge to examine and comprehend the combinatorial role of multiple neurotransmitters, but the task is more tractable than it has been historically.

Although many neuropeptides, hormones or monoamines can modulate motor circuits, our review will focus on the role of dopamine. Dopamine plays an important role in the activation and modulation of the motor system across a wide range of invertebrate and vertebrate species (Harris-Warrick et al., 1998; Svensson et al., 2003; Puhl and Mesce, 2008; Miles and Sillar, 2011; Clemens et al., 2012; Lambert et al., 2012). In humans, movement disorders such as Parkinson's disease and Restless Leg Syndrome provide prime examples of what can happen and how debilitating it can be for the individual when the dopaminergic system is compromised. Because of this, a large degree of effort has

been focused on understanding the contribution and mechanisms of supraspinal dopamine to motor control. Considerably less attention has been paid towards the descending dopaminergic projections to motor networks in the spinal cord compared to the descending serotonergic and noradrenergic systems. We will discuss and contrast current knowledge of the descending dopaminergic system with the descending serotonergic and noradrenergic system in the control of locomotion in a variety of species, with particular focus on work conducted in the rodent.

DOPAMINE'S ROLE IN LOCOMOTION

Across invertebrate and vertebrate species, dopamine has profound and diverse effects on rhythmically active motor networks. These actions are a result of a complex modulation of intrinsic cellular properties and synaptic connectivity (for review see Harris-Warrick et al., 1998). Remarkably, in some species dopamine demonstrates the ability to reconfigure circuits or networks to generate completely different motor behaviors (Puhl and Mesce, 2008; Crisp et al., 2012; Puhl et al., 2012). More recent studies have highlighted the ability of dopamine to shape a motor network during development, producing a change in motor behaviors from immature to more mature adult-like behaviors (Lambert et al., 2012). Furthermore, dopamine also possesses the capacity to promote developmental and adult motor neurogenesis (Reimer et al., 2013).

Early exploration of the role of catecholamines in locomotor behaviors in the 1960's determined that L-DOPA could modulate reflex circuits and promote locomotor activity, although at the time it was thought to be of noradrenergic origin (Jankowska et al., 1967a,b; Grillner and Zangger, 1979). For many years, L-DOPA was used to evoke locomotor activity in spinalized cats. It was later demonstrated that L-DOPA could promote air stepping in the neonatal rodent (Sickles et al., 1992; McCrea et al., 1997; McEwen et al., 1997), an effect that is blocked by both noradrenergic (Taylor et al., 1994) and dopaminergic antagonists (Sickles et al., 1992). This work established that catecholamines act on networks that generate locomotion. This work was unclear on the individual effects of dopamine, or the products of dopamine synthesis (noradrenaline) on locomotion and whether they were acting within the spinal cord. Additional studies now suggest that noradrenaline and dopamine both contribute to the control of locomotion in a different way than the predominant spinal serotonergic system that contributes to both evoking and modulating locomotor behavior (Forssberg and Grillner, 1973; Kiehn et al., 1999; Whelan et al., 2000; Jordan et al., 2008; Humphreys and Whelan, 2012).

DOPAMINE'S ROLE IN RHYTHMICITY: INVERTEBRATES

Work from invertebrate species has largely influenced the way that we view modulation of motor systems. For example, the stomatogastric nervous system is a well-defined series of interconnected ganglia that controls the rhythmic filtering and chewing motor patterns of the crab and lobster foregut. The effector ganglion, specifically known as the stomatogastric ganglion (STG), consists of 26–30 neurons that generate a pacemaker-driven higher frequency pyloric rhythm and a conditionally active and lower frequency gastric mill rhythm. Neuromodulation profoundly reconfigures this small circuit, biasing individual neurons to participate in the different motor behaviors. Within even the simplest of nervous systems rhythmic behaviors are not hard-wired, and neuromodulation imbues networks with the flexibility to generate multiple patterns of motor output (Marder, 2012; Gutierrez et al., 2013). Dopamine modulates every aspect of the pyloric circuit including intrinsic membrane properties such as I_A (Harris-Warrick et al., 1995; Kloppenburg et al., 1999), I_h (Peck et al., 2006), I_{CAN} (Kadiri et al., 2011), $I_{K(V)}$ (Gruhn et al., 2005), the strength and dynamics of graded and spike-dependent synaptic transmission (Johnson and Harris-Warrick, 1990; Johnson et al., 2005, 2011; Kvarta et al., 2012) and even properties of the axon spike initiation zone itself (Bucher et al., 2003). It is pertinent to acknowledge that dopamine produces concentration dependent rhythms in both vertebrates and invertebrates (Clemens et al., 2012) and therefore the state of the network can be affected by neuromodulatory tone. Harris-Warrick and Johnson (2010) have reviewed data demonstrating that dopamine can produce opposing effects on multiple conductances in individual neurons of the circuit. This complex modulation depends on the cell type, but the central idea is that dopamine prevents (at least in the STG) runaway modulation of the circuits. Less is known about the role dopamine plays in the gastric mill rhythm. We still do not fully understand the role of dopamine within this circuit; a sobering realization considering the complexity of spinal cord CPG circuits. What we can learn from work on invertebrates is that a neuromodulator can have a strong influence on individual cells, and even differential effects on individual cells in a circuit. However, if we are to understand the overall modulation of motor behavior we need to consider the combinatorial actions of many neuromodulators on network function and not simply one in isolation. There is another factor at play, namely that network output often remain remarkably robust despite neuromodulatory inputs. Furthermore, a change in network output pattern can be elicited in multiple ways through degenerate mechanisms (Gutierrez et al., 2013). This work is an important extension of previous findings showing that a large number of solutions exist even within a simple circuit for the production of a single behavior (Prinz et al., 2004).

Work from the laboratory of Karen Mesce in the medicinal leech (*Hirudo Medicinalis)* illustrates that dopamine has a profound effect on locomotor behaviors, highlighting its ability to bias the network toward a particular motor output or reinforce an ongoing behavior. Specifically, dopamine acts as a command signal to elicit crawling along with suppression of swimming (Puhl and Mesce, 2008; Crisp et al., 2012; Puhl et al., 2012), and is an excellent example of the ability of dopamine to bias locomotor behavior (Crisp and Mesce, 2004). Dopamine has a similar effect in nematodes (e.g., *Caenorhabditis elegans)* where it also acts to bias locomotor activity to a crawl pattern of activity over swimming (Vidal-Gadea et al., 2011). Similar to neuromodulation of the STG, dopamine could be acting on overlapping populations of neurons that are involved in two separate behaviors (Briggman et al., 2005), biasing circuit configuration output toward one output over the other.

DOPAMINE'S ROLE IN LOCOMOTION: AQUATIC AND AMPHIBIOUS SPECIES

In the lamprey, dopamine elicits a complex modulatory effect on swimming behavior similar in some respects to rhythmically active motor behaviors of invertebrates. Spinal dopamine is released from a number of sources including small cells located around the central canal that send projections into the CSF of the central canal (Ochi et al., 1979; McPherson and Kemnitz, 1994; Pierre et al., 1997), and also in a more ventrally-located plexus of cells that co-release serotonin and interact with the complex dendritic process of motor neurons (Schotland et al., 1995). There are also descending dopaminergic projections from the hypothalamus that may play a role in modulating spinal networks, but their role with respect to locomotion is not well understood (Barreiro-Iglesias et al., 2008). Therefore, the lamprey exhibits both intrinsic and extrinsic dopaminergic neuromodulation of spinal circuits. Because of the co-release of locally-produced spinal dopamine and serotonin, their effect will be discussed in parallel. When bath applied to the spinal cord *in vitro*, serotonin reduces the frequency and increases the amplitude of locomotor bursting activity in a dose dependant manner (Harris-Warrick and Cohen, 1985), an effect that is mediated by 5-HT_{1A} receptors and is readily reproduced in the freely swimming animal (Kemnitz et al., 1995). These effects are elicited by presynaptic inhibition of descending Muller cells which would result in reduced descending excitation of locomotor circuits (Buchanan and Grillner, 1991; Shupliakov et al., 1995). In addition, serotonin also directly reduces the late afterhyperpolarization (AHP) in motor neurons, lateral interneurons (van Dongen et al., 1986), crossed caudal commissural interneurons and giant interneurons (Wallén et al., 1989). Dopamine appears to have more complex effects, whereby low concentrations (0.1–10 μM) result in an increase in locomotor frequency, higher concentrations (10–100 μM) slow the rhythm, and concentrations as high as 100 μM–1 mM can suppress the rhythm (Harris-Warrick and Cohen, 1985; McPherson and Kemnitz, 1994; Schotland et al., 1995; Svensson et al., 2003). The increase in locomotor frequency can be reproduced in freely swimming animals (Kemnitz et al., 1995) and is believed to be mediated by selective D_2 receptor mechanisms (McPherson and Kemnitz, 1994). It was initially proposed that this effect was exerted by decreasing the calcium-dependant potassium channel (SK_{Ca}) and reducing the late component of the AHP in neurons such as edge cells, dorsal cells and giant interneurons, although these cells are not strong contributors to rhythmogenesis. It is more likely that the influence on rhythm frequency is due to a reduction in inhibitory drive from inhibitory commissural interneurons (Kemnitz, 1997). Concentration dependent dopamine effects are also observed in tadpoles, where effects on mainly D_2 receptors were observed at low concentrations with D_1 effects emerging when concentrations of dopamine were increased (Clemens et al., 2012).

The slowing effect of dopamine on locomotor activity at higher concentrations was unclear until recently, but is now believed that it may be acting on convergent mechanisms with that of 5-HT. Both 5-HT_{1A} and D_2 receptors appear to reduce post-inhibitory rebound (PIR) on inhibitory commissural interneurons that generate left-right alternation of swimming by reducing the calcium conductance through the $CaV_{1.3}$ channel (Hill et al., 2003; Wang et al., 2011). Assuming the organization of a classical half center, the authors suggested that the reduction in PIR would decrease the efficiency of the transition from inhibition to excitation, thus reducing the overall frequency of the alternating pattern.

In contrast to the lamprey, spinal dopamine appears to have a transient effect on swimming in the larval zebra fish with depressive effects at 3 days post fertilization (dpf) becoming less potent by 5 dpf (Thirumalai and Cline, 2008). Further work demonstrates that dopamine contributes to the development and maturation of locomotor networks in the spinal cord (Lambert et al., 2012). The sole source of spinal dopamine in the zebrafish is the Orthopedia (transcription factor, *otp*) neurons of the midbrain forming the dopaminergic diencephalospinal tract (DDT) that send descending projections to the spinal cord (McLean and Sillar, 2004a,b). *Otp* transcription factors are conserved in mammalian species and are expressed in the A11 dopaminergic neurons of the mouse, and analogous areas of the zebrafish, which project to the spinal cord (Ryu et al., 2007). In the zebrafish, it appears that these neurons develop around 3 dpf and act on the D_4 receptor to reconfigure the locomotor network to generate a more mature form of locomotor behavior; by 4 dpf the actions have switched, from spontaneous swim episodes consisting of infrequent, long duration bursts to frequent and short duration bursting episodes of swimming (Lambert et al., 2012). Such a modification in locomotor behavior is presumably critical for survival, as it would allow the animal to engage in more active locomotor behaviors associated with foraging whereas the immature form is more directed toward the escape from larger predatory fish. More recent investigation of the spinal dopaminergic system in the zebrafish has demonstrated that the D_{4a} receptor also acts on neural progenitor cells via sonic hedgehog signaling to promote motor neuron generation over V2 interneurons during the first 24–48 h post fertilization; an effect that can be recapitulated to promote neuronal regeneration following spinal cord injury (SCI; Reimer et al., 2013). Dopamine does not work alone in shaping the motor network during development. There is evidence that the descending serotonergic system also plays an important role in the development of a mature swimming pattern after the switch occurs at 4 dpf (Brustein et al., 2003). This points to the possibility that dopamine may play a role in promoting the network shift and serotonin may play more of a role in the modulation of a more mature network in the zebrafish. This is consistent with the idea proposed from work in the STG whereby different monoamines likely act simultaneously and rarely on their own.

More is known with respect to the monoaminergic modulation of locomotor networks by serotonin in the *Xenopus* tadpole. In the larval *Xenopus*, the modulatory role of serotonin on fictive swimming activity is to increase burst duration and intensity with no effect on cycle period, thus promoting a stronger swimming pattern (Sillar et al., 1998). This is in contrast to the noradrenergic system which acts to bias the motor output toward a lower frequency and weaker bursting pattern of activity (Sillar et al.,

1998). Similar to the lamprey (Wang et al., 2011), both serotonin and noradrenaline modulate locomotor activity through converging mechanisms. However, in the X*enopus* they influence the strength of inhibitory post synaptic potentials evoked by glycinergic commissural interneurons projecting to motor neurons via modulation of presynaptic release of glycine from commissural terminals (McDearmid et al., 1997). More specifically, serotonin reduces release of glycine whereas noradrenaline increases glycine release from these terminals onto motor neurons (McDearmid et al., 1997). In the larval X*enopus* locomotor network, dopamine reduces locomotor activity via D_2-like mechanisms at low concentrations (1–5 μM) and promotes locomotor activity at higher concentrations (10–50 μM) via D_1-like mechanisms (Clemens et al., 2012). The precise neuronal populations being acted on are unknown. The development of limbed locomotion in the frog from swimming in the tadpole affords a unique opportunity to study the developmental and potentially evolutionary reconfiguration of the neuronal networks mediating the two different, yet similar, forms of locomotion. In the metamorphosing froglet, with both immature limbs and a tail for swimming still intact, both noradrenaline and serotonin bias locomotor network output controlling either the tail or the developing legs. Specifically, serotonin acts to slow rhythmic tail-associated swimming activity and speeds up the limb-associated activity, whereas noradrenaline will speed up tail-associated swimming activity and slow down limb-associated activity. This provides yet another example of opposing aminergic modulation of distinct spinal locomotor circuits and their functional coupling during amphibian metamorphosis (Rauscent et al., 2009). These are similar to modulatory interactions influencing the pyloric and gastric mill rhythms in the STG (Gutierrez et al., 2013) and expression of swimming and crawling behaviors in the medicinal leech (Crisp and Mesce, 2004; Puhl and Mesce, 2008).

BIOCHEMICAL AND NEUROANATOMICAL ASPECTS OF DESCENDING DOPAMINERGIC SYSTEMS IN MAMMALS

Walking behavior in terrestrial legged mammals recruits up to 80 individual muscles. While the activity of all of them has not been measured during walking, all indications are that each muscle generates a uniquely patterned burst. This adds another layer of complexity to aquatic based species by adding on components of the network that produce not only left-right alternation but also coordination of flexor and extensor muscles within limbs. In mammalian systems, the role of dopamine in rhythmic motor behaviors is less well understood. That said, data collected thus far suggest a modulatory role qualitatively similar to that observed in the lamprey.

CATECHOLAMINE-CONTAINING CELLS

Around the same time that the robust influence of monoamines on long-latency flexor reflex afferents and their relationship to half-center function was being discovered in mammalian motor systems in the early 1960's (Jankowska et al., 1967a,b; Grillner and Zangger, 1979; Baker et al., 1984), nuclei of serotonin and catecholamine-containing cells were described in the mammalian midbrain and hindbrain. These findings were largely based on approaches that utilized histochemical staining against dopamine and noradrenaline (Carlsson et al., 1962; Dahlström and Fuxe, 1964b), later using more modern immunohistochemical approaches to detect the presence of the enzyme tyrosine-hydroxylase (TH; Hökfelt et al., 1984b). Based on such criteria, these cells were coined aminergic cells, or A-cells, and permitted discrete nuclei to be identified and named A1–A17 (Carlsson et al., 1962; Dahlström and Fuxe, 1964a; Hökfelt et al., 1984a). Advances in immunohistochemical methods were later used to identify cells that express other enzymes involved in the synthesis of catecholamines such as aromatic amino acid decarboxylase (AADC), dopamine-β-hydroxylase (DβH) and phenylethanolamine-N-methyl-transferase (PNMT; **Figure 1**). These developments allowed for further characterization between the types of catecholamine-producing cells. The original view that catecholamine-producing cell types consist of dopaminergic, noradrenergic, and adrenergic classes has been expanded and

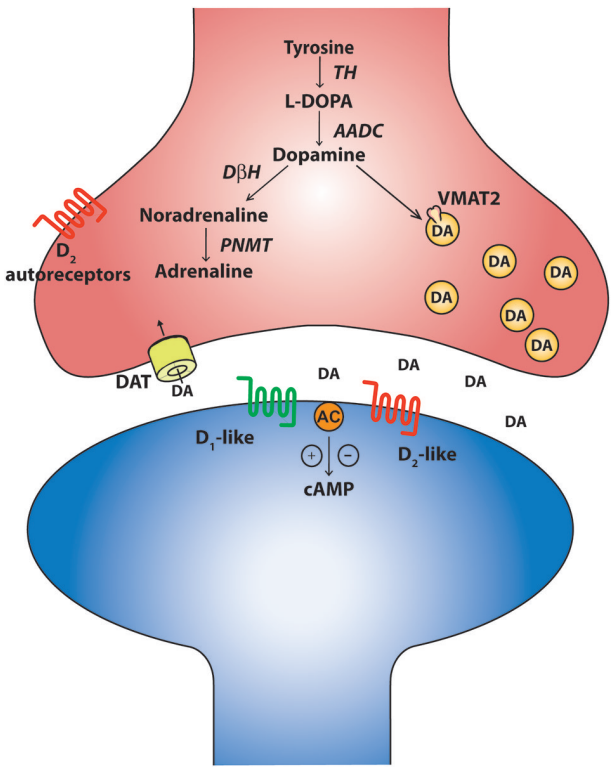

FIGURE 1 | The canonical catecholaminergic neuron is one that generates dopamine, noradrenaline or adrenaline. Catecholamines are synthesized from tyrosine through a series of biosynthetic steps progressing from tyrosine hydroxylase (TH) producing L-DOPA, aromatic amino acid decarboxylase (AADC) producing dopamine, dopamine-β-hydroxylase (DBH) producing noradrenaline and phenylethanolamine-N-methyl-transferase (PNMT) producing adrenaline. These neurons also express the vesicular monoaminergic transporter (VMAT2) to pump catecholamines into synaptic vesicles. A canonical dopaminergic neuron will also express dopamine reuptake transporters (DAT) and inhibitory D_2 autoreceptors to regulate presynaptic release of dopamine. Post synaptic targets of dopamine include the excitatory D_1-like and inhibitory D_2-like receptors that act through G-protein second messenger cascades, increasing and decreasing intracellular cAMP levels respectively.

there is evidence of enzymatic diversity within each class. For example, some TH positive cells lack any enzymes for conversion to traditional catecholamine neurons. Others have a portion, but not all, of the enzymatic machinery. A prime example of these cells are the D-cells which contain AADC but lack TH and are situated around the central canal of the spinal cord (Jaeger et al., 1983). They are similar in location and structure to the dopamine-producing LC cells in the lamprey spinal cord (Ochi et al., 1979; McPherson and Kemnitz, 1994; Pierre et al., 1997) and the $AADC^{+}/TH^{-}$ D cells of the zebrafish spinal cord (Chatelin et al., 2001). While the function of these cells is not clear, the bottom line is that catecholaminergic phenotypes are more complex than originally thought. Nevertheless, cells that express TH, AADC and are DβH negative can still provide a good indication that the cells may be dopaminergic whereas the DβH positive cells could be either noradrenergic or adrenergic. Such criteria permitted for the identification of nine major dopaminergic nuclei in the brain (Björklund and Dunnett, 2007). The canonical dopaminergic cell would also express machinery for dopamine release such as a vesicular monoaminergic transporter (VMAT2) to pump dopamine into vesicles, a reuptake transporter like the dopamine transporter (DAT) and a means of regulating release of dopamine such as presynaptic D_2 autoreceptors (Ugrumov, 2009). It is important to note however that some dopamine neurons differentially express D_2 autoreceptors. The mechanisms of actions at the soma and terminals may also be different (G protein-coupled inwardly-rectifying potassium channel (GIRK) versus Kv1.2 respectively) and some TH^{+} neurons may not even express them at all (Pappas et al., 2008; Ford, 2014). Therefore, expression of D_2 autoreceptors may not be a pre-requisite of a canonical dopamine cell, but may be a common feature. In fact, diversity appears the rule rather than the exception for catecholaminergic cell types (Ugrumov, 2009).

DESCENDING CATECHOLAMINERGIC PROJECTIONS

The predominant sources of spinal dopamine and noradrenaline in mammals are the descending fibers projecting from the dopaminergic A10 and A11 nuclei of the posterior hypothalamus (Björklund and Skagerberg, 1979; Lindvall et al., 1983; Skagerberg and Lindvall, 1985; Qu et al., 2006; Pappas et al., 2008), the A13 of the dorsal hypothalamus (Blessing and Chalmers, 1979) and the pontine noradrenergic A5, A6 (locus coeruleus) and A7 nuclei (Fritschy and Grzanna, 1990; Clark and Proudfit, 1991; Bruinstroop et al., 2012). While these regions appear to be conserved in many mammalian species including primates and humans (Moore and Bloom, 1979; Barraud et al., 2010), we will focus on these regions with emphasis on rodent anatomy as they are best described in these species. The A11 is a small nucleus in the posterior hypothalamus consisting of approximately 150–300 neurons (**Figure 2**) and is the primary source of spinal dopamine in mammalian species. A11 neurons are thought to be L-DOPAergic based on the absence of AADC in non-human primates, although the evidence in rodents is mixed (Barraud et al., 2010). This suggests a possibility that L-DOPA release in the spinal cord of the non-human primate could be converted to dopamine by other cells using spinally located AADC such as that present in the D cells near the central canal (Jaeger et al., 1983). It appears that dopaminergic neurons in the A11 go against what a canonical dopaminergic neuron would look like, since A11 neurons lack the reuptake transporter DAT (Lorang et al., 1994; Ciliax et al., 1999) as well as D_2 auto-receptors (Pappas et al., 2008). The lack of D_2 autoreceptors would presumably increase release probability for dopamine as well as TH enzyme activity (Kehr et al., 1972; Wolf and Roth, 1990; Benoit-Marand et al., 2001; Ford, 2014). One possibility is that the A11 phenotype serves as a compensatory adaptation given their few numbers in order to prolong the release, and increase synthesis, of dopamine to their targets in the ventral horn.

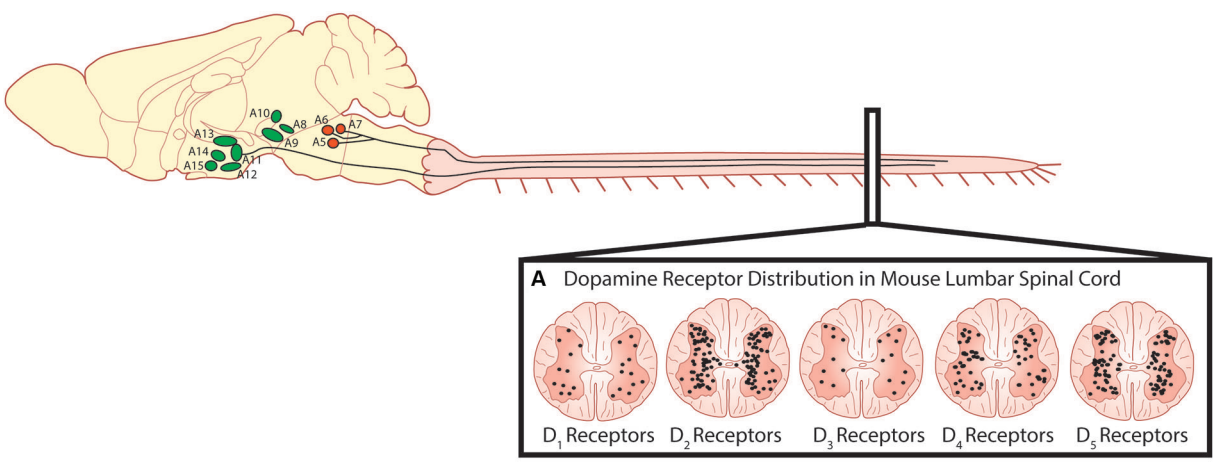

FIGURE 2 | Descending dopaminergic fibres within the spinal cord originate in the A11. (A) Dopamine acts on all five dopamine receptors that are distributed non-uniformly through the dorsal and ventral horns of the spinal cord (Adapted with permission from Zhu et al., 2007). Descending noradrenergic fibres originating in the A5, A6 and A7 nuclei of the pons innervate the spinal cord. Schematic adapted with permission from Björklund and Dunnett (2007).

Although the A11 connectome is not well characterized, it has been shown that the A11 receives inputs from a number of regions including lateral septum, parabrachial nucleus, infralimbic cortex and the bed nucleus of the striata terminalis (Abrahamson and Moore, 2001; Qu et al., 2006). The A11 also receives inputs from the suprachiasmatic nuclei (SCN), suggesting that A11 neurons may be modulated in a circadian pattern (Zhao et al., 2007). There is evidence that this might be the case as the expression of the rate limiting enzyme TH is modulated in a circadian pattern, generating high levels of spinal dopamine during awake periods (Hammar et al., 2004). It has also been suggested that AADC is expressed in a circadian pattern, which may also describe the variable findings to date (Björklund and Dunnett, 2007; Barraud et al., 2010).

The best characterized of the A11 efferent projections are the descending axons that travel through the dorsal longitudinal fasciculus of Schutz, located in the periaqueductal gray, and descend unilaterally through the dorsolateral funiculus into the superficial dorsal horn. A small number of axons also descend in the regions around the central canal and into the ventral horn (Björklund and Skagerberg, 1979; Commissiong et al., 1979; Skagerberg and Lindvall, 1985), with higher densities in the lumbar region relative to the thoracic regions (Pappas et al., 2008). Several examples have been provided by Pappas et al. (2008, 2010), indicating sexual dimorphism of the descending A11 system whereby males have a greater number of dopaminergic neurons and a higher density of descending fibers with no apparent difference in dopamine metabolism (Pappas et al., 2008, 2010). Overall, it has been suggested that this dimorphism, which is androgen-dependant (Pappas et al., 2010), may contribute to the higher female prevalence of restless leg syndrome, a motor disorder associated with impairments in the spinal dopaminergic system (Pappas et al., 2008). To better understand how the A11 exerts its influence on locomotor behavior, the connectivity of the A11 with other locomotor-related regions in the brain needs to be established. In addition to the spinal cord, A11 neurons project collaterals to the dorsal raphe nucleus and the prefrontal cortex (Peyron et al., 1995). It is possible that other targets are likely.

In parallel to A11 projections, noradrenergic fibers of the A5, A6 and A7 travel through the lateral and ventral funiculi and dorsal surface of the dorsal horn. A particularly high density of fibers from the A5 can be found in the lateral region, A6 in the dorsal and ventral regions and A7 mainly in the lateral region. Previously the termination sites of descending noradrenergic projections were controversial (Fritschy and Grzanna, 1990; Clark and Proudfit, 1991). More recent transgenic approaches have revealed that the A5 regions provides the densest innervation of the thoracic sympathetic preganglionic neurons, A6 densest in the dorsal horn at all levels of the spinal cord and the A7 densest in the ventral horn at all levels of the spinal cord (Bruinstroop et al., 2012). Such an organization would suggest that the A5 nucleus would exert more of an influence over autonomic functions, A6 over modulation of sensory input and A7 to motor function. Interestingly, in regards to dopamine innervation of the spinal cord, there is also a clear autonomic innervation of the intermediolateral nucleus of the spinal cord from the A11. Together with noradrenaline this suggests multiple effects of catecholamines on most inputs and outputs of the spinal cord.

Both descending dopaminergic and noradrenergic projections to the spinal cord share similarities in terms of fiber distribution and points of innervation within the gray matter of the spinal cord, but there are differences in terms of fiber density. Dopaminergic fibers appear to develop slower than noradrenergic fibers. Noradrenergic fiber density appears to peak at 14 days after birth with some pruning taking place up to early adulthood. On the other hand dopamine fiber development occurs slowly throughout development in the spinal cord, peaking at early adulthood (Commissiong, 1983). Generally speaking, the descending fibers heavily innervate the dorsal horns where catecholaminergic influence is exerted via diffuse paracrine release, whereas relatively less innervation of the ventral horn is reported, specifically in lamina IX where direct synaptic connections are made on medium to large dendrites with few axosomatic synapses of motor neurons (Yoshida and Tanaka, 1988; Rajaofetra et al., 1992; Ridet et al., 1992). Much more is known about the nature of synaptic interaction of motor neurons by the descending noradrenergic and serotonergic fibers. Noradrenergic and serotonergic input to motor neuron pools have typically been described as diffuse (Heckman et al., 2008; Johnson and Heckman, 2010); however, recently the nature of synaptic targets was further described for noradrenergic and serotonergic neurons in cat splenius motor neurons (Montague et al., 2013) and appear to be concentrated on small diameter distal dendrites of motor neurons with few located on somal targets. This distribution implies compartmentalization of synaptic contacts, which has also been suggested as playing an important role in regulating the input-output properties of motor neurons (Montague et al., 2013).

TARGETS OF SPINAL DOPAMINE

Dopamine receptors are G-protein coupled receptors and are divided into two subfamilies; the D_1-like receptors (D_1 and D_5) and the D_2-like receptors (D_2, D_3, and D_4). Generally, activation of the D_1-like receptor subfamily elicits excitatory effects through a stimulatory G-protein ($G_{s\alpha}$) interacting with adenyl cyclase, subsequently increasing intracellular cAMP levels. In contrast, activation of the D_2- receptor subfamily hyperpolarizes the cell membrane through an inhibitory G-protein ($G_{i\alpha}$) to close calcium channels, open potassium channels and reduce intracellular cAMP levels (Missale et al., 1998). In the rodent, dopamine released from terminals in the lumbar spinal cord can act on all five dopamine receptors (D_1–D_5; **Figure 2**) which are non-uniformly distributed across the transverse lumbar spinal cord (Zhu et al., 2007). Similar to other monoamines, the distribution of receptors is species dependent (Barraud et al., 2010) and their density is likely also developmentally regulated as dopaminergic receptors are in other areas of the brain (Tarazi and Baldessarini, 2000). Little is known in respect of the rostrocaudal distribution but higher levels of dopamine are found in cervical, opposed to lumbar, segments of the spinal cord (Karoum et al., 1981). In the juvenile mouse, D_2-like receptor subtypes are strongly expressed in lamina I-III of the dorsal horn with the predominant subtype being the D_3 receptor

(Levant and McCarson, 2001). This distribution has been suggested to mediate dopamine's anti-nociceptive effects. D_1-like receptors are most strongly expressed in the ventral horn where the motor circuits reside with motor neurons in particular, expressing all five receptor types (Zhu et al., 2007). While *in situ* hybridization techniques have been able to describe the location in the spinal cord where dopamine receptors are expressed, what remains unknown is whether receptors are compartmentalized within different regions of the identified neurons such as is the case for noradrenergic and serotonergic receptors in cat motor neurons (Montague et al., 2013). These factors could contribute to complex modulation of motor neuron input-output properties and network-based effects reflected by dopamine's complex effects on locomotor activity. More recently, when the non-human primate spinal cord was examined for dopaminergic expression, a different distribution pattern from rodents was observed. Specifically, no evidence for D_1 mRNA was observed anywhere in the spinal cord, although D_5 mRNA was found in the dorsal horn. Overall there appears to be a dorsal horn emphasis for dopamine expression in non-human primates compared to rodents (Barraud et al., 2010).

FUNCTIONAL EFFECTS OF SPINAL DOPAMINE ON LOCOMOTOR NETWORKS

Descending catecholamine release has been observed during locomotion (Gerin et al., 1995; Gerin and Privat, 1998) and both dopamine and noradrenaline are capable of promoting locomotion when exogenously introduced to the intact animal (Barbeau and Rossignol, 1991, 1994; Rossignol et al., 1998). That said, a large amount of what we know about the influence of dopamine on the spinal locomotor networks has been derived from *in vitro* investigations of fictive locomotor activity of the neonatal rodent spinal cord studied in isolation (Whelan et al., 2000; Barrière et al., 2004; Gordon and Whelan, 2006; Humphreys and Whelan, 2012; Christie and Whelan, 2005). Unlike serotonin, which is a potent activator of the locomotor central pattern generator (CPG) network across a variety of mammalian species (Harris-Warrick and Cohen, 1985; Schmidt and Jordan, 2000; Madriaga et al., 2004; Liu and Jordan, 2005; Gabriel et al., 2009), dopamine alone can elicit fictive locomotor activity in the rat (Kiehn and Kjaerulff, 1996; Barrière et al., 2004, but not in the mouse, although D_1 agonists alone appear to be sufficient for locomotion (Sharples et al., 2013). Bath application of dopamine on its own is sufficient to evoke rhythmic motor activity. However, the low frequency rhythm does not resemble a functional locomotor pattern characterized by left-right and flexor-extensor alternation (Sqalli-Houssaini and Cazalets, 2000; **Figure 3**). The differential observations with respect to the ability of dopamine and D_1 agonists to evoke locomotor activity are not yet clear. One possibility is that non-specific binding of dopamine to both D_1-like and D_2-like receptor subtypes may act to suppress the full expression of locomotor activity. Noradrenaline does not appear to be capable of eliciting stable locomotor activity in either the isolated rat or mouse spinal cord (Kiehn et al., 1999; Sqalli-Houssaini and Cazalets, 2000). The reasons for the differences between rat and mice in the case of dopamine are unknown but, in the cases where locomotor activity cannot be produced, these two catecholamines generally elicit robust modulatory effects on the lumbar networks (Kiehn et al., 1999; Sqalli-Houssaini and Cazalets, 2000; Gordon and Whelan, 2006; Humphreys and Whelan, 2012).

The behavior of the catecholamines on mammalian motor rhythms should not be surprising in the context of what has been described in the STG where, in all likelihood, neuromodulators influence rhythmic motor behaviors in parallel and rarely in isolation (Marder, 2012). This combinatorial neuromodulation is more evident when ongoing locomotor activity elicited by serotonin and NMA/NMDA is examined where both dopamine and noradrenaline reduce the frequency of the rhythm and increase burst amplitude, resulting in an overall more robust rhythm (Kiehn and Kjaerulff, 1996; Sqalli-Houssaini and Cazalets, 2000; Barrière et al., 2004; Gordon and Whelan, 2006; Humphreys and Whelan, 2012). In other words, these two catecholamines may be promoting ongoing locomotor activity and these qualities are often exploited by incorporating dopamine into the "locomotor cocktail" to elicit robust locomotor activity in investigations that deploy *in vitro* isolated spinal cord models (Whelan et al., 2000).

While neurochemical activation of neonatal locomotor circuits has provided a great amount of insight into the function of locomotor circuits, studies that use models that allow for activation of endogenous neuromodulators are few. In particular, the generation of new models is lacking that allow activation of *in vitro* circuits by direct stimulation of supraspinal nuclei. While these types of studies have been published for serotonin through stimulation of the parapyramidal region of the medulla (Liu and Jordan, 2005), comparable studies have not been performed for catecholamine systems. Furthermore, *in vivo* and developmental studies are required to establish whether dopamine release onto spinal circuits is *necessary* for locomotor activity. This is especially true in neonatal rodents where monoaminergic innervation takes place over 3 weeks following birth (Commissiong, 1983; Bregman, 1987; Rajaofetra et al., 1989).

The receptor mechanisms that mediate the network-based effects of dopamine on locomotor activity are not fully understood. It is likely that dopamine is acting to promote locomotor activity through excitatory influences of D_1-like receptors in rodents, whereas the slowing effect on fictive locomotor frequency is through inhibitory D_2-like receptor mechanisms. Indeed, there is consistent evidence across adult and neonatal preparations that the D_1-like receptor subfamily promotes locomotor activity (Barrière et al., 2004; Lapointe and Guertin, 2008; Lapointe et al., 2009). In contrast, much less is known regarding the role of D_2-like receptors in the control of locomotion (Barrière et al., 2004) despite their presence in the ventral horn (Zhu et al., 2007). Activation of the D_2-like receptor subfamily suppresses recurrent excitatory feedback to the locomotor network, most likely via presynaptic mechanisms (Maitra et al., 1993; Humphreys and Whelan, 2012), but the functional role in the modulation of rhythmically active motor circuits in the mammalian spinal cord remains elusive. Further dissection of these receptor mechanisms requires examination of the dopaminergic influence of individual spinal reflex circuits and known components of the spinal locomotor network. Dopamine does elicit potent effects on spinal

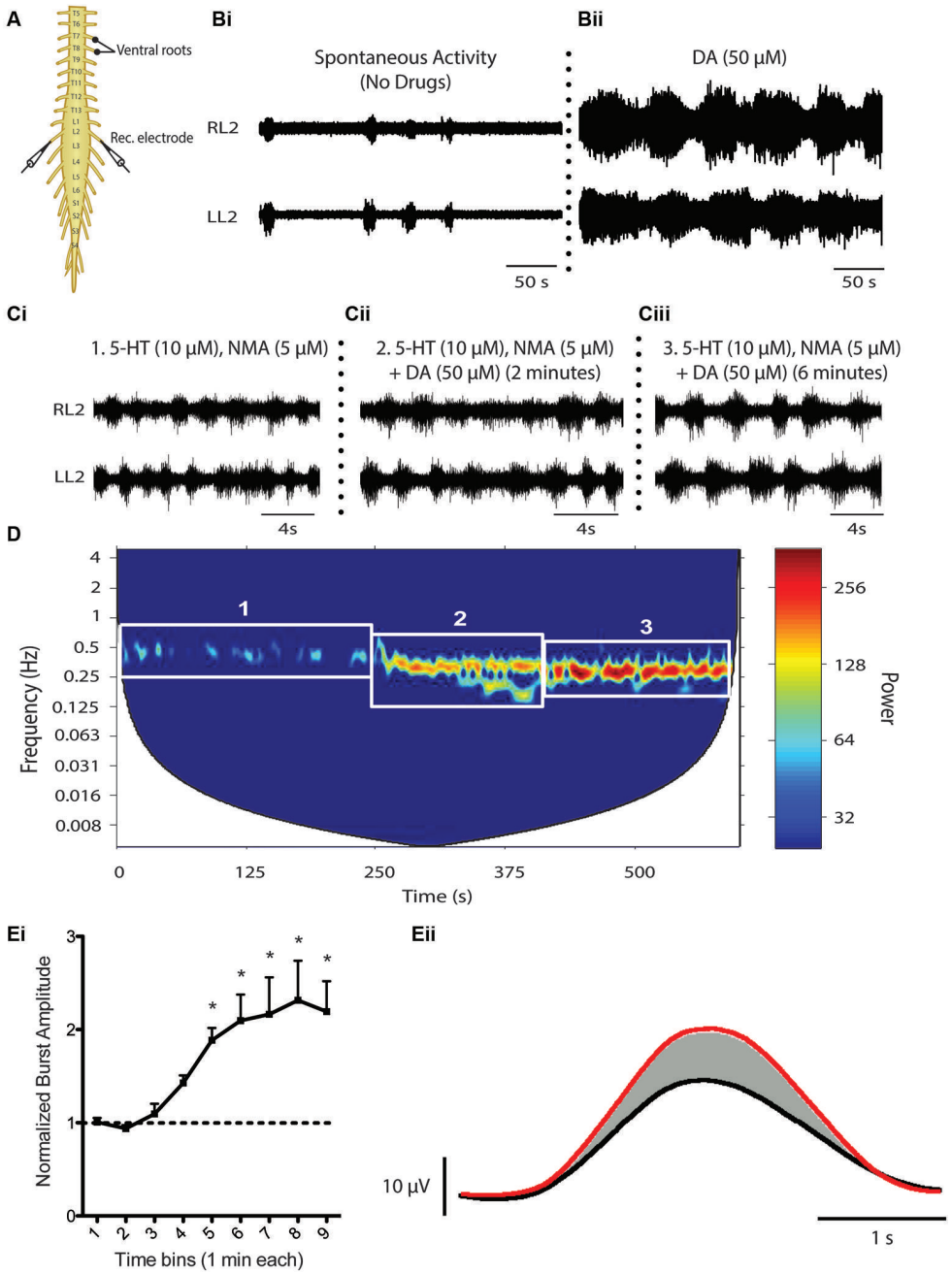

FIGURE 3 | Dopaminergic modulation of spinal CPG activity. **(A)** Schematic of an *in vitro* isolated neonatal mouse spinal cord showing ventral root neurograms from the left and right L2 segment. **(B)** Spontaneous activity **(Bi)** is converted to a rhythmic slow non-locomotor rhythmic pattern **(Bii)** following bath application of dopamine (DA). **(C and D)** When DA is bath-applied during ongoing locomotor activity elicited by 5-HT and NMA it stabilizes and reduces the frequency of the rhythm. The spectrogram **(D)** depicts a cross-wavelet analysis of a locomotor rhythm evoked by 5-HT and NMA (box 1) and effect of dopamine (DA) on the pre-existing rhythm (box 2 and 3). Rhythm frequency is displayed on the *y*-axis and rhythm power displayed as warm or cool colors with warmer colors representing higher power or more stable rhythm. Dopamine also increases burst amplitude **(Ei** and **Eii)**. **(Ei)** Graph displays an increase in burst amplitude over a 10 min period immediately following the addition of dopamine and **(Eii)** shows an average L2 neurogram burst from a representative experiment with bursts evoked by 5-HT and NMA (black) and following addition of dopamine (red) (Adapted from Humphreys and Whelan, 2012).

sensorimotor reflex circuits and has been an area of interest in the context of restless leg syndrome (Carp and Anderson, 1982; Jensen and Yaksh, 1984; Tamae et al., 2005; Barriere et al., 2008; Keeler et al., 2012). D_3 receptor suppression of primary

afferent input to the dorsal horn has been suggested to mediate dopaminergic suppression of the monosynaptic reflex (Hammar et al., 2004) and has been implicated with restless leg syndrome given that D_3 receptor agonists exert potent therapeutic effects at alleviating motor hyperactivity (Manconi et al., 2011). D_2-mediated inhibition of primary afferents and post synaptic neurons in the substantia gelatinosa (Tamae et al., 2005) have been suggested to be anti-nociceptive acting at the dorsal horn; an influence that would work in parallel to excitation of motor circuits to promote motor function. What is not well understood is how dopamine may modulate proprioceptive inputs to spinal cord locomotor circuits. In work by Jankowska on the cat, noradrenergic and serotonergic modulation is complex and consists of both excitatory and inhibitory components onto commissural interneurons (Hammar et al., 2004). Similar heterogeneity of dopamine actions is likely but remains underexplored. What is known is that L-DOPA, whether exerting its effect through dopaminergic or noradrenergic systems, modulates afferent reflex circuits by shutting down short latency reflexes and opening up long latency reflexes (Jankowska et al., 1967a,b). This classic work by Anders Lundberg and colleagues showed systemic injection of L-DOPA could reveal an interneuronal network that could result in flexor-extensor alternating movements when flexor and extensor afferents were stimulated simultaneously. This was taken as cellular evidence for Graham-Brown's half-center concept (Graham-Brown, 1911; Stuart and Hultborn, 2008).

Dopamine has also been shown to modulate synaptic connectivity and intrinsic properties of some known components of the locomotor network (Han et al., 2007; Han and Whelan, 2009). The ability of dopamine to promote ongoing locomotor activity may be due to combined effects exerted on motor neurons, premotor interneurons and Hb9-expressing interneurons that participate at all levels of the spinal locomotor network (**Figure 4**; Han et al., 2007; Han and Whelan, 2009). In the neonatal mouse, dopamine acts to depolarize motor neurons as well as premotor interneurons that project through the ventrolateral funiculus (Han et al., 2007 **Figure 4A**). It appears that dopamine is a necessary component to evoke stable rhythmic bursting activity in classes of genetically identified spinal interneurons (Hb9 cells; Hinckley et al., 2005; Wilson et al., 2005) when introduced with 5-HT and NMDA but is not sufficient to do so on its own (Han et al., 2007); however, other studies suggest that NMDA alone is sufficient (Masino et al., 2012). Dopamine also increases the excitability of motor neurons by reducing the potassium currents I_A and SK_{ca}, but the receptor mechanisms mediating this effect are not known (**Figure 4**; Han et al., 2007). In addition, dopamine increases synaptic AMPA currents onto motor neurons via D_1-like receptor-PKA mechanisms and is analogous to findings in embryonic chick motor neurons mediated by increases in kainite-gated channels (Smith et al., 1995). Together, the combination of increased intrinsic excitability and excitatory synaptic input to motor neurons facilitate motor output; however, it remains unclear why dopamine alone might not be sufficient to evoke locomotor like activity. While these studies are instructive they have only begun to scratch the surface of the function of catecholamines *in vivo*.

TARGETING MONOAMINERGIC SYSTEMS POST SCI TO PROMOTE RECOVERY

Motor impairment is particularly prominent following SCI when the influence from descending serotonergic, noradrenergic and dopaminergic inputs to the lumbar cord are lost or compromised. A number of approaches have been tested experimentally and clinically to promote recovery of motor function following injury and include promoting regeneration of damaged tracts (Hellal et al., 2011), reducing inflammation and subsequent secondary injury (Rowland et al., 2008; Kwon et al., 2011), and promoting plasticity within the spinal motor networks (Boulenguez and Vinay, 2009; Fong et al., 2009; Rossignol and Frigon, 2011). Because monoamines are potent activators of motor networks, they may serve as a suitable target for exciting and promoting recovery following an insult (Rémy-Néris et al., 1999).

Direct application of dopamine or noradrenaline is potentially a useful approach however both would need to be applied intrathecally to spinal segments. For these reasons either agonists or antagonists of catecholamine receptors that can cross the blood brain barrier have been investigated. In chronic spinalized cats, administration of an α_2 agonist, clonidine, was able to elicit walking in acute spinal cats and promote recovery of function in chronic spinal animals (Forssberg and Grillner, 1973; Barbeau and Rossignol, 1987; Chau et al., 1998), but had the opposite effect in spinalized rats (Musienko et al., 2011). In spinalized mice, locomotor movements were elicited following administration of agonists affecting the D_1 receptor system (Lapointe et al., 2009), and recent work demonstrates that administration of the D_2 agonist quinpirole can stabilize gait and facilitate flexion. From these studies we can conclude that dopaminergic agonists potentiate stepping with D_1 agonists in particular boosting extensor activity and weight-bearing support (Lapointe et al., 2009; Musienko et al., 2011). These data support findings of generalized excitation of motor neurons (Han et al., 2007; Han and Whelan, 2009), and stabilization of motor patterns with dopamine using *in vitro* mouse (Jiang et al., 1999; Whelan et al., 2000; Madriaga et al., 2004; Humphreys and Whelan, 2012) or rat (Barrière et al., 2004) preparations. Courtine and colleagues (Musienko et al., 2011) have recently shown that combinations of monoamines including agonists for 5-HT_{1A}, 5-HT_{2A}, D_1 and antagonists for NA (α_2) were particularly successful in producing locomotion that resembled normal stepping in spinalized rats, supported by data from *in vitro* preparations (Madriaga et al., 2004). A cautionary note is that tuning of these monoaminergic combinations will likely need to be performed before translation to humans in a non-human primate where the receptor expression resembles humans (Barraud et al., 2010). A final note on this topic is that there is evidence that monoamine receptors (5-HT_{2C}) are constitutively expressed for months following a SCI in rodents and upregulation of these receptors may contribute to functional improvement in locomotion following injury (Murray et al., 2010). It is not known whether catecholaminergic receptors could contribute in a similar manner but dopamine D_5 receptors do show high constitutive activity (Demchyshyn et al., 2000). D_5 receptors are known to be constitutively active in the rat subthalamic nucleus after 6-OHDA treatment of the medial forebrain

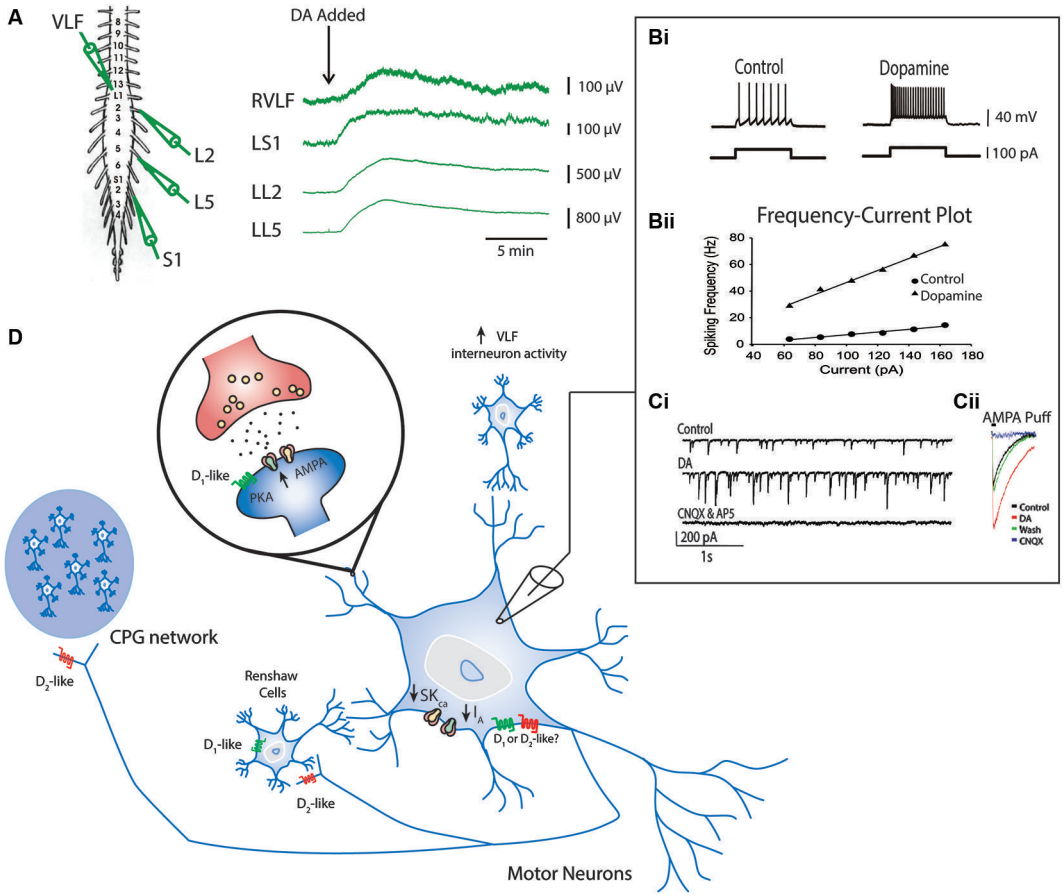

FIGURE 4 | Known effects of dopamine on cellular components of the locomotor network. Ventral root neurograms from the L2, L5 and S1 segments and ventrolateral funiculus (VLF) of a spinal cord isolation preparation with fast synaptic transmission blocked (CNQX, AP5, PTX and Strychnine) indicates that dopamine increases the excitability of motor neurons projecting through ventral roots and interneurons projecting through the VLF (A) (Adapted from Han et al., 2007). Intracellular recordings from motor neurons show that dopamine increases motor neuron excitability indicated by an increase in the slope of the frequency-current relationship **(Bi** and **Bii)**. This effect is in part mediated by a reduction in I_A and SK_{Ca} conductances. Dopamine also increases AMPA conductances **(Ci** and **Cii)** via a D_1-like receptor mechanisms (Adapted with permission from Han and Whelan, 2009). Dopamine reduces recurrent excitatory feedback to the locomotor CPG (Humphreys and Whelan, 2012) and Renshaw cells via D_2-like receptor mechanisms (Maitra et al., 1993). A summary of all these effects are depicted in panel **(D)**.

bundle. D_5 receptors are expressed in the spinal cord and this remains a possibility for intervention (Chetrit et al., 2013). In this regard, an early report of increased dopamine receptor dependent adenyl cyclase following SCI may be relevant (Gentleman et al., 1981).

Another approach is using L-DOPA that is converted to dopamine and noradrenaline in catecholamine axon terminals and within D cells in the spinal cord. Even though descending catecholamine axons tend to degenerate following injury, D-cells may compensate by upregulating AADC (Jaeger et al., 1983; Li et al., 2014). In spinalized mice, administration of L-DOPA and buspirone (dopamine and 5-HT_1 partial agonist) combined with apomorphine reliably elicited locomotor activity in mice with a complete spinal transection (Guertin et al., 2011). Work in decerebrate rats suggests that L-DOPA activation of locomotion is dopamine receptor dependent (Sickles et al., 1992; McCrea et al., 1997). In terms of translation, Maric et al. (2008) showed that oral administration of L-DOPA did not produce any observable change in locomotor recovery following SCI. It is not clear why L-DOPA may have proven ineffective but it is likely that a combination of multiple catecholaminergic agonists will need to be developed for humans in combination with other therapies. In this regard, the use of embryonic cells from the dopaminergic ventral tegmental region may also be a useful strategy, similar to approaches that have used embryonic noradrenergic locus coeruleus (Commissiong, 1984) or serotonergic raphe cells implanted into the injury site (Majczyński et al., 2005; Boido et al., 2009).

It is useful to consider new genetic approaches that target monoaminergic sites. The field of optogenetics has developed methods to target classes of cells such that they can be excited or inhibited with light. This is accomplished by the insertion of light sensitive cation channels (e.g., Channelrhodopsin-2) for activation or proton pumps (e.g., archaerhodopsins) for

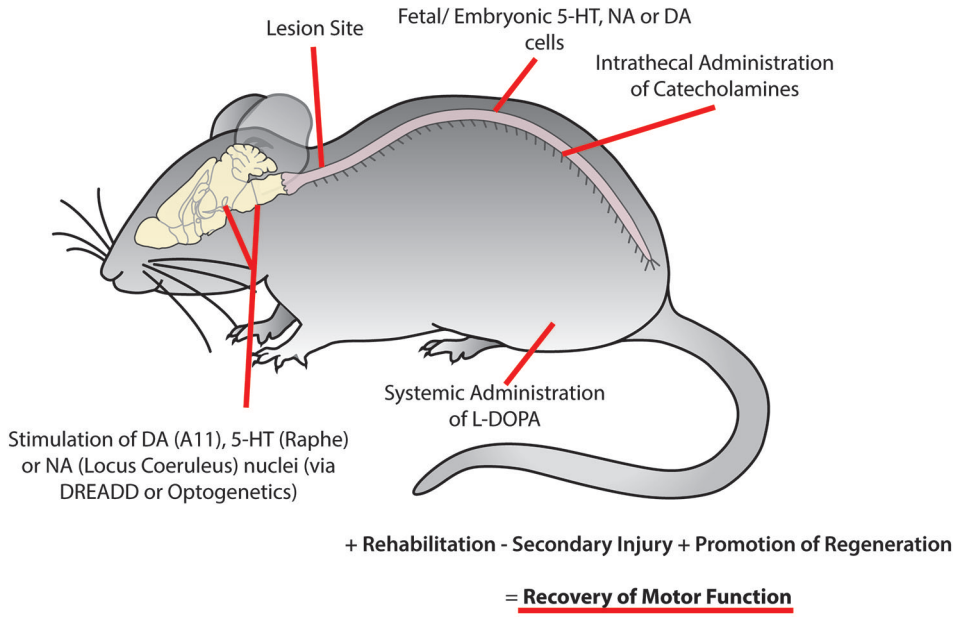

FIGURE 5 | Recovery of motor function following spinal cord injury may be facilitated using combinatorial therapies to promote plasticity within motor networks. This could be accomplished by targeting monoaminergic systems through the activation of descending brain nuclei using optogenetic or pharmacogenetic approaches, implantation of fetal or embryonic cells from the dopaminergic ventral tegmental area, noradrenergic locus coeruleus or serotonergic raphe nucleus caudal to the site of injury, intrathecal injection of catecholamines and systemic injection of L-DOPA. While these approaches may not be optimal in isolation, they may serve as an effective complementary treatment with rehabilitation and other therapies to reduce secondary injury and promote regeneration of damaged descending tracts to promote the recovery of motor function.

silencing of neuronal activity that can be expressed based on cell-specific transcription factors or promoter regions (for review see: Aston-Jones and Deisseroth, 2013). The use of pharmacogenetics which make use of engineered receptors that respond to non-endogenous ligands could address difficulties of translation, as optogenetic approaches require light fibers to be implanted and would most likely need to cover multiple cord segments. In addition, these non-endogenous ligands can be introduced intravenously, intraperitoneally or orally (for review see: Lee et al., 2014). The most commonly utilized form of this technology are the designer receptors exclusively activated by designer drugs (DREADDS), which are modified metabotropic muscarinic receptors that respond to the non-endogenous ligand clozapine-N-oxide (CNO). Similar to optogenetic approaches, these receptors can be inserted into specific cell types under transcriptional control of cell-specific genes. Neuronal activity can be facilitated through insertion of the hm3 receptor form or silenced by insertion of the hm4 receptor form (Shapiro et al., 2012) by administration of CNO. This approach is considerably simpler than optogenetics, since hardware need not be installed and the CNO can be administered systemically. Following administration of the CNO, effects can be observed within tens of minutes and can last for hours. The next day the CNO will be metabolized and one can then repeat the experimental protocol. Using this approach one can target supraspinal nuclei containing monoaminergic cells in patients with incomplete SCI or direct activation of spinal circuits (**Figure 5**). This approach has promise since it allows for the remote activation of targets by administering artificial ligands that bind selectively to the artificial receptors targeted to the monoaminergic nucleus of interest. Other approaches to consider are the use of OptoXRs which activate similar cAMP processes as native α_2 receptors (Airan et al., 2009). The OptoXR approach is light activated at the moment, but offers the opportunity to selectively upregulate α_2 receptors in motor neurons for example. This technique would directly target second messenger pathways in targeted cells. In all likelihood, a combination of supraspinal plus spinal activation and inactivation strategies will need to be deployed to achieve optimal results. In most cases mentioned the tools used combine Cre-driver mice lines coupled with floxxed viral vectors. However, if translation is a consideration then it should be noted that delivery of DREADDs, opsins, and optoXRs can be delivered using a viral vector with a cell specific synthetic promoter. For example, viral vectors with a TH synthetic promoter have been designed to trace dopamine neuron pathways (Oh et al., 2009). This proof-of-principle shows that this approach could be used in different species including, over the long-term, humans.

CONCLUSIONS

This review has focused on the role of dopamine in modulating locomotor centers in the spinal cord. Our knowledge of dopamine's contribution to monoaminergic locomotor drive in mammals is in its infancy. However, parallels to other vertebrate systems, such as the lamprey, clearly exist.

Tools to specifically target dopaminergic and other monoaminergic descending populations now exist. Data from

behaving animals suggests that while monoamines generally act to increase tone, they can act to promote specific patterns on their own. While the temptation is to examine each monoaminergic system in isolation, it will be necessary to examine combinatorial actions to start to understand the state-dependent role of monoamines in the freely moving animal.

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REFERENCES

Abrahamson, E. E., and Moore, R. Y. (2001). The posterior hypothalamic area: chemoarchitecture and afferent connections. *Brain Res.* 889, 1–22. doi: 10.1016/s0006-8993(00)03015-8

Airan, R. D., Thompson, K. R., Fenno, L. E., Bernstein, H., and Deisseroth, K. (2009). Temporally precise in vivo control of intracellular signalling. *Nature* 458, 1025–1029. doi: 10.1038/nature07926

Aston-Jones, G., and Deisseroth, K. (2013). Recent advances in optogenetics and pharmacogenetics. *Brain Res.* 1511, 1–5. doi: 10.1016/j.brainres.2013.01.026

Baker, L. L., Chandler, S. H., and Goldberg, L. J. (1984). L-dopa-induced locomotor-like activity in ankle flexor and extensor nerves of chronic and acute spinal cats. *Exp. Neurol.* 86, 515–526. doi: 10.1016/0014-4886(84)90086-4

Barbeau, H., and Rossignol, S. (1987). Recovery of locomotion after chronic spinalization in the adult cat. *Brain Res.* 412, 84–95. doi: 10.1016/0006-8993(87)91442-9

Barbeau, H., and Rossignol, S. (1991). Initiation and modulation of the locomotor pattern in the adult chronic spinal cat by noradrenergic, serotonergic and dopaminergic drugs. *Brain Res.* 546, 250–260. doi: 10.1016/0006-8993(91)91489-n

Barbeau, H., and Rossignol, S. (1994). Enhancement of locomotor recovery following spinal cord injury. *Curr. Opin. Neurobiol.* 7, 517–524. doi: 10.1097/00019052-199412000-00008

Barraud, Q., Obeid, I., Aubert, I., Barrière, G., Contamin, H., Mcguire, S., et al. (2010). Neuroanatomical study of the A11 diencephalospinal pathway in the non-human primate. *PLoS One* 5:e13306. doi: 10.1371/journal.pone.0013306

Barreiro-Iglesias, A., Villar-Cerviño, V., Anadón, R., and Rodicio, M. C. (2008). Descending brain-spinal cord projections in a primitive vertebrate, the lamprey: cerebrospinal fluid-contacting and dopaminergic neurons. *J. Comp. Neurol.* 511, 711–723. doi: 10.1002/cne.21863

Barriere, G., Leblond, H., Provencher, J., and Rossignol, S. (2008). Prominent role of the spinal central pattern generator in the recovery of locomotion after partial spinal cord injuries. *J. Neurosci.* 28, 3976–3987. doi: 10.1523/jneurosci.5692-07.2008

Barrière, G., Mellen, N., and Cazalets, J.-R. (2004). Neuromodulation of the locomotor network by dopamine in the isolated spinal cord of newborn rat. *Eur. J. Neurosci.* 19, 1325–1335. doi: 10.1111/j.1460-9568.2004.03210.x

Benoit-Marand, M., Borrelli, E., and Gonon, F. (2001). Inhibition of dopamine release via presynaptic D2 receptors: time course and functional characteristics in vivo. *J. Neurosci.* 21, 9134–9141.

Björklund, A., and Dunnett, S. B. (2007). Dopamine neuron systems in the brain: an update. *Trends Neurosci.* 30, 194–202. doi: 10.1016/j.tins.2007.03.006

Björklund, A., and Skagerberg, G. (1979). Evidence for a major spinal cord projection from the diencephalic A11 dopamine cell group in the rat using transmitter-specific fluorescent retrograde tracing. *Brain Res.* 177, 170–175. doi: 10.1016/0006-8993(79)90927-2

Blessing, W. W., and Chalmers, J. P. (1979). Direct projection of catecholamine (presumably dopamine)-containing neurons from hypothalamus to spinal cord. *Neurosci. Lett.* 11, 35–40. doi: 10.1016/0304-3940(79)90052-1

Boido, M., Rupa, R., Garbossa, D., Fontanella, M., Ducati, A., and Vercelli, A. (2009). Embryonic and adult stem cells promote raphespinal axon outgrowth and improve functional outcome following spinal hemisection in mice. *Eur. J. Neurosci.* 30, 833–846. doi: 10.1111/j.1460-9568.2009.06879.x

Boulenguez, P., and Vinay, L. (2009). Strategies to restore motor functions after spinal cord injury. *Curr. Opin. Neurobiol.* 19, 587–600. doi: 10.1016/j.conb.2009.10.005

Bregman, B. S. (1987). Development of serotonin immunoreactivity in the rat spinal cord and its plasticity after neonatal spinal cord lesions. *Brain Res.* 431, 245–263. doi: 10.1016/0165-3806(87)90213-6

Briggman, K. L., Abarbanel, H. D. I., and Kristan, W. B. (2005). Optical imaging of neuronal populations during decision-making. *Science* 307, 896–901. doi: 10.1126/science.1103736

Bruinstroop, E., Cano, G., Vanderhorst, V. G. J. M., Cavalcante, J. C., Wirth, J., Sena-Esteves, M., et al. (2012). Spinal projections of the A5, A6 (locus coeruleus) and A7 noradrenergic cell groups in rats. *J. Comp. Neurol.* 520, 1985–2001. doi: 10.1002/cne.23024

Brustein, E., Saint-Amant, L., Buss, R. R., Chong, M., McDearmid, J. R., and Drapeau, P. (2003). Steps during the development of the zebrafish locomotor network. *J. Physiol. Paris* 97, 77–86. doi: 10.1016/j.jphysparis.2003.10.009

Buchanan, J. T., and Grillner, S. (1991). 5-Hydroxytryptamine depresses reticulospinal excitatory postsynaptic potentials in motoneurons of the lamprey. *Neurosci. Lett.* 122, 71–74. doi: 10.1016/0304-3940(91)90196-z

Bucher, D., Thirumalai, V., and Marder, E. (2003). Axonal dopamine receptors activate peripheral spike initiation in a stomatogastric motor neuron. *J. Neurosci.* 23, 6866–6875.

Carlsson, A., Falck, B., and Hillarp, N. A. (1962). Cellular localization of brain monoamines. *Acta Physiol. Scand. Suppl.* 56, 1–28.

Carp, J. S., and Anderson, R. J. (1982). Dopamine receptor-mediated depression of spinal monosynaptic transmission. *Brain Res.* 242, 247–254. doi: 10.1016/0006-8993(82)90307-9

Chatelin, S., Wehrlé, R., Mercier, P., Morello, D., Sotelo, C., and Weber, M. J. (2001). Neuronal promoter of human aromatic L-amino acid decarboxylase gene directs transgene expression to the adult floor plate and aminergic nuclei induced by the isthmus. *Brain Res. Mol. Brain Res.* 97, 149–160. doi: 10.1016/s0169-328x(01)00318-7

Chau, C., Barbeau, H., and Rossignol, S. (1998). Effects of intrathecal alpha1- and alpha2-noradrenergic agonists and norepinephrine on locomotion in chronic spinal cats. *J. Neurophysiol.* 79, 2941–2963.

Chetrit, J., Taupignon, A., Froux, L., Morin, S., Bouali-Benazzouz, R., Naudet, F., et al. (2013). Inhibiting subthalamic D5 receptor constitutive activity alleviates abnormal electrical activity and reverses motor impairment in a rat model of Parkinson's disease. *J. Neurosci.* 33, 14840–14849. doi: 10.1523/JNEUROSCI.0453-13.2013

Christie, K. J., and Whelan, P. J. (2005). Monoaminergic establishment of rostrocaudal gradients of rhythmicity in the neonatal mouse spinal cord. *J. Neurophysiol.* 94, 1554–1564. doi: 10.1152/jn.00299.2005

Ciliax, B. J., Drash, G. W., Staley, J. K., Haber, S., Mobley, C. J., Miller, G. W., et al. (1999). Immunocytochemical localization of the dopamine transporter in human brain. *J. Comp. Neurol.* 409, 38–56. doi: 10.1002/(sici)1096-9861(19990621)409:1<38::aid-cne4>3.0.co;2-1

Clark, F. M., and Proudfit, H. K. (1991). The projection of locus coeruleus neurons to the spinal cord in the rat determined by anterograde tracing combined with immunocytochemistry. *Brain Res.* 538, 231–245. doi: 10.1016/0006-8993(91)90435-x

Clemens, S., Belin-Rauscent, A., Simmers, J., and Combes, D. (2012). Opposing modulatory effects of D1- and D2-like receptor activation on a spinal central pattern generator. *J. Neurophysiol.* 107, 2250–2259. doi: 10.1152/jn.00366.2011

Commissiong, J. W. (1983). Development of catecholaminergic nerves in the spinal cord of the rat. *Brain Res.* 264, 197–208. doi: 10.1016/0006-8993(83)90817-x

Commissiong, J. W. (1984). Fetal locus coeruleus transplanted into the transected spinal cord of the adult rat: some observations and implications. *Neuroscience* 12, 839–853. doi: 10.1016/0306-4522(84)90174-x

Commissiong, J. W., Gentleman, S., and Neff, N. H. (1979). Spinal cord dopaminergic neurons: evidence for an uncrossed nigrospinal pathway. *Neuropharmacology* 18, 565–568. doi: 10.1016/0028-3908(79)90102-3

Crisp, K. M., Gallagher, B. R., and Mesce, K. A. (2012). Mechanisms contributing to the dopamine induction of crawl-like bursting in leech motoneurons. *J. Exp. Biol.* 215, 3028–3036. doi: 10.1242/jeb.069245

Crisp, K. M., and Mesce, K. A. (2004). A cephalic projection neuron involved in locomotion is dye coupled to the dopaminergic neural network in the medicinal leech. *J. Exp. Biol.* 207, 4535–4542. doi: 10.1242/jeb.01315

Dahlström, A., and Fuxe, K. (1964a). Evidence for the existence of monoamine-containing neurons in the central nervous system. I. Demonstration of monoamines in the cell bodies of brain stem neurons. *Acta Physiol. Scand. Suppl.* 232, 1–55.

Dahlström, A., and Fuxe, K. (1964b). Localization of monoamines in the lower brain stem. *Experientia* 20, 398–399. doi: 10.1007/bf02147990

Demchyshyn, L. L., McConkey, F., and Niznik, H. B. (2000). Dopamine D5 receptor agonist high affinity and constitutive activity profile conferred by carboxyl-terminal tail sequence. *J. Biol. Chem.* 275, 23446–23455. doi: 10.1074/jbc.m000157200

Fong, A. J., Roy, R. R., Ichiyama, R. M., Lavrov, I., Courtine, G., Gerasimenko, Y., et al. (2009). Recovery of control of posture and locomotion after a spinal cord injury: solutions staring us in the face. *Prog. Brain Res.* 175, 393–418. doi: 10.1016/S0079-6123(09)17526-X

Ford, C. P. (2014). The role of D2-autoreceptors in regulating dopamine neuron activity and transmission. *Neuroscience* doi: 10.1016/j.neuroscience.2014.01.025. [Epub ahead of print].

Forssberg, H., and Grillner, S. (1973). The locomotion of the acute spinal cat injected with clonidine i.v. *Brain Res.* 50, 184–186. doi: 10.1016/0006-8993(73)90606-9

Fritschy, J. M., and Grzanna, R. (1990). Demonstration of two separate descending noradrenergic pathways to the rat spinal cord: evidence for an intragriseal trajectory of locus coeruleus axons in the superficial layers of the dorsal horn. *J. Comp. Neurol.* 291, 553–582. doi: 10.1002/cne.902910406

Gabriel, J. P., Mahmood, R., Kyriakatos, A., Söll, I., Hauptmann, G., Calabrese, R. L., et al. (2009). Serotonergic modulation of locomotion in zebrafish: endogenous release and synaptic mechanisms. *J. Neurosci.* 29, 10387–10395. doi: 10.1523/JNEUROSCI.1978-09.2009

Gentleman, S., Parenti, M., Commissiong, J. W., and Neff, N. H. (1981). Dopamine-activated adenylate cyclase of spinal cord: supersensitivity following transection of the cord. *Brain Res.* 210, 271–275. doi: 10.1016/0006-8993(81)90900-8

Gerin, C., Becquet, D., and Privat, A. (1995). Direct evidence for the link between monoaminergic descending pathways and motor activity. I. A study with microdialysis probes implanted in the ventral funiculus of the spinal cord. *Brain Res.* 704, 191–201. doi: 10.1016/0006-8993(95)01111-0

Gerin, C., and Privat, A. (1998). Direct evidence for the link between monoaminergic descending pathways and motor activity: II. A study with microdialysis probes implanted in the ventral horn of the spinal cord. *Brain Res.* 794, 169–173. doi: 10.1016/s0006-8993(98)00278-9

Gordon, I. T., and Whelan, P. J. (2006). Monoaminergic control of cauda-equina-evoked locomotion in the neonatal mouse spinal cord. *J. Neurophysiol.* 96, 3122–3129. doi: 10.1152/jn.00606.2006

Goulding, M. (2009). Circuits controlling vertebrate locomotion: moving in a new direction. *Nat. Rev. Neurosci.* 10, 507–518. doi: 10.1038/nrn2608

Graham-Brown, T. (1911). The intrinsic factors in the act of progression in the mammal. *Proc. R. Soc. Lond. B Biol. Sci.* 84, 308–319. doi: 10.1098/rspb.1911.0077

Grillner, S., and Zangger, P. (1979). On the central generation of locomotion in the low spinal cat. *Exp. Brain Res.* 34, 241–261. doi: 10.1007/bf00235671

Gruhn, M., Guckenheimer, J., Land, B., and Harris-Warrick, R. M. (2005). Dopamine modulation of two delayed rectifier potassium currents in a small neural network. *J. Neurophysiol.* 94, 2888–2900. doi: 10.1152/jn.00434.2005

Guertin, P. A., Ung, R.-V., Rouleau, P., and Steuer, I. (2011). Effects on locomotion, muscle, bone and blood induced by a combination therapy eliciting weight-bearing stepping in nonassisted spinal cord-transected mice. *Neurorehabil. Neural Repair* 25, 234–242. doi: 10.1177/1545968310378753

Gutierrez, G. J., O'Leary, T., and Marder, E. (2013). Multiple mechanisms switch an electrically coupled, synaptically inhibited neuron between competing rhythmic oscillators. *Neuron* 77, 845–858. doi: 10.1016/j.neuron.2013.01.016

Hammar, I., Bannatyne, B. A., Maxwell, D. J., Edgley, S. A., and Jankowska, E. (2004). The actions of monoamines and distribution of noradrenergic and serotoninergic contacts on different subpopulations of commissural interneurons in the cat spinal cord. *Eur. J. Neurosci.* 19, 1305–1316. doi: 10.1111/j.1460-9568.2004.03239.x

Han, P., Nakanishi, S. T., Tran, M. A., and Whelan, P. J. (2007). Dopaminergic modulation of spinal neuronal excitability. *J. Neurosci.* 27, 13192–13204. doi: 10.1523/jneurosci.1279-07.2007

Han, P., and Whelan, P. J. (2009). Modulation of AMPA currents by D(1)-like but not D(2)-like receptors in spinal motoneurons. *Neuroscience* 158, 1699–1707. doi: 10.1016/j.neuroscience.2008.11.040

Harris-Warrick, R. M., and Cohen, A. H. (1985). Serotonin modulates the central pattern generator for locomotion in the isolated lamprey spinal cord. *J. Exp. Biol.* 116, 27–46.

Harris-Warrick, R. M., Coniglio, L. M., Barazangi, N., Guckenheimer, J., and Gueron, S. (1995). Dopamine modulation of transient potassium current evokes phase shifts in a central pattern generator network. *J. Neurosci.* 15, 342–358.

Harris-Warrick, R. M., and Johnson, B. R. (2010). Checks and balances neuromodulation. *Front. Behav. Neurosci.* 4, 1–9. doi: 10.3389/fnbeh.2010.00047

Harris-Warrick, R. M., Johnson, B. R., Peck, J. H., Kloppenburg, P., Ayali, A., and Skarbinski, J. (1998). Distributed effects of dopamine modulation in the crustacean pyloric network. *Ann. N. Y. Acad. Sci.* 860, 155–167.

Heckman, C. J., Hyngstrom, A. S., and Johnson, M. D. (2008). Active properties of motoneurone dendrites: diffuse descending neuromodulation, focused local inhibition. *J. Physiol.* 586, 1225–1231. doi: 10.1113/jphysiol.2007.145078

Hellal, F., Hurtado, A., Ruschel, J., Flynn, K. C., Laskowski, C. J., Umlauf, M., et al. (2011). Microtubule stabilization reduces scarring and causes axon regeneration after spinal cord injury. *Science* 331, 928–931. doi: 10.1126/science.1201148

Hill, R. H., Svensson, E., Dewael, Y., and Grillner, S. (2003). 5-HT inhibits N-type but not L-type Ca(2+) channels via 5-HT1A receptors in lamprey spinal neurons. *Eur. J. Neurosci.* 18, 2919–2924. doi: 10.1111/j.1460-9568.2003.03051.x

Hinckley, C. A., Hartley, R., Wu, L., Todd, A. J., and Ziskind-Conhaim, L. H. (2005). Locomotor-like rhythms in a genetically distinct cluster of interneurons in the mammalian spinal cord. *J. Neurophysiol.* 93, 1439–1449. doi: 10.1152/jn.00647.2004

Hökfelt, T., Johansson, O., and Goldstein, M. (1984a). Chemical anatomy of the brain. *Science* 225, 1326–1334. doi: 10.1126/science.6147896

Hökfelt, T., Martensson, M., Björklund, A., Kleineau, S., and Goldstein, M. (1984b). "Distributional maps of tyrosine-hydroxylase-immunoreactive neurons in the rat brain," in *Handbook of Chemical Neuroanatomy (Classical Transmitters in the CNS, Part 1)* (Vol. 2), eds A. Björklund and T. Hökfelt (Amsterdam: Elsevier Science), 277–379.

Humphreys, J. M., and Whelan, P. J. (2012). Dopamine exerts activation-dependent modulation of spinal locomotor circuits in the neonatal mouse. *J. Neurophysiol.* 108, 3370–3381. doi: 10.1152/jn.00482.2012

Jaeger, C. B., Teitelman, G., Joh, T. H., Albert, V. R., Park, D. H., and Reis, D. J. (1983). Some neurons of the rat central nervous system contain aromatic-L-amino-acid decarboxylase but not monoamines. *Science* 219, 1233–1235. doi: 10.1126/science.6131537

Jankowska, E., Jukes, M. G., Lund, S., and Lundberg, A. (1967a). The effect of DOPA on the spinal cord. 5. Reciprocal organization of pathways transmitting excitatory action to alpha motoneurones of flexors and extensors. *Acta Physiol. Scand.* 70, 369–388. doi: 10.1111/j.1748-1716.1967.tb03636.x

Jankowska, E., Jukes, M. G., Lund, S., and Lundberg, A. (1967b). The effect of DOPA on the spinal cord. 6. Half-centre organization of interneurones transmitting effects from the flexor reflex afferents. *Acta Physiol. Scand.* 70, 389–402. doi: 10.1111/j.1748-1716.1967.tb03637.x

Jensen, T. S., and Yaksh, T. L. (1984). Effects of an intrathecal dopamine agonist, apomorphine, on thermal and chemical evoked noxious responses in rats. *Brain Res.* 296, 285–293. doi: 10.1016/0006-8993(84)90064-7

Jiang, Z., Carlin, K. P., and Brownstone, R. M. (1999). An in vitro functionally mature mouse spinal cord preparation for the study of spinal motor networks. *Brain Res.* 816, 493–499. doi: 10.1016/s0006-8993(98)01199-8

Johnson, B. R., Brown, J. M., Kvarta, M. D., Lu, J. Y. J., Schneider, L. R., Nadim, F., et al. (2011). Differential modulation of synaptic strength and timing regulate synaptic efficacy in a motor network. *J. Neurophysiol.* 105, 293–304. doi: 10.1152/jn.00809.2010

Johnson, B. R., and Harris-Warrick, R. M. (1990). Aminergic modulation of graded synaptic transmission in the lobster stomatogastric ganglion. *J. Neurosci.* 10, 2066–2076.

Johnson, M. D., and Heckman, C. J. (2010). Interactions between focused synaptic inputs and diffuse neuromodulation in the spinal cord. *Ann. N Y Acad. Sci.* 1198, 35–41. doi: 10.1111/j.1749-6632.2010.05430.x

Johnson, B. R., Schneider, L. R., Nadim, F., and Harris-Warrick, R. M. (2005). Dopamine modulation of phasing of activity in a rhythmic motor network: contribution of synaptic and intrinsic modulatory actions. *J. Neurophysiol.* 94, 3101–3111. doi: 10.1152/jn.00440.2005

Jordan, L. M., Liu, J., Hedlund, P. B., Akay, T., and Pearson, K. G. (2008). Descending command systems for the initiation of locomotion in mammals. *Brain Res. Rev.* 57, 183–191. doi: 10.1016/j.brainresrev.2007.07.019

Kadiri, L. R., Kwan, A. C., Webb, W. W., and Harris-Warrick, R. M. (2011). Dopamine-induced oscillations of the pyloric pacemaker neuron rely on release of calcium from intracellular stores. *J. Neurophysiol.* 106, 1288–1298. doi: 10.1152/jn.00456.2011

Karoum, F., Commissiong, J. C., Neff, N. H., and Wyatt, R. J. (1981). Regional differences in catecholamine formation and metabolism in the rat spinal cord. *Brain Res.* 212, 316–366. doi: 10.1016/0006-8993(81)90468-6

Keeler, B. E., Baran, C. A., Brewer, K. L., and Clemens, S. (2012). Increased excitability of spinal pain reflexes and altered frequency-dependent modulation in the dopamine D3-receptor knockout mouse. *Exp. Neurol.* 238, 273–283. doi: 10.1016/j.expneurol.2012.09.002

Kehr, W., Carlsson, A., Lindqvist, M., Magnusson, T., and Atack, C. (1972). Evidence for a receptor-mediated feedback control of striatal tyrosine hydroxylase activity. *J. Pharm. Pharmacol.* 24, 744–747. doi: 10.1111/j.2042-7158.1972.tb09104.x

Kemnitz, C. P. (1997). Dopaminergic modulation of spinal neurons and synaptic potentials in the lamprey spinal cord. *J. Neurophysiol.* 77, 289–298.

Kemnitz, C. P., Strauss, T. R., Hosford, D. M., and Buchanan, J. T. (1995). Modulation of swimming in the lamprey, Petromyzon marinus, by serotonergic and dopaminergic drugs. *Neurosci. Lett.* 201, 115–118. doi: 10.1016/0304-3940(95)12147-1

Kiehn, O., and Kjaerulff, O. (1996). Spatiotemporal characteristics of 5-HT and dopamine-induced rhythmic hindlimb activity in the in vitro neonatal rat. *J. Neurophysiol.* 75, 1472–1482.

Kiehn, O., Sillar, K. T., Kjaerulff, O., and McDearmid, J. R. (1999). Effects of noradrenaline on locomotor rhythm-generating networks in the isolated neonatal rat spinal cord. *J. Neurophysiol.* 82, 741–746.

Kloppenburg, P., Levini, R. M., and Harris-Warrick, R. M. (1999). Dopamine modulates two potassium currents and inhibits the intrinsic firing properties of an identified motor neuron in a central pattern generator network. *J. Neurophysiol.* 81, 29–38.

Kvarta, M. D., Harris-Warrick, R. M., and Johnson, B. R. (2012). Neuromodulator-evoked synaptic metaplasticity within a central pattern generator network. *J. Neurophysiol.* 108, 2846–2856. doi: 10.1152/jn.00586.2012

Kwon, B. K., Casha, S., Hurlbert, R. J., and Yong, V. W. (2011). Inflammatory and structural biomarkers in acute traumatic spinal cord injury. *Clin. Chem. Lab. Med.* 49, 425–433. doi: 10.1515/CCLM.2011.068

Lambert, A. M., Bonkowsky, J. L., and Masino, M. A. (2012). The conserved dopaminergic diencephalospinal tract mediates vertebrate locomotor development in zebrafish larvae. *J. Neurosci.* 32, 13488–13500. doi: 10.1523/jneurosci.1638-12.2012

Lapointe, N. P., and Guertin, P. A. (2008). Synergistic effects of D1/5 and 5-HT1A/7 receptor agonists on locomotor movement induction in complete spinal cord-transected mice. *J. Neurophysiol.* 100, 160–168. doi: 10.1152/jn.90339.2008

Lapointe, N. P., Rouleau, P., Ung, R.-V., and Guertin, P. A. (2009). Specific role of dopamine D1 receptors in spinal network activation and rhythmic movement induction in vertebrates. *J. Physiol.* 587, 1499–1511. doi: 10.1113/jphysiol.2008.166314

Lee, H.-M., Giguere, P. M., and Roth, B. L. (2014). DREADDs: novel tools for drug discovery and development. *Drug Discov. Today* 19, 469–473. doi: 10.1016/j.drudis.2013.10.018

Levant, B., and McCarson, K. E. (2001). D(3) dopamine receptors in rat spinal cord: implications for sensory and motor function. *Neurosci. Lett.* 303, 9–12. doi: 10.1016/s0304-3940(01)01692-5

Li, Y., Li, L., Stephens, M. J., Zenner, D., Murray, K. C., Winship, I. R., et al. (2014). Synthesis, transport, and metabolism of serotonin formed from exogenously applied 5-HTP after spinal cord injury in rats. *J. Neurophysiol.* 111, 145–163. doi: 10.1152/jn.00508.2013

Lindvall, O., Björklund, A., and Skagerberg, G. (1983). Dopamine-containing neurons in the spinal cord: anatomy and some functional aspects. *Ann. Neurol.* 14, 255–260. doi: 10.1002/ana.410140302

Liu, J., and Jordan, L. M. (2005). Stimulation of the parapyramidal region of the neonatal rat brain stem produces locomotor-like activity involving spinal 5-HT7 and 5-HT2A receptors. *J. Neurophysiol.* 94, 1392–1404. doi: 10.1152/jn.00136.2005

Lorang, D., Amara, S. G., and Simerly, R. B. (1994). Cell-type-specific expression of catecholamine transporters in the rat brain. *J. Neurosci.* 14, 4903–4914.

Madriaga, M. A., McPhee, L. C., Chersa, T., Christie, K. J., and Whelan, P. J. (2004). Modulation of locomotor activity by multiple 5-HT and dopaminergic receptor subtypes in the neonatal mouse spinal cord. *J. Neurophysiol.* 92, 1566–1576. doi: 10.1152/jn.01181.2003

Maitra, K. K., Seth, P., Thewissen, M., Ross, H. G., and Ganguly, D. K. (1993). Dopaminergic influence on the excitability of antidromically activated Renshaw cells in the lumbar spinal cord of the rat. *Acta Physiol. Scand.* 148, 101–107. doi: 10.1111/j.1748-1716.1993.tb09538.x

Majczyński, H., Maleszak, K., Cabaj, A., and SŁawińska, U. (2005). Serotonin-related enhancement of recovery of hind limb motor functions in spinal rats after grafting of embryonic raphe nuclei. *J. Neurotrauma* 22, 590–604. doi: 10.1089/neu.2005.22.590

Manconi, M., Ferri, R., Zucconi, M., Clemens, S., Giarolli, L., Bottasini, V., et al. (2011). Preferential D2 or preferential D3 dopamine agonists in restless legs syndrome. *Neurology* 77, 110–117. doi: 10.1212/WNL.0b013e3182242d91

Marder, E. (2012). Neuromodulation of neuronal circuits: back to the future. *Neuron* 76, 1–11. doi: 10.1016/j.neuron.2012.09.010

Maric, O., Zörner, B., and Dietz, V. (2008). Levodopa therapy in incomplete spinal cord injury. *J. Neurotrauma* 25, 1303–1307. doi: 10.1089/neu.2008.0583

Masino, M. A., Abbinanti, M. D., Eian, J., and Harris-Warrick, R. M. (2012). TTX-resistant NMDA receptor-mediated membrane potential oscillations in neonatal mouse Hb9 interneurons. *PLoS One* 7:e47940. doi: 10.1371/journal.pone.0047940

McCrea, A. E., Stehouwer, D. J., and van Hartesveldt, C. (1997). Dopamine D1 and D2 antagonists block L-DOPA-induced air-stepping in decerebrate neonatal rats. *Brain Res. Dev. Brain Res.* 100, 130–132. doi: 10.1016/s0165-3806(97)00027-8

McDearmid, J. R., Scrymgeour-Wedderburn, J. F., and Sillar, K. T. (1997). Aminergic modulation of glycine release in a spinal network controlling swimming in Xenopus laevis. *J. Physiol.* 503, 111–117. doi: 10.1111/j.1469-7793.1997.111bi.x

McEwen, M. L., van Hartesveldt, C., and Stehouwer, D. J. (1997). L-DOPA and quipazine elicit air-stepping in neonatal rats with spinal cord transections. *Behav. Neurosci.* 111, 825–33111, 825–833. doi: 10.1037//0735-7044.111.4.825

McLean, D. L., and Sillar, K. T. (2004a). Divergent actions of serotonin receptor activation during fictive swimming in frog embryos. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 190, 391–402. doi: 10.1007/s00359-004-0504-9

McLean, D. L., and Sillar, K. T. (2004b). Metamodulation of a spinal locomotor network by nitric oxide. *J. Neurosci.* 24, 9561–9571. doi: 10.1523/jneurosci.1817-04.2004

McPherson, D. R., and Kemnitz, C. P. (1994). Modulation of lamprey fictive swimming and motoneuron physiology by dopamine and its immunocytochemical localization in the spinal cord. *Neurosci. Lett.* 166, 23–26. doi: 10.1016/0304-3940(94)90831-1

Miles, G. B., and Sillar, K. T. (2011). Neuromodulation of vertebrate locomotor control networks. *Physiology (Bethesda, Md)* 26, 393–411. doi: 10.1152/physiol.00013.2011

Missale, C., Nash, S. R., Robinson, S. W., Jaber, M., and Caron, M. G. (1998). Dopamine receptors: from structure to function. *Physiol. Rev.* 78, 189–225.

Montague, S. J., Fenrich, K. K., Mayer-Macaulay, C., Maratta, R., Neuber-Hess, M. S., and Rose, P. K. (2013). Nonuniform distribution of contacts from noradrenergic and serotonergic boutons on the dendrites of cat splenius motoneurons. *J. Comp. Neurol.* 521, 638–656. doi: 10.1002/cne.23196

Moore, R. Y., and Bloom, F. E. (1979). Central catecholamine neuron systems: anatomy and physiology of the norepinephrine and epinephrine systems. *Annu. Rev. Neurosci.* 2, 113–168. doi: 10.1146/annurev.ne.02.030179.000553

Murray, K. C., Nakae, A., Stephens, M. J., Rank, M., D'Amico, J., Harvey, P. J., et al. (2010). Recovery of motoneuron and locomotor function after spinal cord

injury depends on constitutive activity in 5-HT2C receptors. *Nat. Med.* 16, 694–700. doi: 10.1038/nm.2160

Musienko, P., van den Brand, R., Märzendorfer, O., Roy, R. R., Gerasimenko, Y., Edgerton, V. R., et al. (2011). Controlling specific locomotor behaviors through multidimensional monoaminergic modulation of spinal circuitries. *J. Neurosci.* 31, 9264–9278. doi: 10.1523/JNEUROSCI.5796-10.2011

Ochi, J., Yamamoto, T., and Hosoya, Y. (1979). Comparative study of the monoamine neuron system in the spinal cord of the lamprey and hagfish. *Arch. Histol. Jpn.* 42, 327–336. doi: 10.1679/aohc1950.42.327

Oh, M. S., Hong, S. J., Huh, Y., and Kim, K.-S. (2009). Expression of transgenes in midbrain dopamine neurons using the tyrosine hydroxylase promoter. *Gene Ther.* 16, 437–440. doi: 10.1038/gt.2008.148

Pappas, S. S., Behrouz, B., Janis, K. L., Goudreau, J. L., and Lookingland, K. J. (2008). Lack of D2 receptor mediated regulation of dopamine synthesis in A11 diencephalospinal neurons in male and female mice. *Brain Res.* 1214, 1–10. doi: 10.1016/j.brainres.2008.03.010

Pappas, S. S., Tiernan, C. T., Behrouz, B., Jordan, C. L., Breedlove, S. M., Goudreau, J. L., et al. (2010). Neonatal androgen-dependent sex differences in lumbar spinal cord dopamine concentrations and the number of A11 diencephalospinal dopamine neurons. *J. Comp. Neurol.* 518, 2423–2436. doi: 10.1002/cne.22340

Peck, J. H., Gaier, E., Stevens, E., Repicky, S., and Harris-Warrick, R. M. (2006). Amine modulation of Ih in a small neural network. *J. Neurophysiol.* 96, 2931–2940. doi: 10.1152/jn.00423.2005

Peyron, C., Luppi, P. H., Kitahama, K., Fort, P., Hermann, D. M., and Jouvet, M. (1995). Origin of the dopaminergic innervation of the rat dorsal raphe nucleus. *Neuroreport* 6, 2527–2531. doi: 10.1097/00001756-199512150-00019

Pierre, J., Mahouche, M., Suderevskaya, E. I., Reperant, J., and Ward, R. (1997). Immunocytochemical localization of dopamine and its synthetic enzymes in the central nervous system of the lamprey Lampetra fluviatilis. *J. Comp. Neurol.* 380, 119–135. doi: 10.1002/(sici)1096-9861(19970331)380:1<119::aid-cne9>3.3.co;2-j

Prinz, A. A., Bucher, D., and Marder, E. (2004). Similar network activity from disparate circuit parameters. *Nat. Neurosci.* 7, 1345–1352. doi: 10.1038/nn1352

Puhl, J. G., Masino, M. A., and Mesce, K. A. (2012). Necessary, sufficient and permissive: a single locomotor command neuron important for intersegmental coordination. *J. Neurosci.* 32, 17646–17657. doi: 10.1523/JNEUROSCI.2249-12.2012

Puhl, J. G., and Mesce, K. A. (2008). Dopamine activates the motor pattern for crawling in the medicinal leech. *J. Neurosci.* 28, 4192–4200. doi: 10.1523/JNEUROSCI.0136-08.2008

Qu, S., Ondo, W. G., Zhang, X., Xie, W. J., Pan, T. H., and Le, W. D. (2006). Projections of diencephalic dopamine neurons into the spinal cord in mice. *Exp. Brain Res.* 168, 152–156. doi: 10.1007/s00221-005-0075-1

Rajaofetra, N., Ridet, J. L., Poulat, P., Marlier, L., Sandillon, F., Geffard, M., et al. (1992). Immunocytochemical mapping of noradrenergic projections to the rat spinal cord with an antiserum against noradrenaline. *J. Neurocytol.* 21, 481–494. doi: 10.1007/bf01186952

Rajaofetra, N., Sandillon, F., Geffard, M., and Privat, A. (1989). Pre- and post-natal ontogeny of serotonergic projections to the rat spinal cord. *J. Neurosci. Res.* 22, 305–321. doi: 10.1002/jnr.490220311

Rauscent, A., Einum, J., Le Ray, D., Simmers, J., and Combes, D. (2009). Opposing aminergic modulation of distinct spinal locomotor circuits and their functional coupling during amphibian metamorphosis. *J. Neurosci.* 29, 1163–1174. doi: 10.1523/JNEUROSCI.5255-08.2009

Reimer, M. M., Norris, A., Ohnmacht, J., Patani, R., Zhong, Z., Dias, T. B., et al. (2013). Dopamine from the brain promotes spinal motor neuron generation during development and adult regeneration. *Dev. Cell* 25, 478–491. doi: 10.1016/j.devcel.2013.04.012

Rémy-Néris, O., Barbeau, H., Daniel, O., Boiteau, F., and Bussel, B. (1999). Effects of intrathecal clonidine injection on spinal reflexes and human locomotion in incomplete paraplegic subjects. *Exp. Brain Res.* 129, 433–440. doi: 10.1007/s002210050910

Ridet, J. L., Sandillon, F., Rajaofetra, N., Geffard, M., and Privat, A. (1992). Spinal dopaminergic system of the rat: light and electron microscopic study using an antiserum against dopamine, with particular emphasis on synaptic incidence. *Brain Res.* 598, 233–241. doi: 10.1016/0006-8993(92)90188-f

Rossignol, S., Chau, C., Brustein, E., Giroux, N., Bouyer, L., Barbeau, H., et al. (1998). Pharmacological activation and modulation of the central pattern generator for locomotion in the cat. *Ann. N Y Acad. Sci.* 860, 346–359. doi: 10.1111/j.1749-6632.1998.tb09061.x

Rossignol, S., and Frigon, A. (2011). Recovery of locomotion after spinal cord injury: some facts and mechanisms. *Annu. Rev. Neurosci.* 34, 413–440. doi: 10.1146/annurev-neuro-061010-113746

Rowland, J. W., Hawryluk, G. W. J., Kwon, B., and Fehlings, M. G. (2008). Current status of acute spinal cord injury pathophysiology and emerging therapies: promise on the horizon. *Neurosurg. Focus* 25:E2. doi: 10.3171/FOC.2008.25.11.E2

Ryu, S., Mahler, J., Acampora, D., Holzschuh, J., Erhardt, S., Omodei, D., et al. (2007). Orthopedia homeodomain protein is essential for diencephalic dopaminergic neuron development. *Curr. Biol.* 17, 873–880. doi: 10.1016/j.cub.2007.04.003

Schmidt, B. J., and Jordan, L. M. (2000). The role of serotonin in reflex modulation and locomotor rhythm production in the mammalian spinal cord. *Brain Res. Bull.* 53, 689–710. doi: 10.1016/s0361-9230(00)00402-0

Schotland, J., Shupliakov, O., Wikström, M. A., Brodin, L., Srinivasan, M., You, Z. B., et al. (1995). Control of lamprey locomotor neurons by colocalized monoamine transmitters. *Nature* 374, 266–268. doi: 10.1038/374266a0

Shapiro, M. G., Frazier, S. J., and Lester, H. A. (2012). Unparalleled control of neural activity using orthogonal pharmacogenetics. *ACS Chem. Neurosci.* 3, 619–629. doi: 10.1021/cn300053q

Sharples, S. A., Humphreys, J. M., Mayr, K., Krajacic, A., Dhoopar, S. A., Delaloye, N., et al. (2013). Dopaminergic contribution to locomotion in the neonatal and adult mouse. (Poster 559.11/ZZ15, Society for Neuroscience, San Diego, California, USA).

Shupliakov, O., Pieribone, V. A., Gad, H., and Brodin, L. (1995). Synaptic vesicle depletion in reticulospinal axons is reduced by 5-hydroxytryptamine: direct evidence for presynaptic modulation of glutamatergic transmission. *Eur. J. Neurosci.* 7, 1111–1116. doi: 10.1111/j.1460-9568.1995.tb01099.x

Sickles, A. E., Stehouwer, D. J., and van Hartesveldt, C. (1992). Dopamine D1 and D2 antagonists block L-dopa-elicited air-stepping in neonatal rats. *Brain Res. Dev. Brain Res.* 68, 17–22. doi: 10.1016/0165-3806(92)90243-p

Sillar, K. T., Reith, C. A., and McDearmid, J. R. (1998). Development and aminergic neuromodulation of a spinal locomotor network controlling swimming in Xenopus larvae. *Ann. N Y Acad. Sci.* 860, 318–332. doi: 10.1111/j.1749-6632.1998.tb09059.x

Skagerberg, G., and Lindvall, O. (1985). Organization of diencephalic dopamine neurones projecting to the spinal cord in the rat. *Brain Res.* 342, 340–351. doi: 10.1016/0006-8993(85)91134-5

Smith, D. O., Lowe, D., Temkin, R., Jensen, P., and Hatt, H. (1995). Dopamine enhances glutamate-activated currents in spinal motoneurons. *J. Neurosci.* 15, 3905–3912.

Sqalli-Houssaini, Y., and Cazalets, J. R. (2000). Noradrenergic control of locomotor networks in the in vitro spinal cord of the neonatal rat. *Brain Res.* 852, 100–109. doi: 10.1016/s0006-8993(99)02219-2

Stuart, D. G., and Hultborn, H. (2008). Thomas Graham Brown (1882–1965), Anders Lundberg (1920-) and the neural control of stepping. *Brain Res. Rev.* 59, 74–95. doi: 10.1016/j.brainresrev.2008.06.001

Svensson, E., Woolley, J., Wikström, M. A., and Grillner, S. (2003). Endogenous dopaminergic modulation of the lamprey spinal locomotor network. *Brain Res.* 970, 1–8. doi: 10.1016/s0006-8993(02)04216-6

Tamae, A., Nakatsuka, T., Koga, K., Kato, G., Furue, H., Katafuchi, T., et al. (2005). Direct inhibition of substantia gelatinosa neurones in the rat spinal cord by activation of dopamine D2-like receptors. *J. Physiol.* 568, 243–253. doi: 10.1113/jphysiol.2005.091843

Tarazi, F. I., and Baldessarini, R. J. (2000). Comparative postnatal development of dopamine D(1), D(2) and D(4) receptors in rat forebrain. *Int. J. Dev. Neurosci.* 18, 29–37. doi: 10.1016/s0736-5748(99)00108-2

Taylor, L. L., Sickles, A. E., Stehouwer, D. J., and Van Hartesveldt, C. (1994). Noradrenergic alpha-1 and alpha-2 antagonists block L-dopa-induced air-stepping in neonatal rats. *Brain Res. Dev. Brain Res.* 79, 242–248. doi: 10.1016/0165-3806(94)90128-7

Thirumalai, V., and Cline, H. T. (2008). Endogenous dopamine suppresses initiation of swimming in prefeeding zebrafish larvae. *J. Neurophysiol.* 100, 1635–1648. doi: 10.1152/jn.90568.2008

Ugrumov, M. V. (2009). Non-dopaminergic neurons partly expressing dopaminergic phenotype: distribution in the brain, development and functional

significance. *J. Chem. Neuroanat.* 38, 241–256. doi: 10.1016/j.jchemneu.2009.08.004

van Dongen, P. A., Grillner, S., and Hokfelt, T. (1986). 5-Hydroxytryptamine (serotonin) causes a reduction in the afterhyperpolarization following the action potential in lamprey motoneurons and premotor interneurons. *Brain Res.* 366, 320–325. doi: 10.1016/0006-8993(86)91310-7

Vidal-Gadea, A., Topper, S., Young, L., Crisp, A., Kressin, L., Elbel, E., et al. (2011). Caenorhabditis elegans selects distinct crawling and swimming gaits via dopamine and serotonin. *Proc. Natl. Acad. Sci. U S A* 108, 17504–17509. doi: 10.1073/pnas.1108673108

Wallén, P., Buchanan, J. T., Grillner, S., Hill, R. H., Christenson, J., and Hokfelt, T. (1989). Effects of 5-hydroxytryptamine on the afterhyperpolarization, spike frequency regulation and oscillatory membrane properties in lamprey spinal cord neurons. *J. Neurophysiol.* 61, 759–768.

Wang, D., Grillner, S., and Wallén, P. (2011). 5-HT and dopamine modulates CaV1.3 calcium channels involved in postinhibitory rebound in the spinal network for locomotion in lamprey. *J. Neurophysiol.* 105, 1212–1224. doi: 10.1152/jn.00324.2009

Whelan, P., Bonnot, A., and O'Donovan, M. J. (2000). Properties of rhythmic activity generated by the isolated spinal cord of the neonatal mouse. *J. Neurophysiol.* 84, 2821–2833.

Wilson, J. M., Hartley, R., Maxwell, D. J., Todd, A. J., Lieberam, I., Kaltschmidt, J. A., et al. (2005). Conditional rhythmicity of ventral spinal interneurons defined by expression of the Hb9 homeodomain protein. *J. Neurosci.* 25, 5710–5719. doi: 10.1523/jneurosci.0274-05.2005

Wolf, M. E., and Roth, R. H. (1990). Autoreceptor regulation of dopamine synthesis. *Ann. N Y Acad. Sci.* 604, 323–343. doi: 10.1111/j.1749-6632.1990.tb32003.x

Yoshida, M., and Tanaka, M. (1988). Existence of new dopaminergic terminal plexus in the rat spinal cord: assessment by immunohistochemistry using anti-dopamine serum. *Neurosci. Lett.* 94, 5–9. doi: 10.1016/0304-3940(88)90261-3

Zhao, H., Zhu, W., Pan, T., Xie, W., Zhang, A., Ondo, W. G., et al. (2007). Spinal cord dopamine receptor expression and function in mice with 6-OHDA lesion of the A11 nucleus and dietary iron deprivation. *J. Neurosci. Res.* 85, 1065–1076. doi: 10.1002/jnr.21207

Zhu, H., Clemens, S., Sawchuk, M. A., and Hochman, S. (2007). Expression and distribution of all dopamine receptor subtypes (D(1)-D(5)) in the mouse lumbar spinal cord: a real-time polymerase chain reaction and non-autoradiographic in situ hybridization study. *Neuroscience* 149, 885–897. doi: 10.1016/j.neuroscience.2007.07.052

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Monoaminergic control of spinal locomotor networks in SOD1^{G93A} newborn mice

Léa Milan[1], Grégory Barrière[1], Philippe De Deurwaerdère[2], Jean-René Cazalets[1] and Sandrine S. Bertrand[1]*

[1] CNRS, Institut de Neurosciences Cognitives et Intégratives d'Aquitaine, UMR5287, Université de Bordeaux, Bordeaux, France
[2] Institut des Maladies Neurodégénératives, Université de Bordeaux, Bordeaux, France

Edited by:
Shawn Hochman, Emory University, USA

Reviewed by:
David Parker, Cambridge University, UK
Brian R. Noga, University of Miami, USA
Patrick John Whelan, University of Calgary, Canada

***Correspondence:**
Sandrine S. Bertrand, CNRS, Institut de Neurosciences Cognitives et Intégratives d'Aquitaine, UMR5287, Université de Bordeaux, Bat 2, 146 Rue Léo Saignat, 33076 Bordeaux cedex, France
e-mail: sandrine.bertrand@u-bordeaux.fr

Mutations in the gene that encodes Cu/Zn-superoxide dismutase (SOD1) are the cause of approximately 20% of familial forms of amyotrophic lateral sclerosis (ALS), a fatal neurodegenerative disease characterized by the progressive loss of motor neurons. While ALS symptoms appear in adulthood, spinal motoneurons exhibit functional alterations as early as the embryonic and postnatal stages in the murine model of ALS, the SOD1 mice. Monoaminergic – i.e., dopaminergic (DA), serotoninergic (5-HT), and noradrenergic (NA) – pathways powerfully control spinal networks and contribute significantly to their embryonic and postnatal maturation. Alterations in monoaminergic neuromodulation during development could therefore lead to impairments in the motoneuronal physiology. In this study, we sought to determine whether the monoaminergic spinal systems are modified in the early stages of development in SOD1 mice. Using a post-mortem analysis by high performance liquid chromatography (HPLC), monoaminergic neuromodulators and their metabolites were quantified in the lumbar spinal cord of SOD1 and wild-type (WT) mice aged one postnatal day (P1) and P10. This analysis underscores an increased content of DA in the SOD1 lumbar spinal cord compared to that of WT mice but failed to reveal any modification of the other monoaminergic contents. In a next step, we compared the efficiency of the monoaminergic compounds in triggering and modulating fictive locomotion in WT and SOD1 mice. This study was performed in P1–P3 SOD1 mice and age-matched control littermates using extracellular recordings from the lumbar ventral roots in the *in vitro* isolated spinal cord preparation. This analysis revealed that the spinal networks of SOD1^{G93A} mice could generate normal locomotor activity in the presence of NMA-5-HT. Interestingly, we also observed that SOD1 spinal networks have an increased sensitivity to NA compared to WT spinal circuits but exhibited similar DA responses.

Keywords: serotonin, dopamine, noradrenaline, spinal cord, ALS, fictive locomotion, HPLC, extracellular recordings

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by the progressive degeneration of M1 neurons in the cerebral cortex and motor neurons in the brainstem and spinal cord. Approximately 20% of familial forms of ALS cases are associated with inherited dominant mutations in the gene that encodes Cu/Zn-superoxide dismutase (SOD1). While ALS syndrome occurs during adulthood in both humans and ALS animal models, a growing body of evidence shows that spinal locomotor networks exhibit functional alterations as early as the embryonic and postnatal stages in mice expressing the human mutated SOD1 protein, the SOD1 mouse model. Behavioral tests performed during the first postnatal week revealed a delay in the maturation processes of sensorimotor modalities such as righting and hind-paw grasping responses in mutant SOD1^{G85R} mice (Amendola et al., 2004). At the cellular level, developing SOD1^{G85R} and SOD1^{G93A} motoneurons have been shown to exhibit dendritic abnormalities and alterations in both excitability and synaptic inputs compared to the motoneurons of age-matched wild-type (WT) mice (Bories et al., 2007; Pambo-Pambo et al., 2009; Chang and Martin, 2011; Filipchuk and Durand, 2012; Martin et al., 2013). Differences in neurochemical sensitivity have also been reported between newborn WT and SOD1 mice. Indeed, the classical rhythmogenic drug cocktail (a mixture of glutamate agonist plus serotonin) used to activate the locomotor central pattern generators (CPGs) in the isolated spinal cord preparation of newborn rodents has been shown to be inefficient in inducing fictive locomotion in SOD1^{G85R} mice (Amendola et al., 2004). As such early alterations could prime neuronal circuits and make them more permissive to pathological changes later in life, it appears of importance to further decipher the changes undergone by the spinal motor networks in early developmental stages in the SOD1 models of ALS.

Motoneurons are targeted by numerous extra- and intraspinal neuromodulatory systems that control both their intrinsic membrane properties and incoming synaptic inputs (for review see Miles and Sillar, 2011). In addition to their role in excitability control, neuromodulatory influences contribute significantly to the embryonic and postnatal development of the spinal cord motor networks (for review see Vinay et al., 2002; Miles and Sillar, 2011).

Amongst neuromodulatory systems, monoaminergic – i.e., dopaminergic (DA), serotoninergic (5-HT), and noradrenergic (NA) – pathways have been shown to initiate and facilitate the expression of spinal motor outputs, to control segmental reflexes and to play a major role in the maturation of spinal locomotor networks (Barbeau and Rossignol, 1991; Crick and Wallis, 1991; Cazalets et al., 1992; Sqalli-Houssaini et al., 1993; Kiehn and Kjaerulff, 1996; Sqalli-Houssaini and Cazalets, 2000; Whelan et al., 2000; Vinay et al., 2002; Barrière et al., 2004, 2008; Han et al., 2007; Han and Whelan, 2009; Tartas et al., 2010; Miles and Sillar, 2011; Humphreys and Whelan, 2012; Pearlstein, 2013). Alterations in monoaminergic controls during development could therefore lead to significant reorganizations of the spinal locomotor circuits and contribute to the developmental impairments described in SOD1 motoneurons. However, to the best of our knowledge, data concerning the possible changes in the spinal monoaminergic inputs in the early developmental stages of SOD1 mice are not currently available. In the present study, we sought to determine whether spinal monoaminergic content and sensitivity is modified in newborn SOD1 mice. Specifically, we performed a high performance liquid chromatography (HPLC) analysis of the spinal monoamine contents and compared the effects of 5-HT, DA, and NA on the locomotor activity recorded extracellularly from isolated spinal cord preparations from newborn SOD1 and age-matched control littermates. This study found that well-organized locomotor-like activity could be generated in the isolated spinal cord preparation from SOD1^{G93A} mice by classical pharmacological activation. We also reported an increased content of spinal DA content in the second postnatal week, as well as an increased sensitivity to NA, in SOD1 spinal networks compared to those of WT mice.

MATERIALS AND METHODS

ETHICS STATEMENTS AND ANIMALS

All procedures were conducted in accordance with the local ethics committee of the University of Bordeaux and the European Committee Council Directive. All efforts were made to minimize animal suffering and to reduce the number of animals used in the experiments. Transgenic mice expressing a mutated human SOD-1 gene from the B6SJL-TgN (SOD1^{G93A}) 1 Gur/J line (SOD1 mice) were purchased from Jackson Laboratories. These mice were bred in our laboratory and maintained as hemizygotes by mating transgenic males with wild-type B6SJL females. Two hundred thirty-eight newborn mice were used in this study. All of the experiments and analyses presented here have been performed blind to the genotype of the animals. Mice were genotyped from genomic DNA purified from tail biopsies by PCR using the following primers: 5′ CATCAGCCCTAATCCATCTGA 3′ (forward), 5′ CGCGACTAACAATCAAAGTGA 3′ (reverse).

TISSUE PROCESSING FOR POST-MORTEM ANALYSIS

After decapitation, the lumbar spinal cord of postnatal 1 (P1) or P10 SOD1 and age-matched control littermate male mice was quickly removed by a laminectomy, placed in dry ice and stored at −80°C until experiment processing. When needed, the lumbar spinal cord was placed on the side and cut in the middle of the dorso-ventral axis with micro dissecting knives to separate the ventral and dorsal part of the cord.

POST-MORTEM HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) MEASUREMENTS

On the day of the biochemical analysis, after weighing the samples, tissue was homogenized in 0.1 N perchloric acid ($HClO_4$), sonicated and centrifuged at 13,000 rpm for 30 min at 4°C. The tissue contents of monoamines (DA, NA, 5-HT) and their metabolites (5-hydroxyindole-3-acetic: 5-HIAA a 5-HT metabolite and 3,4-dihydroxyphenylacetic acid: DOPAC, a DA metabolite) were measured by a sensitive HPLC-electrochemical detection (ECD) system. Aliquots of the sample supernatant were placed in an automated autosampler (Shimadzu, SIL-20A, Paris, France) at 4°C to be injected into the HPLC column (Hypersyl C18, 150 mm × 4.6 mm, 5 μm; C.I.L.-Cluzeau, Sainte-Foy-La-Grande, France) protected by a Brownlee–Newguard precolumn (RP-8, 15 × 3.2 mm, 7 μm; C.I.L.-Cluzeau). The mobile phase was delivered at a flow rate of 1.3 ml/min using a HPLC pump (LC20-AD, Shimadzu, France) and was composed as follows (in millimoles): 60 NaH_2PO_4, 0.1 disodium EDTA and 2 octane sulfonic acid plus 7% methanol, adjusted to a pH of 3.9 with orthophosphoric acid and filtered through a 0.22 mm Millipore filter. Monoamines and their metabolites were detected using a coulometric cell (Analytical cell 5011, Coulochem) coupled to a programmable detector (Coulochem II, ESA, Paris, France). The potential of the electrodes was set at +350 mV for the oxidation and −270 mV for the reduction. Output signals were recorded on a computer (Beckman, system GOLD). Under these conditions, the sensitivity for NA, DA, 5-HT, DOPAC, and 5-HIAA was 3, 1, 8, 6, and 5 pg/10 μl, respectively, with a signal/noise ratio of 3:1. The tissue content of monoamines was expressed in pg/mg of tissue and corresponded to the mean ± SEM values in each group. Differences of the monoamine content between P1/P10 SOD1 mice and control littermates were analyzed using repeated measures two-way analyses of variance (ANOVA) with Sidak's multiple comparison tests (Graph Pad Prism). Statistical significance was set at $p < 0.05$.

ISOLATED SPINAL CORD PREPARATION

Newborn SOD1 and WT littermate mice aged P1–P3 were deeply anesthetized with 4% isoflurane, decapitated and eviscerated. A laminectomy was then performed to expose and remove the spinal cord. All dissections and recording procedures were performed under continuous superperfusion with artificial cerebrospinal fluid (aCSF) containing (in milimoles): NaCl 130, KCl 3, $CaCl_2$ 2.5, $MgSO_4$ 1.3, NaH_2PO_4 0.58, $NaHCO_3$ 25, and glucose 10, with a pH of 7.4 when bubbled with 95% O_2 + 5% CO_2 at room temperature (24–26°C).

PHARMACOLOGY

All drugs (*N*-methyl-D, L-aspartate: NMA; 5-HT, DA, and NA) were obtained from Sigma (St. Louis, MO, USA). Stock solutions of 5-HT and NMA were prepared at 0.1 mM in distilled water and stored at −20°C. Fresh drug solutions of DA and NA were prepared daily and protected from light exposure. Pharmacological compounds were bath-applied using a peristaltic pump (flow rate 7 ml/min).

EXTRACELLULAR RECORDINGS AND ANALYSIS

Motor outputs were recorded extracellularly from the lumbar ventral roots using glass suction electrodes. In each *in vitro* spinal cord preparation, motor outputs from the right and left lumbar 2 (rL2, lL2, respectively) and one L5 ventral root were simultaneously recorded to investigate both the bilateral segmental alternation and the flexor/extensor activity (Cazalets et al., 1992; Kiehn and Kjaerulff, 1996). The neurograms were amplified (×10000) using high impedance AC amplifiers (200–3000 Hz) built at the laboratory and digitized at 5 kHz (Axograph, Sydney, NSW, Australia) for future analysis. Pharmacologically induced locomotor rhythms were analyzed using non-stationary analysis techniques in a Matlab-based software developed at the laboratory. This custom-made software is similar to SpinalCore, a software developed by the Lev-Tov group (Mor and Lev-Tov, 2007), and is based on the MatLab wavelet coherence package provided by Aslak Grinsted (http://noc.ac.uk/using-science/crosswavelet-wavelet-coherence; Torrence and Compo, 1998; Grinsted et al., 2004). Two-minute sections of pairs of neurograms were analyzed using cross wavelet transform and wavelet coherence. These methods were applied to high-pass (50 Hz), rectified and low-pass filtered (5–10 Hz) signals. For convenience, cross wavelet spectrum and wavelet coherence maps were combined into a mixed cross/coherence map (**Figure 2B1**) highlighting coherent, common high power frequency regions. In these maps, the evolution of the frequency components of the extracellular signals (*y*-axis, logarithmic scale) is represented as a function of time (*x*-axis), and the power of each frequency is color-coded with warm colors assigned to high power regions and cool colors to low power regions. The asymptotic lines in the mixed cross/coherence maps indicate the cone of influence. This cone delimits the region where edge effect becomes too important. The values outside of this cone were thus excluded from the statistical analysis (Torrence and Compo, 1998; Mor and Lev-Tov, 2007). The high power band of the generated time/frequency map was selected as a region of interest (ROI) and arbitrarily segmented into 1 s bins to compute the mean frequency, coherence, and phase relationship between pairs of neurograms. The critical level of statistical significance of the wavelet coherence was calculated using Monte-Carlo simulations (Mor and Lev-Tov, 2007). The power and phase of the mean vector of pairs of ventral root recordings were extracted for each experiment with this procedure. Using the Igor Pro software (Wavemetrics), angular distribution tests and Watson nonparametric circular two sample U2 tests were performed to compare circular data between WT and SOD1 mice in the different pharmacological conditions tested.

Motor burst amplitudes were computed in a custom-made Matlab-based software. For each preparation, the burst amplitude values were normalized to the amplitude measured in the presence of 10 μM 5-HT for experiments with increasing doses of 5-HT or to the amplitude observed during bath-applications of NMA-5-HT (16 μM each; Sqalli-Houssaini et al., 1993) alone prior to the addition of NA or DA to the bath. Repeated measures two-way analyses of variance (ANOVA) with Sidak's multiple comparison tests were performed to evaluate monoamines and mouse genotype effects (Graph Pad Prism). All data are expressed as means ± SEM. Asterisks in the Figures and Tables indicate positive significance levels of *post hoc* analysis ($p < 0.05$).

RESULTS

SPINAL MONOAMINERGIC CONTENTS IN NEWBORN WILD-TYPE AND SOD1 MICE

In rats, the first connections between brainstem monoaminergic cells and spinal neurons are established during the last week of gestation and mature sequentially along the rostrocaudal and ventro-dorsal axes until the second postnatal week (Commissiong, 1983; Bregman, 1987; Rajaofetra et al., 1989, 1992; Giménez y Ribotta et al., 1998; Clarac et al., 2004). To get an overview of the spinal monoaminergic innervation, we first conducted an HPLC analysis of the endogenous spinal content of biogenic amines and their metabolites in SOD1 mice and WT littermates (**Figure 1A**). To assess the impact of the descending pathway maturation on monoamine contents, we performed these procedures on the lumbar spinal cord samples from both P1 and P10 animals. This HPLC analysis revealed that the NA and 5-HT contents were about 20 times greater than the DA content in the lumbar spinal cord of both P1 and P10 mice. We also observed that regardless of the mouse genotype, the contents of NA (**Figure 1B1**), DA (**Figure 1C1**) and 5-HT (**Figure 1D1**) were significantly higher in P10 animals compared to P1 mice. In contrast, DOPAC and 5-HIAA were not significantly different between the two developmental stages tested (**Table 1**). *Post hoc* pairwise comparisons conducted between WT and SOD1 mice revealed no significant change in NA, 5-HT, DOPAC, or 5-HIAA. However, we observed that the DA content was significantly enhanced in the whole lumbar spinal cord of SOD1 P10 mice compared to age-matched WT animals.

Monoaminergic pathways densely innervate both dorsal and ventral spinal circuits. To specifically look for changes in the monoaminergic contents in spinal motor networks, the same HPLC analysis was repeated on the ventral half of the lumbar spinal cord (**Figures 1B2,C2,D2**). In these experimental conditions, we observed that the contents of all of the monoaminergic compounds tested (**Figures 1B2,C2,D2**) except 5-HIAA (**Table 1**) significantly increased during development. In contrast to the aforementioned results obtained using the whole spinal cord, the mouse genotype had no detectable influence on the monoaminergic contents measured in the ventral part of the cord.

RHYTHMOGENIC CAPABILITIES OF SOD1^{G93A} SPINAL NETWORKS AND SEROTONINERGIC MODULATION

As previously mentioned, Amendola et al. (2004) reported that NMA (10–20 μM) plus 5-HT (5–20 μM), a mixture known to generate locomotor-like activity in the *in vitro* spinal cord preparation of newborn rodents, induces only tonic activity when bath-applied to SOD1^{G85R} mouse spinal networks (Amendola et al., 2004). Dramatically different alterations in the motoneuronal physiology have been reported depending on the SOD1 mouse model used (see for example Meehan et al., 2010; Delestrée et al., 2014). The question then arises as to whether NMA-5-HT effects are similar in the SOD1^{G85R} and SOD1^{G93A} high expressor line mouse strains. To address this question, we compared the efficiency of

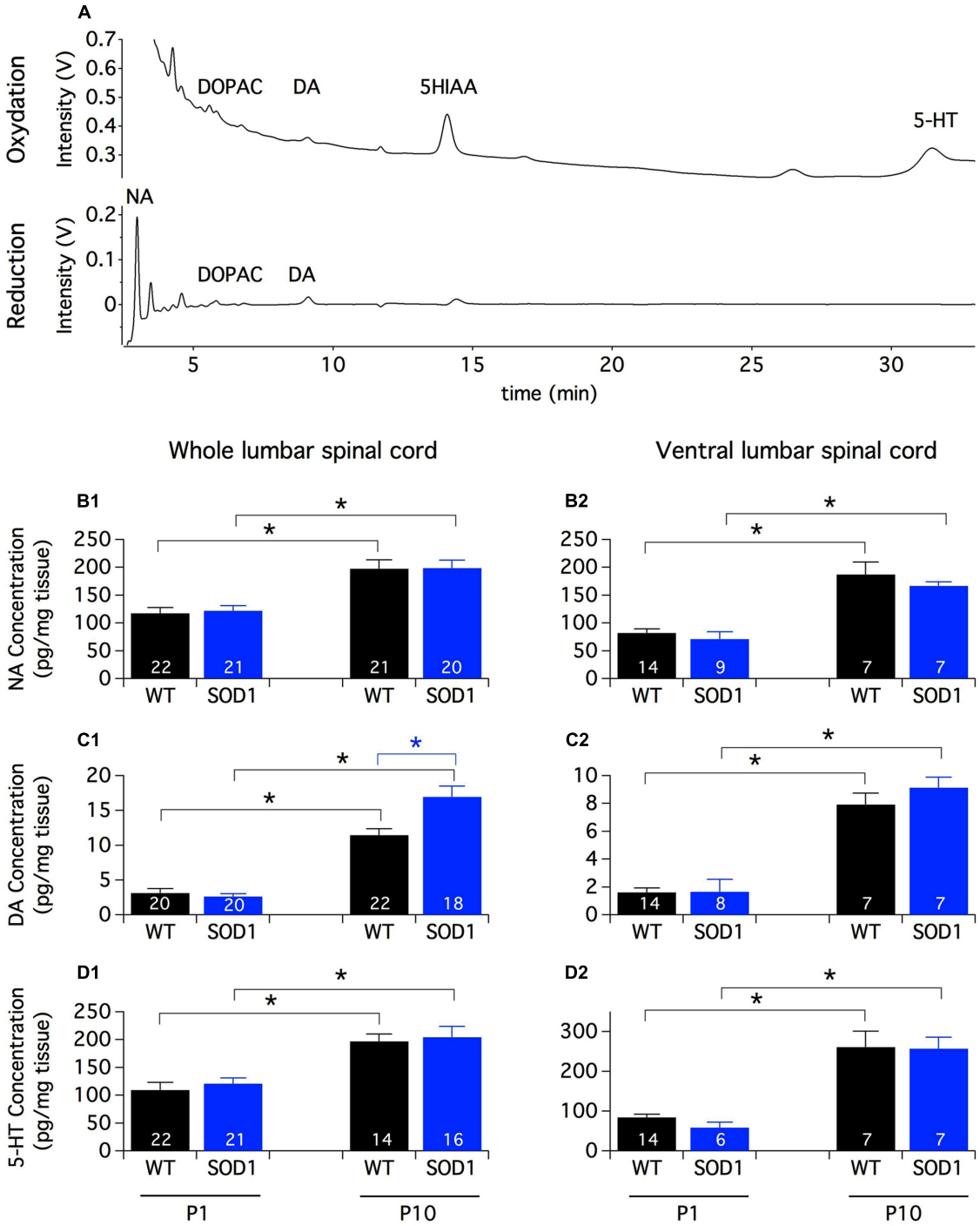

FIGURE 1 | Monoamine contents in the lumbar spinal cord of newborn mice. (A) Example of HPLC chromatograms of a whole spinal cord sample from a P10 SOD1 mouse. The chromatograms represent the output Coulometric signals (nanoampere converted in ±1 V output by the recorder) produced after their separation by the compounds at the level of the electrode of oxidation (upper trace; +350 mV) and/or the electrode of reduction (lower trace; −270 mV) of the Coulometric cell. Most of the quantitative analyses are performed using the oxidation channel (all compounds of interest except NA which is often confounded in the solvent front) and NA, DOPAC and DA quantities can be also analyzed using the reduction channel. **(B–D)** Contents of NA **(B1,B2)**, DA **(C1,C2)**, and 5-HT **(D1,D2)** measured by HPLC assays in the whole lumbar spinal cord **(B1–D1)** or in its ventral half **(B2–D2)** from P1 and P10 WT (black bars) and SOD1 (blue bars) mice. Note the increased concentration of monoamines with age and the significant difference in DA content between WT and SOD1 P10 mice. Asterisks indicate positive significance levels and the numbers in histogram bars refer to the number of samples tested. NA: noradrenaline, DA: dopamine, 5-HT: serotonin, DOPAC: 3,4-dihydroxyphenylacetic acid, 5-HIAA: 5-hydroxyindole-3-acetic.

Table 1 | HPLC measurements of 3,4-dihydroxyphenylacetic acid (DOPAC) and 5-hydroxyindole-3-acetic (5-HIAA) contents in the whole or ventral part of the lumbar spinal cord from P1 and P10 WT and SOD1 mice.

		Wild-type		SOD1	
		P1	**P10**	**P1**	**P10**
DOPAC	Whole lumbar	14.3 ± 4 (14)	27.8 ± 3 (18)	33.4 ± 10 (17)	26.9 ± 4 (17)
	Ventral lumbar	8.8 ± 1 (13)	28.2 ± 8 (7)*	11.5 ± 3 (6)	34.8 ± 9 (7)*
5-HIAA	Whole lumbar	148.9 ± 14	174.4 ± 18	154.7 ± 11	176.1 ± 17
	Ventral lumbar	99.4 ± 11	122.8 ± 15	100.9 ± 22	128.7 ± 19

The numbers in brackets correspond to the number of samples tested. Asterisks indicate significant differences between the P1 and P10 developmental stages.

NMA (16 μm) in the presence of increasing concentrations of 5-HT in activating the spinal locomotor CPGs in SOD1^{G93A} and age-matched littermate controls. **Figure 2A** shows that, regardless of the 5-HT concentration tested, the bath-application of NMA-5-HT triggered an alternating bursting activity of the right and left L2 and homolateral flexor L2 and extensor L5 ventral roots in both WT (**Figure 2A1**) and SOD1 mice (**Figure 2A2**). This motor pattern, with a mean period ranging from 3 to 4 s (**Figure 2D1**), is characteristic of a locomotor-like activity (Cazalets et al., 1992; Kiehn and Kjaerulff, 1996). Regardless of the animal genotype, raising the 5-HT concentration from 10 to 12.5 or 15 μm did not significantly affect the rhythm phase relationships (**Figures 2B,C**; **Table 1**), period (**Figure 2D1**) or motor burst duration (L2: **Figure 2D2**, Data not shown for L5). In contrast, the locomotor burst amplitude was significantly increased in the presence of 12.5 or 15 μm 5-HT compared to the 10 μm 5-HT condition (**Figure 2D3** for L2 bursts, data not shown for L5). This 5-HT-induced amplification was, however, not significantly different between the WT and SOD1 mice.

Overall, these data suggest that the spinal networks of newborn SOD1^{G93A} mice could generate well-organized locomotor patterns under NMA-5-HT chemical stimulation and present a WT-like serotoninergic sensitivity.

NORADRENERGIC MODULATION

In a first series of experiments, the effects of the bath-application of NA alone (10^{-5}, $5 \times 10^{-}5$, and 10^{-4} M (Sqalli-Houssaini and Cazalets, 2000) or in combination with NMA were tested. Under these conditions, tonic or very irregular motor activities were generated in both WT and SOD1 preparations, preventing accurate measurements from being performed (data not shown). In this context, we decided to restrict the analysis of the noradrenergic effects to the modulation of the NMA-5-HT-induced rhythm. After inducing control fictive locomotion with NMA-5-HT (16 μm each), WT and SOD1 spinal cords were challenged with increasing NA concentrations in the presence of NMA-5-HT (**Figure 3**). Regardless of the NA concentration and mouse genotype, the phase relationships of the NA+NMA/5-HT-induced rhythms were similar to those computed in the absence of NA (**Table 2**; **Figure 3B**; Data not shown for the L2/L5 alternation). In contrast, the locomotor rhythm was strongly slowed down in the presence of NA (**Figure 3A**). In WT animals (**Figure 3A1**), all of the NA concentrations tested (10, 50, and 100 μm) induced a significant increase in the locomotor parameters (period, **Figure 3C1**; L2 burst duration, **Figure 3C2**, and amplitude, L2: **Figure 3C3**, black bars, L5: data not shown) compared to the NMA-5-HT control conditions. The rhythm period and L2 burst duration values computed in the presence of 50 μm NA were also significantly different from the ones obtained with 10 μm NA in WT animals. In SOD1 mice (**Figure 3A2**), both the L2 burst durations and amplitude values were significantly enhanced compared to the NMA-5-HT condition for all of the NA concentrations tested (**Figures 3C2,C3**, blue bars). In contrast, the locomotor rhythm period was only significantly different from the control condition in the presence of 50 μm NA (**Figure 3C1**).

Interestingly, a *post hoc* analysis revealed that the NA-induced amplification of the L2 (**Figure 3C3**) and L5 (data not shown) burst amplitude values observed in the presence of 10 and 50 μm NA was significantly higher in SOD1 mice compared to WT animals. This result underscores the differences in the NA sensitivity between WT and SOD1 spinal locomotor networks.

DOPAMINERGIC MODULATION

As previously described for NA, the superfusion of DA by itself (10^{-4} and 5×10^{-4} M) or in combination with NMA (DA: 10^{-5} M, 5×10^{-5} M and 10^{-4} M (Barrière et al., 2004; Spalloni et al., 2011) on *in vitro* spinal cord preparations failed to generate regular motor activities that could be accurately analyzed in both WT and SOD1 preparations (data not shown; see also (Jiang et al., 1999). The comparison of the dopaminergic modulation between WT and SOD1 spinal cord networks was therefore restricted to the NMA-5-HT-induced locomotor like activity. For this purpose, control fictive locomotion was first acquired in the presence of NMA-5-HT (16 μm each, **Figure 4A**) and subsequent DA bath-applications of increasing concentration (50 and 500 μm) were realized. Similar to the other two amines tested, DA did not affect the phase relationships of the NMA-5-HT-induced locomotor activity (**Table 2** and **Figure 4B**, data not shown for the L2/L5 alternation) in both WT and SOD1 spinal cord preparations. However, irrespective of the mouse genotype, 50 or 500 μm DA significantly increased the values of all locomotor parameters (period, **Figure 4C1**; L2 burst duration, **Figure 4C2** and burst amplitude values, **Figure 4C3**, data not shown for L5) compared to the control NMA-5-HT condition. The period of the locomotor rhythm in WT animals (**Figure 4C1**) as well as the L2 burst amplitudes in SOD1 mice (**Figure 4C3**) computed in the presence of

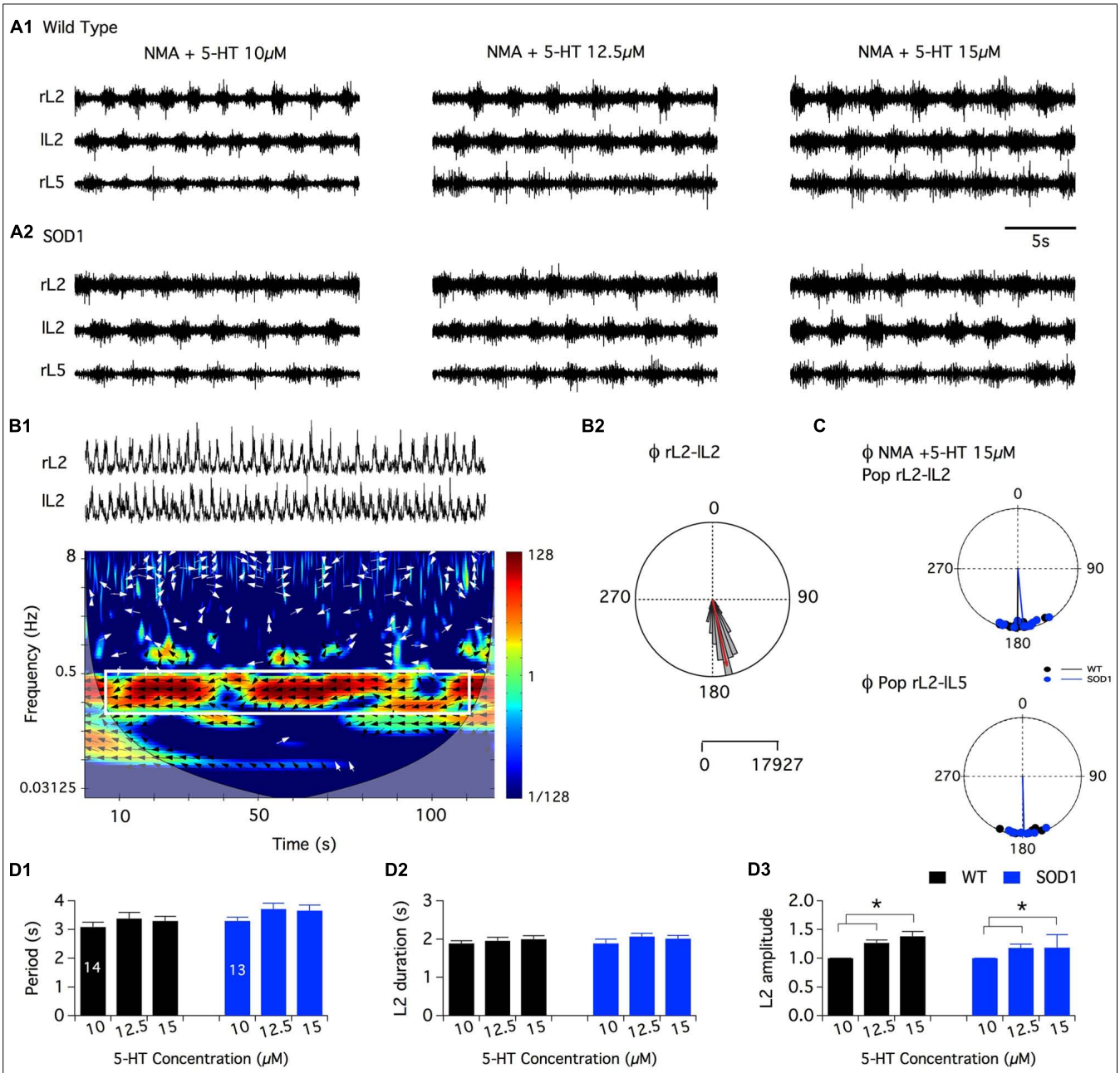

FIGURE 2 | Serotoninergic modulation of SOD1^{G93A} spinal locomotor networks. (A) Representative traces of the fictive locomotion recorded from the right and left lumbar 2 (rL2 and lL2) and right lumbar 5 (rL5) ventral roots in the presence of 16 μm NMA and increasing concentrations of serotonin in WT **(A1)** and SOD1 **(A2)** newborn mice. **(B)** Mixed cross-coherence time frequency map **(B1)** computed from the high pass, rectified and low pass filtered traces presented in the upper panel recorded in the presence of 16 μm NMA+ 15 μm 5-HT. The white box on the map represents the region of interest selected for further analysis. Black and white arrows indicate phase relationships with a left direction indicating an out of phase relationship. Polar graph **(B2)** of the phase (Φ) relationships between rL2 and lL2 extracted from the graph in **B1**. Calibration bars indicate the number of values in the largest histogram of repartition (5°bins). The evolution of the frequency components of the extracellular signals (*y*-axis, logarithmic scale) is represented as a function of time (*x*-axis). The power of the frequency is color-coded with warm colors assigned to high power regions and cool colors to low power regions. **(C)** Polar graphs of the mean phase relationships between rL2 and lL2 (upper panel) and rL2-lL5 (lower panel) for all the WT (black dots and line, n = 14) and SOD1 (blue dots and line, n = 13) spinal cord preparations tested in the presence of NMA+5-HT 15 μm. Pop: population. **(D)** Plots of the period **(D1)**, L2 burst duration **(D2)** and amplitude **(D3)** in the presence of increasing concentrations of 5-HT in WT (black bars) and SOD1 (blue bars) mice. This analysis reveals that SOD1 spinal cords exhibit a WT-like serotoninergic sensitivity. Asterisks indicate positive significance levels and the numbers in histogram bars refer to the number of spinal cord preparations tested.

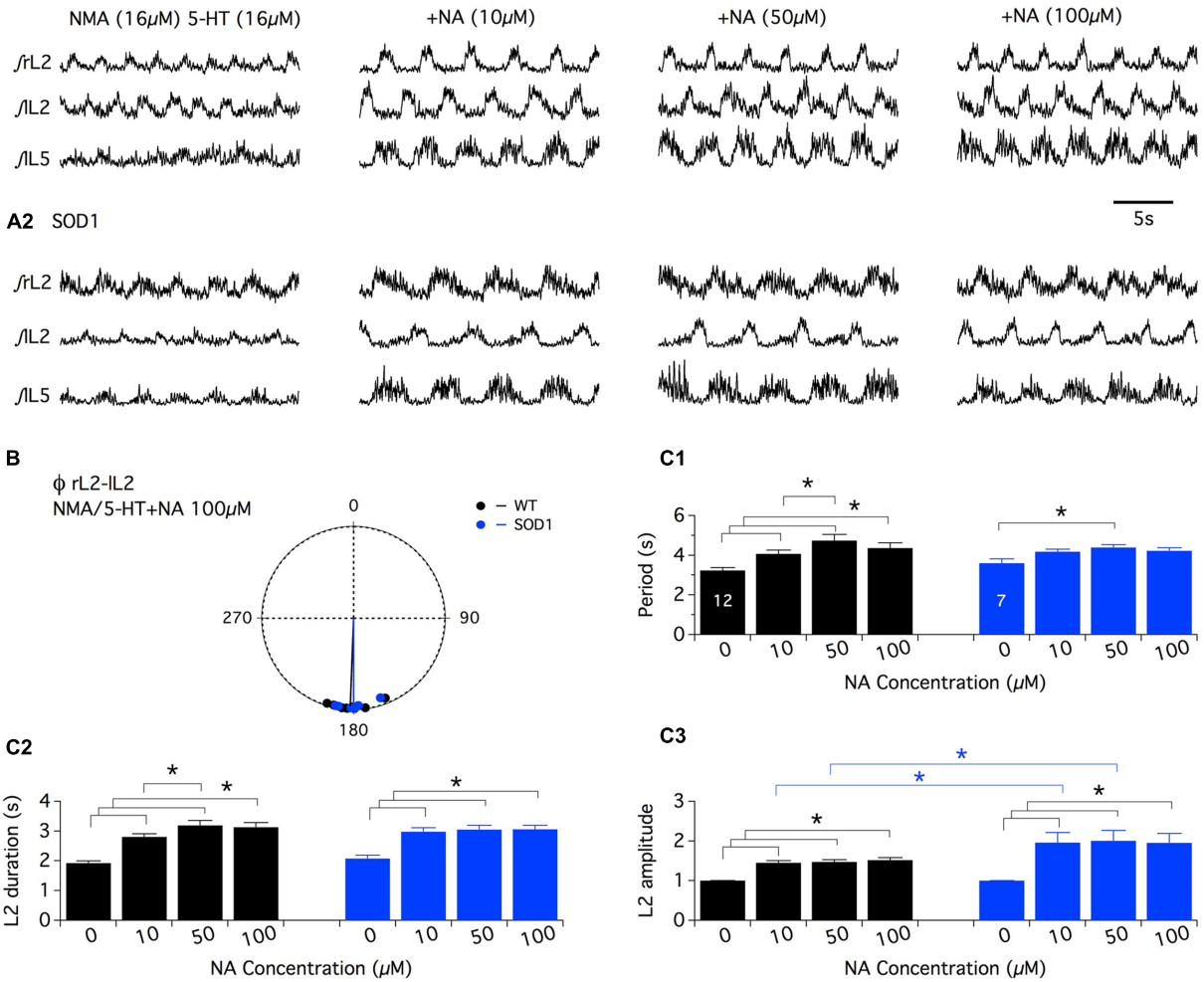

FIGURE 3 | Neuromodulatory actions of noradrenaline on NMA+5-HT-induced fictive locomotion in SOD1 and age-matched control mice. (A) Representative integrated (∫) extracellular recordings from the right, left L2 and left L5 ventral roots (rL2, lL2, and lL5) in the presence of NMA+5-HT (16 μm each) alone or with noradrenaline (NA; 10, 50, or 100 μm) in WT animals **(A1)** and SOD1 mice **(A2)**. **(B)** Polar graph of the rL2–lL2 phase (Φ) relationships computed in WT (black dots and line, $n = 12$) and SOD1 (blue dots and line, $n = 7$) spinal cords in the presence of 100 μm NA. **(C)** Plots of the period **(C1)**, L2 burst duration **(C2)** and amplitude **(C3)** in the absence or presence of NA bath-applied at increasing concentrations on WT (black bars) and SOD1 (blue bars) spinal cord preparations. Note the significant difference in L2 motor burst modulation between WT and SOD1 mice. Asterisks indicate positive significance levels and the numbers in histogram bars refer to the number of spinal cord preparations tested.

50 μm DA were significantly enhanced when DA concentration was raised to 500 μm. In contrast, the other locomotor parameters tended to increase in the presence of 500 μm DA but were not significantly different from those computed in the presence of 50 μm DA (**Figure 4C**). These results indicate that DA neuromodulatory processes are similar in WT and SOD1 spinal motor networks. These results are in agreement with the HPLC analysis of spinal DA content that failed to reveal differences between WT and SOD1 mice at this developmental stage.

DISCUSSION

FICTIVE LOCOMOTION GENERATION IN SOD1 SPINAL NETWORKS

Our data show that in contrast to what has been previously described in the $SOD1^{G85R}$ mouse line (Amendola et al., 2004), coordinated fictive locomotion could be efficiently triggered by the bath-application of NMA plus 5-HT in the $SOD1^{G93A}$ mouse spinal cord. Several mutant SOD1 mouse models that serve as invaluable tools to understand the pathophysiology of ALS have been developed. To date, 12 lines of transgenic mouse expressing different human mutated SOD1 proteins are available. Transgenic $SOD1^{G93A}$ mice are principally used in ALS research, followed by $SOD1^{G37R}$, $SOD1^{G85R}$, and $SOD1^{G86R}$ mice. All of these models exhibit slightly different time courses of the disease and associated neurodegenerative processes depending on the SOD1 mutation site, related enzymatic activity, transgene copy number and genetic background (Jonsson et al., 2006; Turner and Talbot, 2008; Van Den Bosch, 2011). $SOD1^{G93A}$ proteins, for example, are about 10 times more active than the native SOD1 proteins

Table 2 | Circular statistics of the phase relationships between right and left L2s in the presence of the different monaminergic concentrations.

	NMA 16 μm					
	+ 5-HT 10 μm		+ 5-HT 12.5 μm		+ 5-HT 15 μm	
	WT	SOD1	WT	SOD1	WT	SOD1
Mean vector	0.901	0.875	0.893	0.89	0.942 (14)	0.924 (13)
Mean angle	−3.11	3.11	−3.12	2.99	−3.13	3.04
	NMA + 5-HT		+ DA 50 μm		+ DA 500 μm	
	WT	SOD1	WT	SOD1	WT	SOD1
Mean vector	0.893	0.876	0.960	0.954	0.957 (16)	0.910 (11)
Mean angle	−3.09	2.98	3.02	−3.10	3.02	−3.02

	NMA + 5-HT		+ NA 10 μm		+ NA 50 μm		+ NA 100 μm	
	WT	SOD1	WT	SOD1	WT	SOD1	WT	SOD1
Mean vector	0.950	0.931	0.971	0.975	0.968	0.964	0.961 (12)	0.954 (7)
Mean angle	−3.09	3.03	3.13	3.08	3.06	3.08	−3.105	3.139

16 μm NMA and 16 μm 5-HT were used to induce control locomotor-like activity. The number of preparations tested in each condition is indicated in brackets. Circular two sample test analysis revealed no significant differences between the experimental conditions tested.

while in contrast, SOD1^{G85R} mutant proteins are almost inactive. Striking discrepancies between SOD1 models have been previously reported concerning motoneuron excitability (Meehan et al., 2010; Delestrée et al., 2014). Pambo-Pambo et al. (2009) have also shown that motoneurons are more immature in the *SOD1*^{G93A} low expressor line (*SOD1*$^{G93A\ low}$) compared to *SOD1*^{G85R}. Indeed, in newborn mice, SOD1$^{G93A\ low}$ motoneurons have a more depolarized resting membrane potential and appear to be more excitable than SOD1^{G85R} and WT motoneurons. The difference between SOD1^{G93A} and SOD1^{G85R} mouse lines in NMA/5-HT's effectiveness in triggering fictive locomotion further emphasizes the heterogeneity of SOD1 mouse mutants. This observed heterogeneity certainly parallels the complex etiology of ALS and stresses the importance of the complementary use of the different SOD1 mouse models to explore the different aspects of this motor disease.

In the present study, the NMA-5-HT-evoked rhythm was neither qualitatively nor quantitatively different between SOD1 and WT animals. These results suggest that whereas different groups have described early developmental alterations in motoneuron functioning, the locomotor outputs recorded from motoneuron axons in SOD1 mice are similar to the WT locomotor outputs. In the SOD1^{G93A} model, motoneurons at birth have been shown to be more immature, more excitable than WT motoneurons and to present a different dendritic branching pattern (Kuo, 2003; Pieri et al., 2003; Kuo et al., 2005; Pambo-Pambo et al., 2009). The question then arises as to whether these changes have a real impact on motoneuron output or whether the whole spinal network in charge of locomotion generation operates in such a way that these alterations are compensated. For example, it is well known that after a lesion, the spinal locomotor networks undergo a restructuring and can, after training, adapt their functioning and produce almost the same locomotor pattern that existed before the lesion (Barbeau and Rossignol, 1987; Rossignol et al., 2008). Such compensatory mechanisms may occur in newborn SOD1 spinal networks to ensure normal locomotor network function. A limitation of the present study is that locomotor activity was assessed using extracellular recordings. Further research is needed to further decipher the impact, at the cellular level, of the previously reported impairments in motoneuron excitability and morphology on the motor circuits.

SPINAL MONOAMINERIGIC NEUROMODULATION

Our HPLC data of either whole or ventral half spinal samples show that the monoaminergic rates rose between birth and the second postnatal week in the lumbar enlargement. This result is in agreement with developmental studies that have reported a progressive rostrocaudal gradient of the monaminergic innervation associated with an increase in axonal density (Commissiong, 1983; Rajaofetra et al., 1989, 1992; Giménez y Ribotta et al., 1998; Pappas et al., 2008; Pearlstein, 2013). Interestingly, we report an increased content of DA in the whole lumbar spinal cord of P10 SOD1 mice compared to WT animals. As this discrepancy was not observed in ventral spinal cord samples, our results provide insights into changes in the DA contents in the dorsal part of the SOD1 lumbar cord. This area is densely innervated and controlled by dopaminergic pathways (for review see Millan, 2002). Significant damages in the sensory system have been described in the presymptomatic stages in SOD1 models (Guo et al., 2009; Filali et al., 2011). Altered sensorimotor development characterized by a delay in maturation processes has also been reported in newborn SOD1 mice (Amendola et al., 2004). The increased

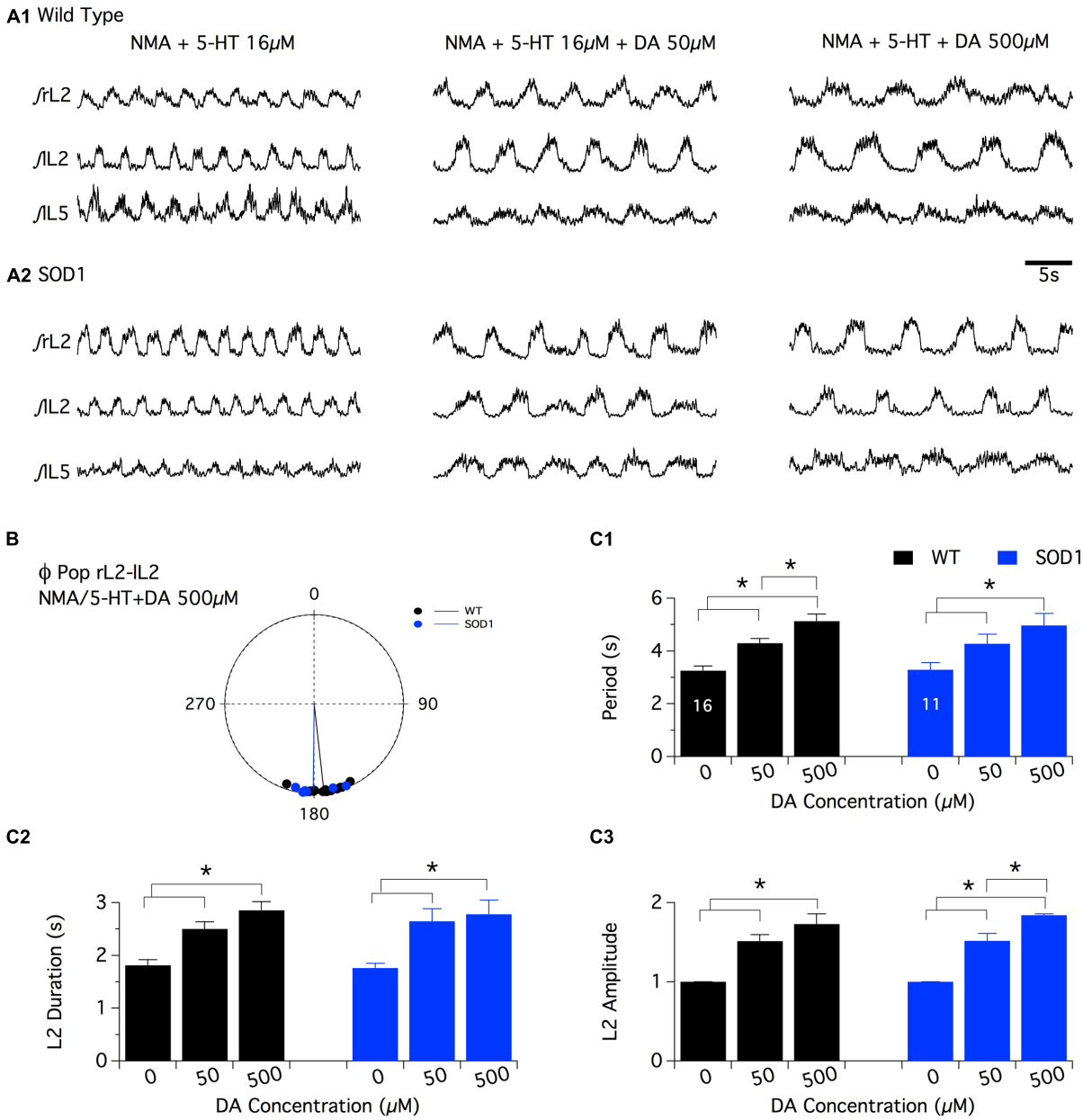

FIGURE 4 | Effects of dopamine on NMA+5-HT-induced fictive locomotion in SOD1 and age-matched control mice. (A) Representative integrated (∫) traces of NMA + 5-HT (16 μm each)-induced fictive locomotion recorded extracellularly from the right, left L2 and left L5 ventral roots (rL2, lL2, and lL5) in the absence or presence of dopamine (DA, 50 or 500 μm). Upper panels present traces obtained in WT animals **(A1)** and lower panels in SOD1 mice **(A2)**. **(B)** Polar graph of the rL2-lL2 phase (Φ) relationships computed in WT (black dots and line, $n = 16$) and SOD1 (blue dots and line, $n = 11$) spinal cords in the presence of 500 μm DA. **(C)** Plots of the period **(C1)**, L2 burst duration **(C2)** and amplitude **(C3)** in the absence (NMA + 5-HT alone) or presence of increasing concentrations of DA in WT (black bars) and SOD1 (blue bars) mice. These data show that the SOD1 genotype has no effect on the DA neuromodulation when investigated at the extracellular level. Asterisks indicate positive significance levels and the numbers in histogram bars refer to the number of spinal cord preparations tested.

DA content in the SOD1 dorsal spinal cord found in this study may contribute to these early alterations. It has been shown that both a reduction or an amplification of the monaminergic spinal content leads to a delay in spinal circuit maturation (Nakajima et al., 1998; Cazalets et al., 2000; Vinay et al., 2002), suggesting that a precise intraspinal level of these compounds is required for normal spinal cord network development. In the present study, we observed that regardless of the mouse genotype, the three monoamines tested potentiate locomotor activity by boosting the amplitude of the ventral root bursts and increasing the locomotor period. While DA contents were increased in the P10 SOD1 lumbar spinal cords, we failed to report any modification

in DA neuromodulation in SOD1 mice. This discrepancy could be explained by the fact that extracellular recordings were performed on spinal cord preparations from P1-P3 mice, ages where DA rates are similar between SOD1 and WT mice. The sustainable locomotor activities necessary for neuromodulatory studies are difficult to achieve in *in vitro* preparations from P10 mice (but see (Jiang et al., 1999). To assess the impact of the DA content increase on both basal membrane properties and DA sensitivity of the P10 lumbar motoneurons, patch-clamp recordings in spinal cord slices will be needed to investigate the effects of DA antagonists and agonists on these neurons. Dose-response curves will also have to be performed to assess and compare DA sensitivity in WT and SOD1 motoneurons.

It is generally acknowledged that changes in the period and/or phase relationships of the locomotor rhythm reflects effects on the locomotor CPG while modifications in the burst amplitudes are associated with changes in the motoneuron or last order interneuron excitability (Miles and Sillar, 2011). In SOD1 mice, the period and phase relationships of the rhythm expressed in the presence of the three monoamines tested were similar to those found in WT animals. The modulation of burst amplitude was also comparable between SOD1 and WT mice in the presence of 5-HT and DA. In contrast, the amplitude values of the bursts recorded in the presence of NA were more amplified in SOD1 mice compared to WT littermates. These results suggest that the monoaminergic neuromodulation of the locomotor CPG is preserved and normal in newborn $SOD1^{G93A}$ mice but that SOD1 motoneurons exhibit an increased sensitivity to NA compared to WT motoneurons. As the spinal cord size is not different between SOD1 and WT mice (personal unpublished observation), this effect could not be explained by differences in NA penetration into and the final concentrations attained within the spinal tissue (see for example Brumley et al., 2007). Lumbar motoneurons express the α_1, α_{2a}, and β_1 receptors at birth (Rekling et al., 2000; Tartas et al., 2010). We have previously shown in newborn rats that the activation of these receptors increased the lumbar motoneuron excitability partly via the inhibition of the inwardly rectifying K^+ current, K_{IR}, a key determinant of neuronal excitability (Tartas et al., 2010). In addition, NA, through the activation of presynaptic α1 and β receptors, enhanced the synaptic transmission originating from the upper lumbar segments in motoneurons (Tartas et al., 2010). Possible alterations in the expression of Kir channels and/or noradrenergic receptors could sustain part of the increased sensitivity to NA in SOD1 spinal cord by modifying both the intrinsic membrane properties of motoneurons and the excitatory synaptic inputs they receive. Up-regulation of NA receptor number, for example, could lead to NA supersensitivity in SOD1 motoneurons. This kind of phenomenon has been described for DA receptors in diverse pathological conditions (see for examples: Briand et al., 2008; Seeman, 2013).

Due to their lack of Ca^{2+} buffering proteins, motoneurons are more prone to excitotoxicity than other neurons. An excess of excitatory inputs has been hypothesized to play a major role in the neuronal degeneration observed in ALS (Turner et al., 2013). The NA hypersensitivity we reported in the present study could trigger aberrant depolarizations and subsequent Ca^{2+} entries in the lumbar motoneurons leading to progressive damage to the intracellular machinery. It is thus of interest to further decipher the cellular basis of the NA neuromodulation in newborn SOD1 motoneurons and to investigate the NA antagonist effects on the SOD1 mouse life span.

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REFERENCES

Amendola, J., Verrier, B., Roubertoux, P., and Durand, J. (2004). Altered sensorimotor development in a transgenic mouse model of amyotrophic lateral sclerosis. *Eur. J. Neurosci.* 20, 2822–2826. doi: 10.1111/j.1460-9568.2004.03745.x

Barbeau, H., and Rossignol, S. (1987). Recovery of locomotion after chronic spinalization in the adult cat. *Brain Res.* 412, 84–95. doi: 10.1016/0006-8993(87)91442-9

Barbeau, H., and Rossignol, S. (1991). Initiation and modulation of the locomotor pattern in the adult chronic spinal cat by noradrenergic, serotonergic and dopaminergic drugs. *Brain Res.* 546, 250–260. doi: 10.1016/0006-8993(91)91489-N

Barrière, G., Mellen, N., and Cazalets, J.-R. (2004). Neuromodulation of the locomotor network by dopamine in the isolated spinal cord of newborn rat. *Eur. J. Neurosci.* 19, 1325–1335. doi: 10.1111/j.1460-9568.2004.03210.x

Barrière, G., Tartas, M., Cazalets, J.-R., and Bertrand, S. S. (2008). Interplay between neuromodulator-induced switching of short-term plasticity at sensorimotor synapses in the neonatal rat spinal cord. *J. Physiol. (Lond.)* 586, 1903–1920. doi: 10.1113/jphysiol.2008.150706

Bories, C., Amendola, J., Lamotte d'Incamps, B., and Durand, J. (2007). Early electrophysiological abnormalities in lumbar motoneurons in a transgenic mouse model of amyotrophic lateral sclerosis. *Eur. J. Neurosci.* 25, 451–459. doi: 10.1111/j.1460-9568.2007.05306.x

Bregman, B. S. (1987). Development of serotonin immunoreactivity in the rat spinal cord and its plasticity after neonatal spinal cord lesions. *Brain Res.* 431, 245–263. doi: 10.1016/0165-3806(87)90213-6

Briand, L. A., Flagel, S. B., Seeman, P., and Robinson, T. E. (2008). Cocaine self-administration produces a persistent increase in dopamine D2High receptors. *Eur. Neuropsychopharmacol.* 18, 551–556. doi: 10.1016/j.euroneuro.2008.01.002

Brumley, M. R., Hentall, I. D., Pinzon, A., Kadam, B. H., Blythe, A., Sanchez, F. J., et al. (2007). Serotonin concentrations in the lumbosacral spinal cord of the adult rat following microinjection or dorsal surface application. *J. Neurophysiol.* 98, 1440–1450. doi: 10.1152/jn.00309.2007

Cazalets, J.-R., Gardette, M., and Hilaire, G. (2000). Locomotor network maturation is transiently delayed in the MAOA-deficient mouse. *J. Neurophysiol.* 83, 2468–2470.

Cazalets, J. R., Sqalli-Houssaini, Y., and Clarac, F. (1992). Activation of the central pattern generators for locomotion by serotonin and excitatory amino acids in neonatal rat. *J. Physiol. (Lond.)* 455, 187–204.

Chang, Q., and Martin, L. J. (2011). Glycine receptor channels in spinal motoneurons are abnormal in a transgenic mouse model of amyotrophic lateral sclerosis. *J. Neurosci.* 31, 2815–2827. doi: 10.1523/JNEUROSCI.2475-10.2011

Clarac, F., Brocard, F., and Vinay, L. (2004). The maturation of locomotor networks. *Prog. Brain Res.* 143, 57–66. doi: 10.1016/S0079-6123(03)43006-9

Commissiong, J. W. (1983). Development of catecholaminergic nerves in the spinal cord of the rat. *Brain Res.* 264, 197–208. doi: 10.1016/0006-8993(83)90817-X

Crick, H., and Wallis, D. I. (1991). Inhibition of reflex responses of neonate rat lumbar spinal cord by 5-hydroxytryptamine. *Br. J. Pharmacol.* 103, 1769–1775. doi: 10.1111/j.1476-5381.1991.tb09861.x

Delestrée, N., Manuel, M., Iglesias, C., Elbasiouny, S. M., Heckman, C. J., and Zytnicki, D. (2014). Adult spinal motoneurones are not hyperexcitable in a mouse model of inherited amyotrophic lateral sclerosis. *J. Physiol. (Lond.)* 592, 1687–1703. doi: 10.1113/jphysiol.2013.265843

Filali, M., Lalonde, R., and Rivest, S. (2011). Behavioural brain research. *Behav. Brain Res.* 225, 215–221. doi: 10.1016/j.bbr.2011.07.034

Filipchuk, A. A., and Durand, J. (2012). Postnatal dendritic development in lumbar motoneurons in mutant superoxide dismutase 1 mouse model of amyotrophic lateral sclerosis. *Neuroscience* 209, 144–154. doi: 10.1016/j.neuroscience.2012.01.046

Giménez y Ribotta, M., Sandillon, F., and Privat, A. (1998). Influence of hypergravity on the development of monoaminergic systems in the rat spinal cord. *Brain Res. Dev. Brain Res.* 111, 147–157. doi: 10.1016/S0165-3806(98)00132-1

Grinsted, A., Moore, J. C., and Jevrejeva, S. (2004). Application of the cross wavelet transform and wavelet coherence to geophysical time series. *Nonlinear Process. Geophys.* 11, 561–566. doi: 10.5194/npg-11-561-2004

Guo, Y.-S., Wu, D.-X., Wu, H.-R., Wu, S.-Y., Yang, C., Li, B., et al. (2009). Sensory involvement in the SOD1-G93A mouse model of amyotrophic lateral sclerosis. *Exp. Mol. Med.* 41, 140–150. doi: 10.3858/emm.2009.41.3.017

Han, P., Nakanishi, S. T., Tran, M. A., and Whelan, P. J. (2007). Dopaminergic modulation of spinal neuronal excitability. *J. Neurosci.* 27, 13192–13204. doi: 10.1523/JNEUROSCI.1279-07.2007

Han, P., and Whelan, P. J. (2009). Modulation of AMPA currents by D 1-like but not D 2-like receptors in spinal motoneurons. *Neuroscience* 158, 1699–1707. doi: 10.1016/j.neuroscience.2008.11.040

Humphreys, J. M., and Whelan, P. J. (2012). Dopamine exerts activation-dependent modulation of spinal locomotor circuits in the neonatal mouse. *J. Neurophysiol.* 108, 3370–3381. doi: 10.1152/jn.00482.2012

Jiang, Z., Carlin, K. P., and Brownstone, R. M. (1999). An in vitro functionally mature mouse spinal cord preparation for the study of spinal motor networks. *Brain Res.* 816, 493–499. doi: 10.1016/S0006-8993(98)01199-8

Jonsson, P. A., Graffmo, K. S., Andersen, P. M., Brännström, T., Lindberg, M., Oliveberg, M., et al. (2006). Disulphide-reduced superoxide dismutase-1 in CNS of transgenic amyotrophic lateral sclerosis models. *Brain* 129, 451–464. doi: 10.1093/brain/awh704

Kiehn, O. O., and Kjaerulff, O. O. (1996). Spatiotemporal characteristics of 5-HT and dopamine-induced rhythmic hindlimb activity in the in vitro neonatal rat. *J. Neurophysiol.* 75, 1472–1482.

Kuo, J. J. (2003). Hyperexcitability of cultured spinal motoneurons from presymptomatic ALS mice. *J. Neurophysiol.* 91, 571–575. doi: 10.1152/jn.00665.2003

Kuo, J. J., Siddique, T., Fu, R., and Heckman, C. J. (2005). Increased persistent Na(+) current and its effect on excitability in motoneurones cultured from mutant SOD1 mice. *J. Physiol. (Lond.)* 563, 843–854. doi: 10.1113/jphysiol.2004.074138

Martin, E., Cazenave, W., Cattaert, D., and Branchereau, P. (2013). Embryonic alteration of motoneuronal morphology induces hyperexcitability in the mouse model of amyotrophic lateral sclerosis. *Neurobiol. Dis.* 54, 116–126. doi: 10.1016/j.nbd.2013.02.011

Meehan, C. F., Moldovan, M., Marklund, S. L., Graffmo, K. S., Nielsen, J. B., and Hultborn, H. (2010). Intrinsic properties of lumbar motor neurones in the adult G127insTGGG superoxide dismutase-1 mutant mouse in vivo: evidence for increased persistent inward currents. *Acta Physiol. (Oxf.)* 200, 361–376. doi: 10.1111/j.1748-1716.2010.02188.x

Miles, G. B., and Sillar, K. T. (2011). Neuromodulation of vertebrate locomotor control networks. *Physiology* 26, 393–411. doi: 10.1152/physiol.00013.2011

Millan, M. J. (2002). Descending control of pain. *Prog. Neurobiol.* 66, 355–474. doi: 10.1016/S0301-0082(02)00009-6

Mor, Y., and Lev-Tov, A. (2007). Analysis of rhythmic patterns produced by spinal neural networks. *J. Neurophysiol.* 98, 2807–2817. doi: 10.1152/jn.00740.2007

Nakajima, K., Matsuyama, K., and Mori, S. (1998). Prenatal administration of para-chlorophenylalanine results in suppression of serotonergic system and disturbance of swimming movements in newborn rats. *Neurosci. Res.* 31, 155–169. doi: 10.1016/S0168-0102(98)00034-0

Pambo-Pambo, A., Durand, J., and Gueritaud, J. P. (2009). Early excitability changes in lumbar motoneurons of transgenic SOD1G85R and SOD1G93A-low Mice. *J. Neurophysiol.* 102, 3627–3642. doi: 10.1152/jn.00482.2009

Pappas, S. S., Behrouz, B., Janis, K. L., Goudreau, J. L., and Lookingland, K. J. (2008). Lack of D2 receptor mediated regulation of dopamine synthesis in A11 diencephalospinal neurons in male and female mice. *Brain Res.* 1214, 1–10. doi: 10.1016/j.brainres.2008.03.010

Pearlstein, E. (2013). Role of descending aminergic pathways in the development of locomotion. *Curr. Pharm. Des.* 19, 4332–4340. doi: 10.2174/13816128113192040002

Pieri, M., Albo, F., Gaetti, C., Spalloni, A., Bengtson, C. P., Longone, P., et al. (2003). Altered excitability of motor neurons in a transgenic mouse model of familial amyotrophic lateral sclerosis. *Neurosci. Lett.* 351, 153–156. doi: 10.1016/j.neulet.2003.07.010

Rajaofetra, N., Poulat, P., Marlier, L., Geffard, M., and Privat, A. (1992). Pre- and postnatal development of noradrenergic projections to the rat spinal cord: an immunocytochemical study. *Brain Res. Dev. Brain Res.* 67, 237–246. doi: 10.1016/0165-3806(92)90224-K

Rajaofetra, N., Sandillon, F., Geffard, M., and Privat, A. (1989). Pre- and post-natal ontogeny of serotonergic projections to the rat spinal cord. *J. Neurosci. Res.* 22, 305–321. doi: 10.1002/jnr.490220311

Rekling, J. C., Funk, G. D., Bayliss, D. A., Dong, X. W., and Feldman, J. L. (2000). Synaptic control of motoneuronal excitability. *Physiol. Rev.* 80, 767–852.

Rossignol, S., Barrière, G., Frigon, A., Barthélemy, D., Bouyer, L., Provencher, J., et al. (2008). Plasticity of locomotor sensorimotor interactions after peripheral and/or spinal lesions. *Brain Res. Rev.* 57, 228–240. doi: 10.1016/j.brainresrev.2007.06.019

Seeman, P. (2013). Are dopamine D2 receptors out of control in psychosis? *Prog. Neuropsychopharmacol. Biol. Psychiatry* 46, 146–152. doi: 10.1016/j.pnpbp.2013.07.006

Spalloni, A., Origlia, N., Sgobio, C., Trabalza, A., Nutini, M., Berretta, N., et al. (2011). Postsynaptic alteration of nr2a subunit and defective autophosphorylation of alphaCaMKII at threonine-286 contribute to abnormal plasticity and morphology of upper motor neurons in presymptomatic SOD1G93A mice, a murine model for amyotrophic lateral sclerosis. *Cereb. Cortex* 21, 796–805. doi: 10.1093/cercor/bhq152

Sqalli-Houssaini, Y., and Cazalets, J. R. (2000). Noradrenergic control of locomotor networks in the in vitro spinal cord of the neonatal rat. *Brain Res.* 852, 100–109. doi: 10.1016/S0006-8993(99)02219-2

Sqalli-Houssaini, Y., Cazalets, J. R., and Clarac, F. (1993). Oscillatory properties of the central pattern generator for locomotion in neonatal rats. *J. Neurophysiol.* 70, 803–813.

Tartas, M., Morin, F., Barrière, G., Goillandeau, M., Lacaille, J.-C., Cazalets, J.-R., et al. (2010). Noradrenergic modulation of intrinsic and synaptic properties of lumbar motoneurons in the neonatal rat spinal cord. *Front. Neural Circuits* 4:4. doi: 10.3389/neuro.04.004.2010

Torrence, C., and Compo, G. P. (1998). A practical guide to wavelet analysis. *Bull. Am. Meteorol. Soc.* 79, 61–78. doi: 10.1175/1520-0477(1998)079<0061:APGTWA>2.0.CO;2

Turner, B. J., and Talbot, K. (2008). Transgenics, toxicity and therapeutics in rodent models of mutant SOD1-mediated familial ALS. *Prog. Neurobiol.* 85, 94–134. doi: 10.1016/j.pneurobio.2008.01.001

Turner, M. R., Hardiman, O., Benatar, M., Brooks, B. R., Chio, A., de Carvalho, M., et al. (2013). Controversies and priorities in amyotrophic lateral sclerosis. *Lancet Neurol.* 12, 310–322. doi: 10.1016/S1474-4422(13)70036-X

Van Den Bosch, L. (2011). Genetic rodent models of amyotrophic lateral sclerosis. *J. Biomed. Biotechnol.* 2011:348765. doi: 10.1155/2011/348765

Vinay, L., Brocard, F., Clarac, F., Norreel, J. C., Pearlstein, E., and Pflieger, J. F. (2002). Development of posture and locomotion: an interplay of endogenously generated activities and neurotrophic actions by descending pathways. *Brain Res. Brain Res. Rev.* 40, 118–129. doi: 10.1016/S0165-0173(02)00195-9

Whelan, P., Bonnot, A., and O'Donovan, M. J. (2000). Properties of rhythmic activity generated by the isolated spinal cord of the neonatal mouse. *J. Neurophysiol.* 84, 2821–2833.

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Inter- and intralimb adaptations to a sensory perturbation during activation of the serotonin system after a low spinal cord transection in neonatal rats

Misty M. Strain[1], Sierra D. Kauer[2], Tina Kao[3,4,5] and Michele R. Brumley[2]*

[1] *Department of Psychology, Texas A&M University, College Station, TX, USA*
[2] *Department of Psychology, Idaho State University, Pocatello, ID, USA*
[3] *Department of Psychology, City University of New York, Brooklyn, NY, USA*
[4] *Department of Psychology, New York University, New York, NY, USA*
[5] *Department of Neuroscience, Columbia University, New York, NY, USA*

Edited by:
Brian R. Noga, University of Miami, USA

Reviewed by:
Laurent Vinay, CNRS and Aix-Marseille Université, France
Claire Wyart, Brain and Spinal Cord Institute (ICM), France

***Correspondence:**
Michele R. Brumley, Department of Psychology, Idaho State University, 921 S. 8th Ave., Stop 8112, Pocatello, ID 83209-8112, USA
e-mail: brummich@isu.edu

Activation of the serotonin system has been shown to induce locomotor activity following a spinal cord transection. This study examines how the isolated spinal cord adapts to a sensory perturbation during activation of the serotonergic system. Real-time and persistent effects of a perturbation were examined in intact and spinal transected newborn rats. Rats received a spinal surgery (sham or low thoracic transection) on postnatal day 1 and were tested 9 days later. At test, subjects were treated with the serotonergic receptor agonist quipazine (3.0 mg/kg) to induce stepping behavior. Half of the subjects experienced range of motion (ROM) restriction during stepping, while the other half did not. Differences in stepping behavior (interlimb coordination) and limb trajectories (intralimb coordination) were found to occur in both intact and spinal subjects. Adaptations were seen in the forelimbs and hindlimbs. Also, real-time and persistent effects of ROM restriction (following removal of the perturbation) were seen in ROM-restricted subjects. This study demonstrates the sensitivity of the isolated spinal cord to sensory feedback in conjunction with serotonin modulation.

Keywords: quipazine, stepping, range of motion, locomotion, development

INTRODUCTION

The role of serotonin (5-HT) in the activation and modulation of spinal locomotor circuits is well known, and has been demonstrated at the anatomical, neurophysiological, and behavioral levels of analysis. 5-HT receptors are found in high concentrations in locomotor regions of the spinal cord such as the cervical and lumbar enlargements (Jankowska et al., 1995, 1999), and bath application of 5-HT induces fictive locomotion in the spinal cord and spinal cord slices *in vitro* (e.g., Cowley and Schmidt, 1994; Garraway and Hochman, 2001; Hayes et al., 2008). During fictive locomotion induced by stimulation of the mesencephalic locomotor region, serotonergic boutons containing 5-HT_7, 5-HT_{2A}, and 5-HT_{1A} receptors were found to form synapses with or were in close proximity to activated lumbar motor neurons in the cat (Noga et al., 2009). *In vivo* behavioral studies further support the role of 5-HT in activating locomotor circuits. Treatment with 5-HT receptor agonists has been shown to produce alternated stepping behavior in intact and spinal transected fetal and newborn rats (McEwen et al., 1997; Brumley and Robinson, 2005; Brumley et al., 2012), adult rats (Kao et al., 2006), and adult mice (Lapointe and Guertin, 2008; Ung et al., 2008). Further, 5-HT receptor antagonists reduce locomotor activity produced by 5-HT treatment or electrical stimulation of the parapyramidal region (Cazalets et al., 1992; Liu and Jordan, 2005; Kao et al., 2006). Taken together, these studies point to an active role of the serotonergic system in modulating locomotor circuit activity.

Additionally, evidence suggests that sensory stimulation in conjunction with activation of 5-HT receptors increases locomotor function in spinal injured animals. For example, walking is improved in spinal injured mice following administration of the 5-HT_{2A} receptor agonist quipazine and robotic hindlimb training (Fong et al., 2005). Because 5-HT_{2A} receptor antagonists block quipazine-induced activity, quipazine is believed to act at the 5-HT_{2A} receptor (Ung et al., 2008). Quipazine and 8-OH-DPAT (a 5-$HT_{1A/7}$ agonist) also facilitate stepping on a treadmill in a posture-dependent manner (Slawinska et al., 2012). Barbeau and Rossignol (1990) reported increases in step length and flexor and extensor responses to treadmill training in spinal cats following treatment with 5-HT substances. The degree to which such improvements in locomotor activity are due to activation of the 5-HT system, sensory stimulation, or both, is difficult to disentangle. However, it is important to understand how malleable these isolated or injured spinal circuits remain, particularly for the development of treatments aimed at functional recovery of locomotor behavior.

Sensory stimulation below the site of a spinal cord injury (SCI) is often used in rehabilitation efforts in individuals with SCI or other spinal disorders (i.e., spina bifida) where spinal sensorimotor systems have disrupted supraspinal regulation (Field-Fote,

2001; Pepin et al., 2003; Teulier et al., 2009). Treadmill training is a common form of sensory stimulation used to improve locomotor function after injury. The dramatic influence of sensory stimulation can be seen most clearly in research examining how animals adapt their behavior during and after exposure to stimulation. For example, studies examining trip responses on a treadmill or interlimb training have shown that training with these forms of sensory experiences can produce both real-time and persistent effects on limb activity and coordination (Pang et al., 2003; Brumley and Robinson, 2010; Zhong et al., 2012). Changes in response to sensory feedback also have been reported in an *in vitro* spinal cord-hindlimb preparation (Hayes et al., 2012). These studies point to the role of sensory feedback in modulating locomotor recovery.

Understanding how activation of spinal locomotor circuits, modulation of neural pathways, and sensory feedback interact may help shed light on why some rehabilitation strategies help and others seem to have no effect or even be harmful. The current study attempts to examine this complex interaction by investigating how the isolated spinal cord adapts to a sensory perturbation during activation of the 5-HT system in rats 9 days after a complete low thoracic spinal cord transection. In this study, rats received a spinal transection on postnatal day 1 (P1) and were tested on P10. During testing, stepping behavior was induced with the 5-HT agonist quipazine. Half of the rats received range of motion (ROM) restriction during stepping. By comparing interlimb and intralimb coordination in sham and spinal subjects, we examined the real-time and persistent effects of the sensory perturbation (ROM restriction) on quipazine-induced stepping behavior. We expected both sham and spinal subjects to show real-time and persistent responses to the sensory perturbation, as research has shown that neonatal transected rats retain considerable spinal plasticity (Weber and Stelzner, 1980; Stelzner et al., 1986). Thus, we expected subjects to keep their limbs more proximal to the body not only during ROM restriction, but also following removal of the perturbation.

MATERIALS AND METHODS

SUBJECTS

Thirty-two Sprague–Dawley male rats received a low thoracic spinal cord transection or a sham operation on postnatal day 1 (P1; 24 h after birth) and were tested on P10. Adult rats were obtained from Simonsen Laboratories and mated in the PI's laboratory. Pregnant females were pair-housed until a couple days before birth, and then were housed individually. Animals were kept on a 12-h light:dark cycle with food and water available *ad libitum*. Animals were maintained in accordance with guidelines on animal care and use established by the NIH and Institutes of Laboratory Animal Resources (2011) and the Institutional Animal Care and Use Committee at Idaho State University.

STUDY DESIGN

A total of six subjects were in each of the four groups. Subjects received spinal surgery (half received a complete low thoracic spinal transection; the other half underwent a sham spinal operation) on P1. To control for maternal behavior within each litter, all pups from the same litter received the same operation. Subjects were tested on P10: all subjects were treated with quipazine and either experienced ROM restriction or no ROM restriction. Each subject was tested in only one condition. Litters were culled to 6–8 pups on P1. Each subject within a group was selected from a different dam to avoid litter effects. Only males were used to avoid confounding group differences with sex effects.

SPINAL SURGERY

Spinal surgery was performed on P1. Subjects were voided before surgery commenced and showed evidence of recent feeding by the presence of a milk band across the abdomen. Subjects were anesthetized by hypothermia. The spinal transection technique used was that of Kao et al. (2006). Briefly, a partial laminectomy exposed the spinal cord from T8 to T10. For subjects that received a spinal cord transection, the spinal cord was physically cut at the T9–T10 level and a collagen matrix was injected into the transection site. Muscle and skin on the back was sutured. For subjects that received the sham operation, all procedures were the same except that the transection was not performed and no collagen matrix was injected.

Following surgery, subjects were administered a 50 μl subcutaneous injection of both Buprenex (0.1 ml of 0.04 mg/kg solution) and 0.9% (wt/vol) saline. Each subject was then placed with littermates and bedding from their home cage inside an infant incubator maintained at 35°C. When subjects recovered from anesthesia and looked healthy (i.e., their color was pink and behavior was normal), they were returned to the home cage with the dam. They remained with the dam until the day of testing (P10). To ensure that the dam would take care of her pups equally, pups from the same litter received the same type of spinal surgery (i.e., spinal transection or sham operation); litters were not mixed. Subjects were checked daily to ensure no complications or infections. Subcutaneous injections of saline were injected as needed on P5 to help with weight gain and hydration.

BEHAVIORAL TESTING

Behavioral testing took place on P10. Subjects showed evidence of recent feeding. A minimum body weight cut-off of 15.50 g for inclusion in the study was used to ensure that subjects were within a fairly healthy weight range. However, comparison of body mass between the two surgery groups revealed a significant difference [$F_{(1,\ 22)} = 26.54$, $p < 0.001$], with spinal subjects (mean ± *SD*: 20.41 g ± 2.7) having lower body weights than sham subjects (26.93 g ± 3.4).

Subjects were individually tested inside an infant incubator that controlled temperature (30°C) and humidity. They were manually voided, and then acclimated to incubator conditions in a small plastic dish with up to two pups 30 min prior to testing. To start the test session, subjects were secured in the prone posture to a vinyl-coated horizontal bar using a jacket with adjustable straps across the neck and abdomen. The jacket did not impede limb movement; limbs hung pendantly in the air. Following a 5-min baseline, subjects were given an intraperitoneal injection of quipazine (3.0 mg/kg; Brumley et al., 2012) with volume of injection based on body weight (25 μl/5 g of body weight). Following injection, a 15-min ROM restriction period

was imposed for half of the subjects. To induce ROM restriction (and thus alter cutaneous and proprioceptive feedback), a piece of Plexiglas was placed beneath the subject's limbs at 50% limb length when the limbs were fully extended. For the remaining half of the subjects, no ROM restriction was imposed. After the 15-min period of ROM restriction, the Plexiglas was removed and the subject was recorded for a 15-min post-ROM restriction period. In the no ROM restriction condition, subjects continued to be recorded for the 15-min post-ROM restriction period. **Figure 1** shows the experimental timeline. The entire 35-min test session (5-min baseline, 15-min ROM restriction, and 15-min post-ROM restriction) was recorded using microcameras placed lateral and underneath the subject so that all limbs were visible. All sessions were recorded onto DVD for later behavioral scoring.

BEHAVIORAL SCORING

Interlimb coordination: stepping behavior

Stepping was scored during DVD playback using the underneath camera view with the software program JWatcher. Alternated and synchronized steps, along with non-stepping limb movements (e.g., twitches), were scored. Alternated steps were defined as occurring when the pup's homologous limbs exhibited sequential extension and flexion in one limb immediately followed by sequential extension and flexion in the other limb (Brumley et al., 2012). Synchronized steps were defined as occurring when the pup's homologous limbs exhibited simultaneous flexion and extension in both legs. Forelimbs and hindlimbs were scored in separate viewing passes. The scorer was blind to surgery condition. Intra- and interreliability for scoring was >90%.

Intralimb coordination: limb trajectories

Limb position was scored at specific time points to examine dorsal-ventral and rostral-caudal changes in the trajectory of limb movements, during DVD playback of the lateral camera view. The dorsal-ventral trajectory space was divided into a proximal and distal area, while the rostral-caudal trajectory space was divided into a front, center and back area (see **Figure 2**). These areas were calculated separately for the forelimbs and hindlimbs, for each subject.

The right forelimb and hindlimb were scored for each subject. Maximum limb length for each subject was determined from the baseline or post-ROM restriction period. To find maximum length, the ventrum of the subject and the tip of the toenail of the longest digit when the limb was fully extended were used as proximal and distal points on the forelimb, respectively. For the hindlimb, the base of the tail on the ventral side and the tip of the toenail of the longest digit when the limb was fully extended were used as proximal and distal points, respectively. Once the maximum length was found, a semicircle was made using the maximum length as the radius, with the center of the circle being placed on the appropriate proximal point (i.e., ventrum for forelimbs or base of tail for hindlimbs). An inner semicircle was then drawn within the aforementioned semicircle, with a radius equal to two-thirds the maximum length to

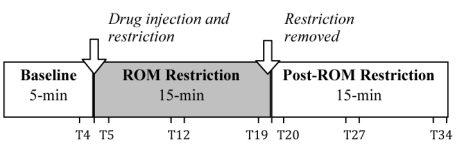

FIGURE 1 | Testing timeline. After a 5-min baseline, subjects were injected with the serotonergic receptor agonist quipazine to induce stepping behavior. This was followed by a 15-min period of either ROM restriction (Plexiglas placed at 50% of maximum limb length) or no ROM restriction. Testing continued for a 15-min Post-ROM restriction period to look at the persistent effects of ROM restriction. One-minute time points beginning at T4, T5, T12, T19, T20, T27, and T34 were used to analyze the effects of ROM restriction on limb trajectories.

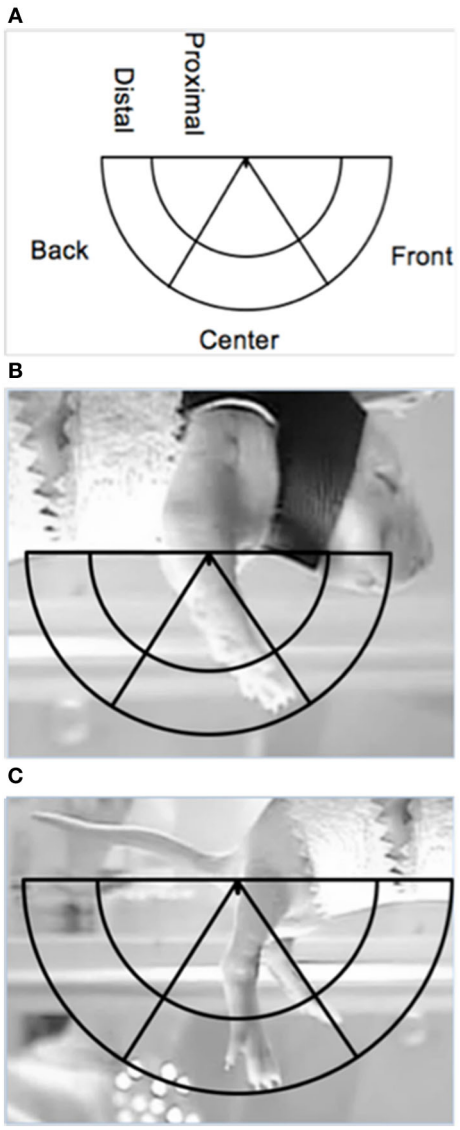

FIGURE 2 | Depiction of the limb trajectory space with the different limb trajectory areas labeled (A) along with illustrations of the placement of the trajectory area on proximal points for the (B) forelimbs and the (C) hindlimbs (the ventrum of the pup and the base of the tail, respectively).

create two dorsal-ventral limb trajectory areas: proximal (2/3 the distance of the maximum extension length) and distal (outer 1/3 the distance of the maximum extension length). To calculate the rostral-caudal trajectory areas, two lines were drawn from the appropriate proximal point (i.e., ventrum or base of tail) at 60- and 120-degrees of the entire 180-degree semicircle, thus creating a front (0–60 degrees), center (60–120 degrees), and back (120–180 degrees) trajectory area. **Figure 2** depicts the trajectory areas and their placement on the forelimb and hindlimb proximal points. Due to the time-consuming nature of this analysis and preplanned comparisons, specific 1-min sections were scored: the last minute of baseline (T4), beginning of ROM restriction (T5), middle of ROM restriction (T12), end of ROM restriction (T19), beginning of the post-ROM restriction period (T20), middle of post-ROM restriction (T27), and end of the post-ROM restriction (T34) (see **Figure 1**). The scorer was blind to surgery condition.

HISTOLOGY

After testing, subjects were euthanized and preserved in 10% (wt/vol) formalin. Specimens were later examined under low magnification to verify complete spinal cord separation between spinal segments T9 and T10 (for spinal transected subjects) or an intact spinal cord (for shams).

DATA ANALYSIS

Data were analyzed using SPSS statistical software. A significance level of $p \leq 0.05$ was adopted. *Post-hoc* tests used Tukey's Honestly Significant Difference.

Stepping behavior

Forelimb and hindlimb stepping was compared during the 35-min test session (5-min baseline, 15-min ROM restriction, and 15-min post-ROM restriction period). Frequencies of alternated steps and synchronized steps, along with the percentage of alternated and synchronized steps (calculated as a function of total limb movements) were summarized into 5-min time bins. Data were analyzed using repeated measures ANOVA, with surgery and ROM restriction condition as between subjects variables and time as a within subjects (repeated measure) variable. Forelimb and hindlimb data were analyzed separately.

Limb trajectories

Total time in each trajectory area (proximal, distal, front, center, and back) per 1-min section (as described above) was scored for hindlimbs and forelimbs. Data were analyzed using repeated measures ANOVA, with surgery and ROM restriction condition as between subjects variables and time as the within subjects (repeated measure) variable. Preplanned comparisons examined differences between baseline trajectories (T4) and those seen at the beginning of ROM restriction (T5), the end of ROM restriction (T19), after removal of ROM restriction (T20), and end of post-ROM restriction (T34) (see **Figure 1**). Additionally, comparison of sections within the ROM restriction period (T5, T12, and T19) was performed to determine real-time changes due to the perturbation. The time bin preceding (T19) and following (T20) post-ROM restriction were compared to determine immediate adaptations to removal of the perturbation. Comparison of all time bins during the post-ROM restriction period (T20, T27, and T34) was conducted to look for lasting changes. Forelimb and hindlimb data were analyzed separately.

RESULTS

Because spinal and sham subjects differed in body weight, we examined the correlation between body weight and total forelimb and hindlimb movements. There was no correlation (p = n.s.) between body weight and fore- or hindlimb activity. Therefore, we did not examine the influence of body weight further.

INTERLIMB COORDINATION: STEPPING BEHAVIOR

Forelimb stepping

Alternated forelimb steps. For frequency of alternated forelimb steps, there was a main effect of time [$F_{(6,\ 120)} = 12.49, p < 0.001$]. Follow-up analysis did not reveal any significant differences among time points; however, as shown in **Figure 3A**, there was a slight increase in forelimb steps after baseline followed by a reduction 15-min later. Because individual subjects may differ in their amount of total limb activity and thus the proportion of steps among subjects may vary, the percentage of alternated steps as a function of total movements (all steps + non-stepping movements) was examined. For percentage of alternated forelimb steps, there were main effects of surgery [$F_{(1,\ 20)} = 6.10, p = 0.02$] and time [$F_{(6,\ 120)} = 17.73, p < 0.001$]. The percentage of alternated forelimb steps significantly increased after baseline, and spinal subjects showed a significantly higher percentage of alternated steps compared to shams (**Figure 3B**).

Synchronized forelimb steps. For frequency of synchronized forelimb steps, there were effects of surgery [$F_{(1,\ 20)} = 4.98, p = 0.04$] and ROM restriction condition [$F_{(1,\ 20)} = 4.98, p = 0.04$], and a two-way interaction between surgery and time [$F_{(6,\ 120)} = 3.55, p = 0.002$]. As shown in **Figure 3C**, sham subjects expressed significantly more synchronized steps compared to spinal subjects at T20 and T30, with T25 approaching significance ($p = 0.07$). Additionally, subjects that received no ROM restriction expressed significantly more synchronized steps compared to subjects that did receive ROM restriction. The results for percentage of synchronized forelimb steps are very similar to those for frequency. There were main effects of surgery [$F_{(1,\ 20)} = 7.94,\ p = 0.01$] and ROM restriction condition [$F_{(1,\ 20)} = 5.80, p = 0.03$], and a two-way interaction between surgery and time [$F_{(6,\ 120)} = 4.41, p < 0.001$]. As shown in **Figure 3D**, sham subjects showed a significantly higher percentage of synchronized steps during the last 15 min of the test session (T15–T30) compared to spinal subjects, and subjects that did not receive ROM restriction showed a significantly higher percentage of synchronized forelimb steps compared to ROM-restricted subjects.

Hindlimb stepping

Alternated hindlimb steps. For alternated hindlimb steps there were effects of surgery [$F_{(1,\ 20)} = 21.77,\ p < 0.001$] and time [$F_{(6,\ 120)} = 20.89,\ p < 0.001$], two-way interactions between surgery and time [$F_{(6,\ 120)} = 11.51, p < 0.001$] and ROM restriction condition and time [$F_{(6,\ 120)} = 3.22,\ p = 0.006$], and a three-way interaction between all factors [$F_{(6,\ 120)} = 6.27,$

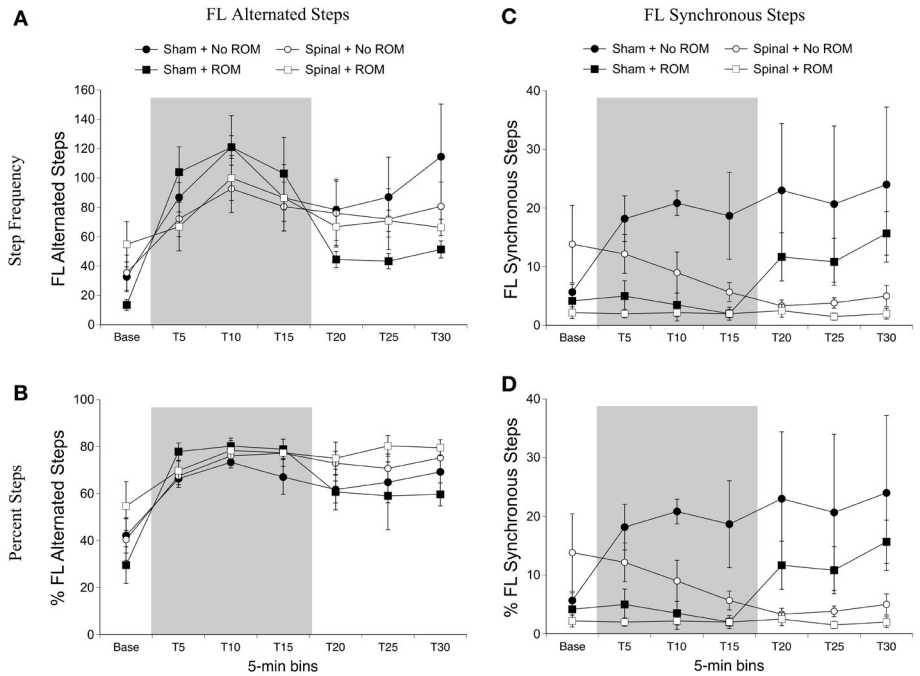

FIGURE 3 | Step frequency and percentage of forelimb steps for sham and spinal P10 rats by ROM condition. Frequency **(A)** and percentage **(B)** of alternated forelimb steps, and frequency **(C)** and percentage **(D)** of synchronous forelimb steps are shown. The shaded gray region reflects the period of ROM restriction. Points show means; vertical lines are s.e.m.

$p < 0.001$]. Follow-up analyses showed that significantly more alternated hindlimb steps occurred in spinal subjects compared to shams, and that alternated hindlimb stepping significantly increased after baseline (**Figure 4A**). To examine the three-way interaction, ROM restriction condition and time were examined within the two surgery conditions. As shown in **Figure 4A**, spinal subjects that experienced ROM restriction showed significantly fewer alternated steps at T10 and T15 compared to spinal subjects that did not experience ROM restriction. No significant differences were seen in shams. For percentage of alternated hindlimb steps there was a main effect of time [$F_{(6,\ 120)} = 68.78$, $p < 0.001$], a two-way interaction between surgery and time [$F_{(6,\ 120)} = 6.06$, $p < 0.001$], and a three-way interaction between surgery, ROM restriction condition and time [$F_{(6,\ 120)} = 2.66$, $p = 0.02$]. The percentage of alternated hindlimb steps increased after baseline and remained elevated throughout testing, and spinal subjects that received ROM restriction showed a significantly lower percentage of alternated steps at T10 compared to spinal subjects that did not experience ROM restriction (**Figure 4B**). Again we found no differences in sham subjects.

Synchronized hindlimb steps. For synchronized hindlimb steps there were main effects of surgery [$F_{(1,\ 20)} = 5.36$, $p = 0.03$] and time [$F_{(6,\ 120)} = 5.57$, $p < 0.001$], two-way interactions between surgery and time [$F_{(6,\ 120)} = 2.47$, $p = 0.03$], and ROM restriction condition and time [$F_{(6,\ 120)} = 3.26$, $p = 0.005$], and a three-way interaction between all factors [$F_{(6,\ 120)} = 2.59$, $p = 0.02$]. Significantly more synchronized steps occurred in spinal subjects compared to shams, and synchronized hindlimb stepping significantly increased after baseline (**Figure 4C**). To examine the three-way interaction, ROM condition and time were examined within the two surgery conditions. In spinal subjects, significantly fewer synchronized steps occurred at T15 for subjects receiving ROM restriction compared to those that did not receive restriction. There were no differences between ROM restriction conditions in the sham group. For percentage of synchronized hindlimb steps, there was a main effect of time [$F_{(6,\ 120)} = 5.33$, $p < 0.001$]. Follow-up analyses did not reveal any significant differences among the different time points. However, as can be seen in **Figure 4D**, most groups showed a slightly higher percentage of synchronized steps during the last half of the test session.

INTRALIMB COORDINATION: LIMB TRAJECTORIES

Forelimb trajectories

We first examined time spent in the dorsal-ventral limb trajectory space. The *proximal* area was within 2/3 distance of maximum limb extension, whereas the *distal* area was the outer 1/3 distance (see **Figure 2**). We only analyzed time spent in one area (distal), as the pattern of effects are mirrored in the opposite (proximal) area. For time spent in the distal area, there was a main effect of time [$F_{(6,\ 120)} = 3.94$, $p < 0.001$]. Significantly less time was spent in the distal area for the forelimbs at T19 compared to baseline, and significantly more time was spent in the distal area at T34 compared to T19 (**Figure 5**).

For the rostral-caudal limb trajectory space, we divided it into three equal areas: *front*, *center*, and *back* (see **Figure 2**). Time spent in each of these areas was analyzed separately. For time in the front area for the forelimbs there were effects of ROM restriction

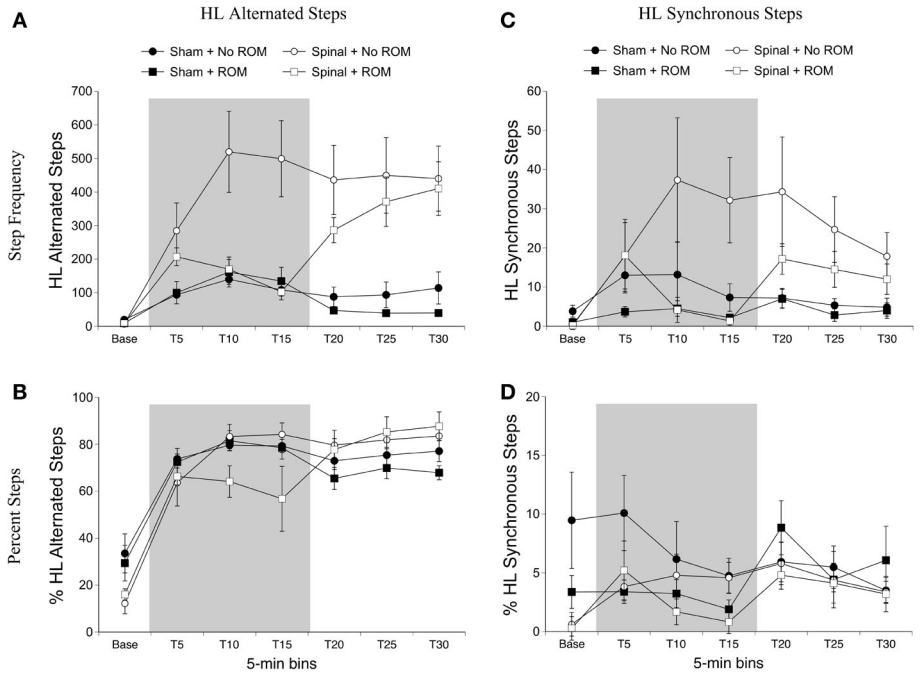

FIGURE 4 | Step frequency and percentage of hindlimb steps for sham and spinal P10 rats by ROM restriction condition. Frequency **(A)** and percentage **(B)** of alternated hindlimb steps, and frequency **(C)** and percentage **(D)** of synchronous hindlimb steps are shown. The shaded gray region reflects the period of ROM restriction. Points show means; vertical lines are s.e.m.

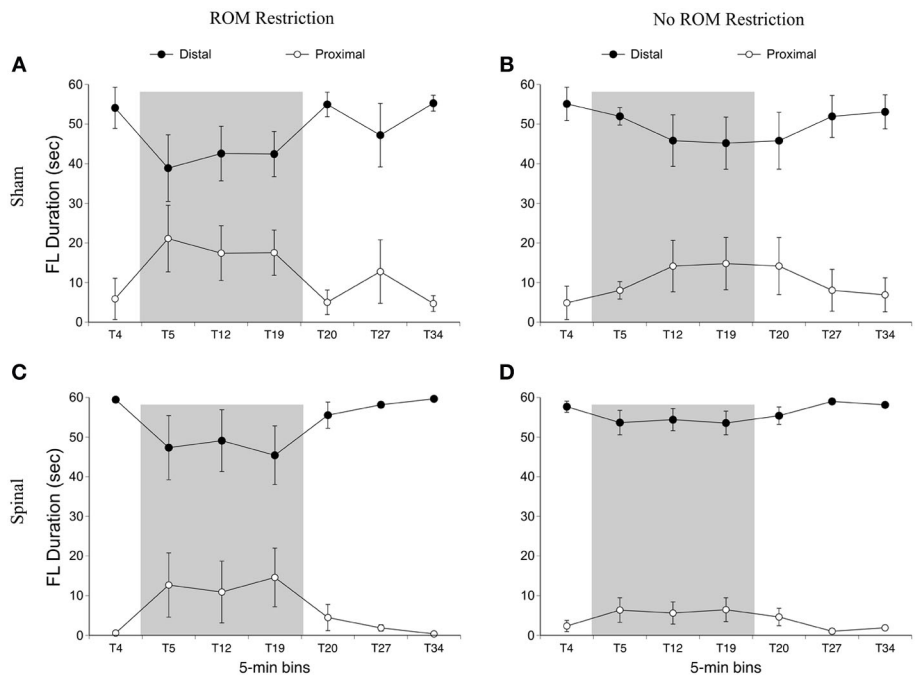

FIGURE 5 | Dorsal-ventral forelimb trajectories for sham and spinal P10 rats by ROM restriction condition. Graphs show duration of time spent in distal and proximal trajectory areas for 1-min sections during baseline, the ROM restriction period, and the post-ROM restriction period for **(A)** sham subjects that received ROM restriction, **(B)** sham subjects that did not receive ROM restriction, **(C)** spinal subjects that received ROM restriction, and **(D)** spinal subjects that did not receive ROM restriction. The shaded gray region reflects the period of ROM restriction. Points show means; vertical lines are s.e.m.

condition [$F_{(1, 20)} = 14.52$, $p = 0.001$] and time [$F_{(6, 120)} = 28.86$, $p < 0.001$], a two-way interaction between ROM restriction condition and time [$F_{(6, 120)} = 7.67$, $p < 0.001$], and a three-way interaction between all factors [$F_{(6, 120)} = 2.24$, $p = 0.04$]. ROM-restricted subjects spent significantly more time in the front compared to subjects that did not receive ROM restriction, and time in the front increased significantly after baseline. For ROM-restricted sham subjects, significantly more time was spent in the front at T5 and T12 and at T19 approached significance ($p = 0.07$), compared to sham subjects that did not receive ROM restriction. For ROM-restricted spinal subjects, significantly more time was spent in the front with the forelimbs from T5 to T27 compared to spinal subjects that did not receive ROM restriction. When surgery and time were examined within the two ROM restriction conditions, ROM-restricted spinal subjects showed significantly more time in the front at T20–27 than ROM-restricted shams. These effects can be seen in **Figure 6**.

For time in the center area with the forelimbs there were effects of ROM restriction condition [$F_{(1, 20)} = 14.65, p = 0.001$] and time [$F_{(6, 120)} = 31.07$, $p < 0.001$], a two-way interaction between ROM restriction condition and time [$F_{(6, 120)} = 7.37$, $p < 0.001$], and a three-way interaction between all factors [$F_{(6, 120)} = 2.29$, $p = 0.04$]. As shown in **Figure 6**, ROM-restricted subjects spent significantly less time in the center with their forelimbs compared to subjects that did not experience ROM restriction, and that time in center decreased after baseline. For ROM-restricted shams, significantly less time was spent in center at T5 and T12, with T19 approaching significance ($p = 0.06$), compared to shams that did not experience ROM restriction. For ROM-restricted spinal subjects, significantly less time was spent in center from T5 to T27 compared to spinal subjects that did not receive ROM restriction. Also, spinal subjects that experienced ROM restriction showed significantly less time in center at T20–27 than shams that received ROM restriction. Thus, both surgery groups decreased the amount of time in center with their forelimbs during the period of ROM restriction. Significant decreases in the center area also were seen in the forelimbs of spinal subjects during the first 10 min of post-ROM restriction (**Figure 6**).

For time in the back area with the forelimbs there were effects of surgery [$F_{(1, 20)} = 4.61$, $p = 0.04$] and ROM restriction condition [$F_{(1, 20)} = 4.47$, $p = 0.05$]. Sham subjects spent significantly more time in the back area compared to spinal subjects, and ROM-restricted subjects spent significantly less time in back compared to subjects that did not experience restriction (**Figure 6**).

Summary. No effects were seen for time spent in the distal area for the forelimbs as a result of ROM restriction. From T5 to T12, sham subjects that experienced ROM restriction decreased time in center and increased time in the front area. From T5 to T27, spinal subjects that experienced ROM restriction decreased time in center and increased time in the front area as well.

Preplanned comparisons. For sham and spinal subjects that experienced ROM restriction, there were significant decreases in

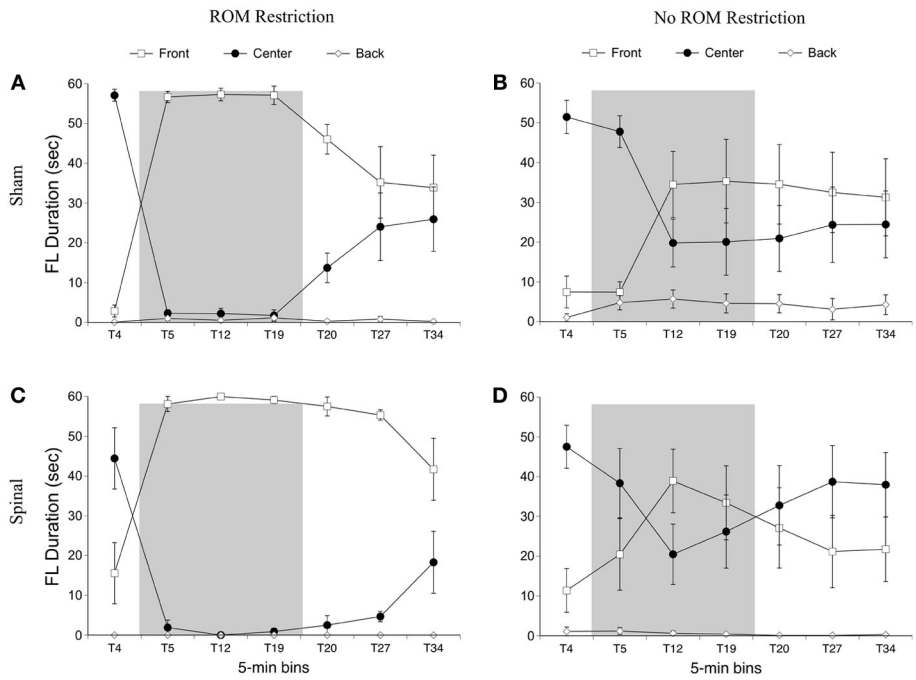

FIGURE 6 | Rostral-caudal forelimb trajectories for sham and spinal P10 rats by ROM restriction condition. Graphs show duration of time spent in front, center, and back trajectory areas for 1-min sections during baseline, the ROM restriction period, and the post-ROM restriction period for **(A)** sham subjects that received ROM restriction, **(B)** sham subjects that did not receive ROM restriction, **(C)** spinal subjects that received ROM restriction, and **(D)** spinal subjects that did not receive ROM restriction. The shaded gray region reflects the period of ROM restriction. Points show means; vertical lines are s.e.m.

the center and significant increases in the front area with the forelimbs between baseline (T4) and the following time points: beginning of ROM restriction (T5), end of ROM restriction (T19), start of post-ROM restriction (T20), and end of post-ROM restriction (T34). These effects are summarized in **Figure 7**. Other comparisons were not significantly different between groups.

Hindlimb trajectories

For time in the distal area with the hindlimbs, there was an effect of time [$F_{(6,\ 120)} = 19.12$, $p < 0.001$] and an interaction between ROM restriction condition and time [$F_{(6,\ 120)} = 17.22$, $p < 0.001$]. For ROM-restricted subjects, significantly less time was spent in the distal area during ROM restriction (T5–T19), compared to subjects that did not experience ROM restriction. This can be seen in **Figure 8**.

For time in the front area there were effects of surgery [$F_{(1,\ 20)} = 8.01, p = 0.01$] and time [$F_{(6,\ 120)} = 8.54, p < 0.001$], and interactions between surgery and time [$F_{(6,\ 120)} = 7.31, p < 0.001$] and ROM restriction condition and time [$F_{(6,\ 120)} = 2.44$, $p = 0.03$]. Spinal subjects showed significantly more time in the front with their hindlimbs compared to shams, and significantly more time in the front after baseline (see **Figure 9**). Follow-up analysis of the two-way interaction between surgery and time revealed that spinal subjects spent significantly more time in the front from T12 to T27 compared to shams. Follow-up analysis of the interaction between ROM restriction condition and time showed that ROM-restricted subjects spent significantly more time in the front at T5 and T19 compared to subjects that did not receive ROM restriction.

For time in the center area with the hindlimbs there were effects of ROM restriction condition [$F_{(1,\ 20)} = 10.28, p = 0.004$] and time [$F_{(6,\ 120)} = 23.79$, $p < 0.001$], interactions between surgery and time [$F_{(6,\ 120)} = 5.30, p < 0.001$] and ROM restriction condition and time [$F_{(6,\ 120)} = 6.32$, $p < 0.001$], and a three-way interaction between all factors [$F_{(6,\ 120)} = 3.05$, $p = 0.008$; **Figure 9**]. ROM-restricted subjects spent significantly less time in center compared to subjects that did not experience restriction, and time in center significantly decreased after baseline. For shams that experienced ROM restriction, less time was spent in center at T5 compared to subjects that did not receive restriction. For spinal subjects, less time was spent in center at T19 and approached significance at T12 ($p = 0.07$), compared to subjects that did not receive ROM restriction. Also, for spinal subjects that experienced ROM restriction, less time was spent in center from T19 to T27 compared to shams that experienced ROM restriction. These effects can be seen in **Figure 9**.

For time in the back area with the hindlimbs, there were effects of surgery [$F(6, 120) = 6.46, p = 0.02$] and time [$F(6, 120) = 7.10, p < 0.001$], an interaction between surgery and time [$F(6, 120) = 3.71, p = 0.002$] and ROM restriction condition and time [$F(6, 120) = 2.84, p = 0.01$], and an interaction between all factors [$F(6, 120) = 2.445, p = 0.04$]. Shams spent significantly more time than spinal subjects in back with their hindlimbs, and time in back significantly increased after baseline. ROM-restricted shams showed significantly more time in back at T5 compared to shams that did not experience restriction. Also, ROM-restricted spinal subjects showed significantly more time in back at T5 compared to ROM-restricted shams. For subjects that did not experience restriction, spinal subjects showed significantly more time in the back area at T20 with T19 approaching significance ($p = 0.06$), compared to shams.

Summary. ROM-restricted subjects spent less time in the distal area with the hindlimbs during the restriction period. Additionally, for shams that experienced restriction, less time was spent in the center and more time was spent in the back at T5. For spinal subjects that experienced restriction, less time was spent in the center and more time was spent in the front at T19.

Preplanned comparisons. For sham subjects that experienced ROM restriction, significantly less time was spent in the center and distal areas with the hindlimbs between baseline (T4) and beginning of ROM restriction (T5) and end of ROM restriction (T19). Other comparisons were not significant in shams. For spinal subjects that experienced ROM restriction, there were significant decreases for time spent in the distal area with the hindlimbs between the baseline (T4) and beginning and end of ROM restriction (T5 and T19). There were significant decreases in center between baseline (T4) and

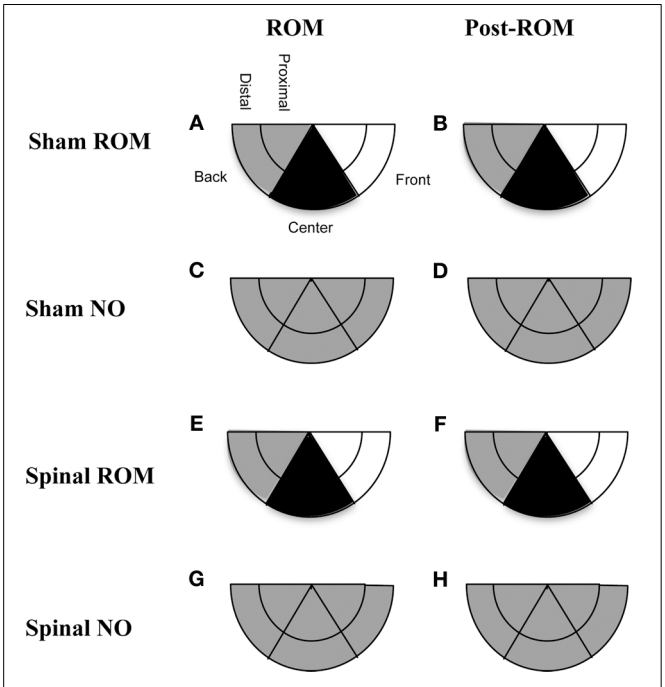

FIGURE 7 | Summary of changes in forelimb trajectories for sham and spinal P10 rats by ROM restriction condition. Changes in limb trajectories are show for sham subjects that experienced ROM restriction **(A,B)**, shams that did not receive ROM restriction **(C,D)**, spinal subjects that received ROM restriction **(E,F)**, and spinal subjects that did not receive ROM restriction **(G,H)**. The shaded regions reflect an increase (white), maintenance (gray), or decrease (black) from baseline within that trajectory area during the designated time period.

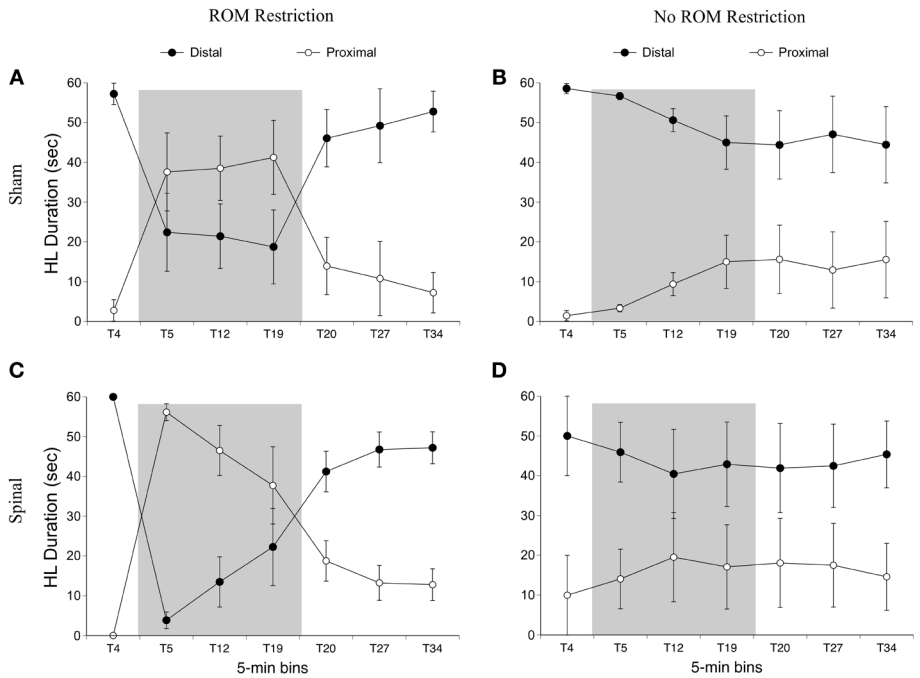

FIGURE 8 | Dorsal-ventral hindlimb trajectories for sham and spinal rats by ROM restriction condition. Graphs show duration of time spent in distal and proximal trajectory areas for 1-min sections during baseline, ROM restriction, and the post-ROM restriction period for **(A)** sham subjects that received ROM restriction, **(B)** sham subjects that did not receive ROM restriction, **(C)** spinal subjects that received ROM restriction, and **(D)** spinal subjects that did not receive ROM restriction. The shaded gray region reflects the period of ROM restriction. Points show means; vertical lines are s.e.m.

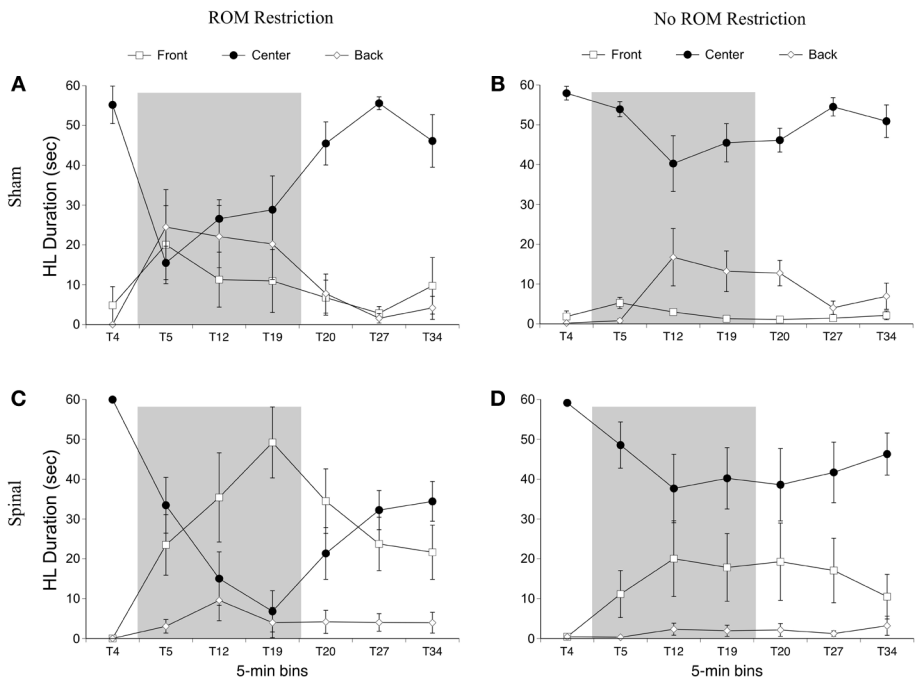

FIGURE 9 | Rostral-caudal hindlimb trajectories for sham and spinal P10 rats by ROM restriction condition. Graphs show duration of time spent in front, center, and back trajectory areas for 1-min sections during baseline, ROM restriction, and the post-ROM restriction period for **(A)** sham subjects that received ROM restriction, **(B)** sham subjects that did not receive ROM restriction, **(C)** spinal subjects that received ROM restriction, and **(D)** spinal subjects that did not receive ROM restriction. The shaded gray region reflects the period of ROM restriction. Points show means; vertical lines are s.e.m.

the following times: end of ROM restriction (T19), beginning of post-ROM restriction (T20), and end of post-ROM restriction (T34). There also was a significant decrease in center between the beginning and end of ROM restriction (T5 and T19). For sham and spinal subjects that did not receive ROM restriction, there were no significant differences within the different trajectory areas. These effects are summarized in **Figure 10**.

DISCUSSION

This study demonstrates the sensitivity of the isolated spinal cord to sensory (cutaneous and proprioceptive) feedback in conjunction with 5-HT modulation. The 5-HT_{2A} receptor agonist quipazine induced forelimb and hindlimb stepping in both sham and spinal transected P10 rats. However, only spinal subjects modulated their hindlimb stepping during ROM restriction. Both sham and spinal subjects showed real-time and persistent effects of ROM restriction on forelimb trajectories during stepping. However, only spinal subjects showed persistent effects of ROM restriction (on hindlimb activity). This corresponds with previous research showing that animals can adapt to and show persistent changes in behavior following a spinal cord transection (Viala et al., 1986; Grau, 2001; Wolpaw, 2006, 2007; Brumley and Robinson, 2010).

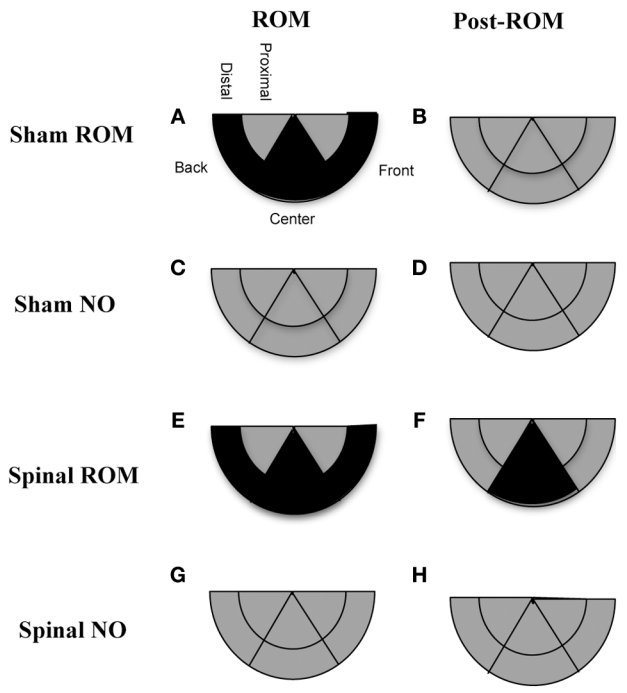

FIGURE 10 | Summary of changes in hindlimb trajectories for sham and spinal P10 rats by ROM restriction condition. Changes in limb trajectories are show for sham subjects that experienced ROM restriction **(A,B)**, shams that did not receive ROM restriction **(C,D)**, spinal subjects that received ROM restriction **(E,F)**, and spinal subjects that did not receive ROM restriction **(G,H)**. The shaded regions reflect a maintenance (gray) or decrease (black) from baseline within that trajectory area during the designated time period.

EFFECTS OF ROM RESTRICTION ON INTERLIMB COORDINATION (STEPPING)

ROM restriction influenced fore- and hindlimb step frequency during the period of restriction. While a decrease in synchronized forelimb stepping corroborates a previous study in 1-day old intact rats (Brumley et al., 2012), ROM-restricted spinal pups in the current study also exhibited a reduction in alternated hindlimb stepping during the restriction period. As shown in **Figure 4A**, non-ROM-restricted spinal pups showed a 3-fold increase in alternated hindlimb steps following quipazine treatment. However, ROM restriction suppressed this effect. Because ROM restriction suppressed hindlimb stepping starting 10-min after restriction, but not within the first 5-min, perhaps the initial increase in stepping gave spinal subjects more "trials" with the perturbation and hence more sensory feedback to respond to (compared to shams). Thus, additional steps may have helped facilitate responsiveness to ROM restriction. Another possibility is that quipazine had different neuromodulatory effects on sensory responsiveness in sham vs. spinal subjects. For example, Chopek et al. (2013) showed that quipazine preferentially increases the monosynaptic reflex of flexor nerves in spinal-transected compared to spinal-intact adult rats. Thus, sensory processing of the perturbation was likely different in the two surgery conditions.

Despite subjects showing real-time effects, no persistent effects of ROM restriction (following removal of the perturbation) were seen on step frequency. One possibility for lack of persistent changes may be a result of limited exposure to ROM restriction. Previous studies of interlimb motor learning after spinal transection, such as conjugate limb yoking studies, typically expose subjects to the sensory perturbation (interlimb yoke) for around 30 min (Brumley and Robinson, 2010). Thus, extending the exposure time in the current study may have allowed persistent effects to emerge. However, given that quipazine markedly increases motor activity in neonatal rats, we assumed that more motor activity might provide more sensory feedback and thus a shorter exposure time might be sufficient to induce persistent effects. Another possibility is that persistent effects are age-dependent. In a study with human infants, persistent adaptations to trip training on a treadmill were not reliably seen until about 9 months of age, despite infants showing real-time changes (Pang et al., 2003). Given research that equates P10 rats with 9–10 month old human infants in terms of locomotor development (Vinay et al., 2005), but only the late-term human fetus in terms of brain development (Clancy et al., 2001), our subjects may be too immature to show reliable lasting effects. However, studies examining interlimb yoke training have found persistent changes in motor coordination in rat fetuses and newborns (Robinson, 2005; Robinson et al., 2008; Brumley and Robinson, 2010), suggesting that persistent interlimb changes should be possible. Therefore, future studies should examine key features necessary to produce reliable, persistent coordinative changes following a spinal cord transection.

It is curious that ROM restriction leads to a decrease in step frequency, given the large body of research showing that sensory feedback often facilitates locomotor recovery after SCI (e.g., Carhart et al., 2004; Teulier et al., 2009). However, as observed in the current study, subjects that received ROM restriction changed

their intralimb trajectories (discussed below). This alteration in intralimb coordination may have compromised the ability of the pup to maintain interlimb coordination during stepping.

EFFECTS OF ROM RESTRICTION ON INTRALIMB COORDINATION (LIMB TRAJECTORIES)

During ROM restriction, the perturbation blocked part of the center and distal limb trajectory areas. Consequently, ROM-restricted pups produced fewer limb movements in these areas. To adapt, pups tended to move their forelimbs mostly to the front area during ROM restriction. For the hindlimbs, pups moved more-or-less equally to front and back areas, while avoiding the center during restriction. Further, during restriction, intralimb adaptations in spinal subjects appeared to be more drastic in the hindlimbs compared to forelimbs (i.e., much less time was spent in center and distal areas). As noted above, these intralimb adaptations may have interfered with interlimb coordination. One reason that the limbs may have adapted by mainly moving to the front of the subject rather than behind, may be related to the differential effect of serotonergic stimulation on flexor and extensor motor output. Quipazine has been shown to preferentially increase flexor motor output in spinal rats (Chopek et al., 2013), and likewise a lack of serotonin increases limb extension (Pflieger et al., 2002). In the current study, the limbs could be relatively flexed and remain in the front of the animal, but to move to the back the limbs would require significant extensor activity. Thus, perhaps the movement of the limbs mainly to the front trajectory area is indicative of a stronger effect on flexor activity rather than extensor activity, following treatment with a 5-HT receptor agonist.

Persistent effects of ROM restriction were seen immediately after the perturbation was removed in both fore- and hindlimbs. Persistent forelimb effects lasted for a longer period of time in spinal pups. One possibility for longer lasting persistent effects in spinal pups may be due to the isolation of the forelimbs from the hindlimbs. Blocking ascending input from the caudal spinal cord, including propriospinal neurons, may make sensory feedback from the forelimbs more salient since there is less input into the rostral cord (compared to shams). Blocking caudal input may be especially important given research suggesting that quipazine-induced hindlimb activity may help drive the forelimbs (McEwen et al., 1997; Brumley and Robinson, 2005). Another possibility is changes in somatosensory cortex processing. Hindlimb areas within the cortex failed to respond to hindlimb stimulation but instead responded to forelimb stimulation, in adult rats transected as neonates (Kao et al., 2011). Thus, feedback from the forelimbs may be activating more brain regions in spinal animals and facilitate lasting intralimb adaptations. Alternatively, given that spinal pups in the current study had been living with a spinal cord transection for 9 post-operative days, they may have adopted novel posture and movement strategies during this period. The duration of such intralimb adaptations, or whether or not such changes might persist from one test session to the next, remains to be determined.

Besides changes in intralimb coordination for ROM-restricted subjects, quipazine also altered intralimb coordination for pups in both surgery conditions. Specifically, pups showed a decrease in the center and distal areas following treatment with quipazine. This is likely the result of quipazine increasing the amount of stepping behavior, as stepping involves both limb flexion and extension, with the limbs typically showing locomotor-like swing and stance limb excursions. Thus, it is not surprising that pups treated with quipazine (but not ROM restriction) utilized their movement space differently from baseline.

QUIPAZINE-INDUCED STEPPING IN SPINAL SUBJECTS

As mentioned above, P10 rats that received a low thoracic spinal cord transection on P1 showed three times as many hindlimb steps following treatment with quipazine, compared to shams. Researchers looking at what has been termed "hindlimb supersensitivity" have found changes in the spinal cord following a transection that may help account for this apparent sensitivity to stimulation at 5-HT receptors. For example, in chronic transected rats the concentration of 5-HT_2 receptors has been shown to increase 3 to 5-fold throughout motor neuronal somata and dendrite regions, within the caudal cord (Kong et al., 2010). A study that examined the time course of changes in 5-HT receptors after a complete spinal transection reported an increase in 5-HT_2 receptors beginning 24 h after surgery (Kong et al., 2011). Another study examined changes in motor neuron excitability and found that small doses (10–50 μM) of a 5-HT_2 agonist produced cell depolarization, increased input resistance, and large persistent inward currents in adult spinal rats (Harvey et al., 2006). Specifically, 5-HT_{2A} receptor stimulation has been shown to restore hyperpolarizing inhibition in spinal motor neurons via upregulating activity and expression of the K-Cl cotransporter KCC2 in the isolated neonatal rat spinal cord *in vitro* (Bos et al., 2013). This permits endogenous reciprocal inhibition necessary for maintaining left-right alternation seen during locomotion, which can be activated in the isolated spinal cord *in vitro* and *in vivo* with 5-HT_{2A}receptors (Norreel et al., 2003; Pearlstein et al., 2005). Thus, it is possible that spinal pups in the current study experienced an up-regulation of 5-HT_2 receptors, motor neuronal hyperexcitability responses to quipazine, and enhanced reciprocal inhibition, and therefore the effects of quipazine (a 5-HT_{2A}receptor agonist) were much more pronounced in these pups compared to shams. Further support for the role of serotonergic stimulation in regulating lumbar networks during early development comes from studies that have demonstrated impaired locomotor coordination, posture, and motor neuron excitability following serotonin depletion (Myoga et al., 1995; Nakajima et al., 1998; Pflieger et al., 2002).

An interesting finding in the current study was the occurrence of alternated forelimb stepping in spinal pups treated with quipazine (although the amount of forelimb stepping was much lower than hindlimb stepping). Previous studies looking at quipazine-induced stepping in perinatal rats have reported robust hindlimb stepping, but very little forelimb stepping after a mid-thoracic spinal transection (McEwen et al., 1997; Brumley and Robinson, 2005). However, the current study differs from these studies in a couple of important ways, which may help to explain this difference in forelimb behavior. First, the previous studies performed behavioral testing within 24 h after spinal transection, whereas in the current study pups were allotted 9 days

after surgery to recover before testing. Therefore, longer recovery time may have allowed pups to fully recover from any spinal shock rostral to the injury site. Second, the current study used a low thoracic spinal cord transection, whereas the previous studies used mid-thoracic transections. Thus, it is possible that the neural circuitry for forelimb stepping extends into the high thoracic area, and therefore a mid-thoracic transection interferes with or damages forelimb stepping circuitry. However, with a low thoracic transection, perhaps we missed that circuitry altogether, and therefore animals could easily show stepping behavior in the forelimbs.

CONCLUSIONS

Findings from this study suggest that the immature, isolated spinal cord modulates inter- and intralimb coordination in response to sensory feedback during locomotor activity induced by serotonergic stimulation. Furthermore, we found that the spinal cord is able to support persistent behavioral changes after exposure to a sensorimotor perturbation. A number of studies have shown the importance of pairing sensory input with spinal cord circuitry activation. In these studies, the addition of 5-HT receptor stimulation is often shown to modulate changes in recovery. For example, subjects given motor training typically do not recover to the same extent as subjects given motor training plus treatment with a 5-HT agonist. Interestingly, a study that examined mice and rats with a chronic spinal cord contusion found that increases in behavioral recovery were correlated with an increase in 5-HT receptor expression in the spinal cord (Wang et al., 2011). Thus, 5-HT stimulation is not just a tool to activate locomotor circuits but is likely part of a dynamic system involved in the production, modulation, and recovery of functional movement. By understanding how 5-HT modulates locomotor behavior and how sensory feedback and supraspinal input changes 5-HT expression, we can gain a more accurate picture of how to tailor therapies toward better recovery. Such therapeutic strategies can already be seen in studies with humans where sensory feedback (i.e., treadmill training) is paired with activation of local spinal circuits (e.g., epidural stimulation) (Carhart et al., 2004).

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REFERENCES

Barbeau, H., and Rossignol, S. (1990). The effects of serotonergic drugs on the locomotor pattern and on cutaneous reflexes of the adult chronic spinal cat. *Brain Res.* 514, 55–67. doi: 10.1016/0006-8993(90)90435-E

Bos, R., Sadlaoud, K., Boulenguez, P., Buttigieg, D., Liabeuf, S., Brocard, C., et al. (2013). Activation of 5-HT2A receptors upregulates the function of the neuronal K-Cl cotransporter KCC2. *Proc. Natl. Acad. Sci. U.S.A.* 110, 348–353. doi: 10.1073/pnas.1213680110

Brumley, M. R., Roberto, M. E., and Strain, M. M. (2012). Sensory feedback modulates quipazine-induced stepping behavior in the newborn rat. *Behav. Brain Res.* 229, 257–264. doi: 10.1016/j.bbr.2012.01.006

Brumley, M. R., and Robinson, S. R. (2005). The serotonergic agonists quipazine, CGS-12066A, and α-methylserotonin alter motor activity and induce hindlimb stepping in the intact and spinal rat fetus. *Behav. Neurosci.* 119, 821–833. doi: 10.1037/0735-7044.119.3.821

Brumley, M. R., and Robinson, S. R. (2010). "Experience in the perinatal development of action systems," in *Oxford Handbook of Developmental Behavioral Neuroscience*, eds M. S. Blumberg, J. H. Freeman,and S. R. Robinson (New York, NY: Oxford University Press), 181–209.

Carhart, M. R., He, J., Herman, R., D'Luzansky, S., and Willis, W. T. (2004). Epidural spinal-cord stimulation facilitates recovery of function walking following incomplete spinal-cord injury. *IEEE Trans. Neural Syst. Rehabil. Eng.* 12, 32–42. doi: 10.1109/TNSRE.2003.822763

Cazalets, J. R., Squalli-Houssaini, Y., and Clarac, F. (1992). Activation of the central pattern generators for locomotion by serotonin and excitatory amino acids in neonatal rat. *J. Physiol.* 455, 187–204.

Chopek, J. W., MacDonell, C. W., Power, K. E., Gardiner, K., and Gardiner, P. (2013). Removal of supraspinal input reveals a difference in the flexor and extensor monosynaptic reflex response to quipazine independent of motoneuron excitation. *J. Neurophysiol.* 109, 2056–2063. doi: 10.1152/jn.00405.2012

Clancy, B., Darlington, R. B., and Finay, B. L. (2001). Translating developmental time across mammalian species. *Neuroscience* 105, 7–17. doi: 10.1016/S0306-4522(01)00171-3

Cowley, K. C., and Schmidt, B. J. (1994). A comparison of motor patterns induced by N-methyl-D-aspartate, acetylcholine and serotonin in the *in vitro* neonatal rat spinal cord. *Neurosci. Lett.* 171, 147–150. doi: 10.1016/0304-3940(94)26-2

Field-Fote, E. C. (2001). Combined use of body weight support, functional electric stimulation, and treadmill training to improve walking ability in individuals with chronic incomplete spinal cord injury. *Arch. Phys. Med. Rehabil.* 82, 818–824. doi: 10.1053/apmr.2001.23752

Fong, A. J., Cai, L. L., Otoshi, C. K., Reinkensmeyer, D. J., Burdick, J. W., Roy, R. R., et al. (2005). Spinal cord-transected mice learn to step in response to quipazine treatment and robotic training. *J. Neurosci.* 25, 11738–11747. doi: 10.1523/JNEUROSCI.1523-05.2005

Garraway, S. M., and Hochman, S. (2001). Pharmacological characterization of serotonin receptor subtypes modulating primary afferent input to seep dorsal horn neurons in the neonatal rat. *Br. J. Pharmacol.* 132, 1789–1798. doi: 10.1038/sj.bjp.0703983

Grau, J. W. (2001). "Learning and memory without a brain," in *The Cognitive Animal*, eds M. Bekoff, C. Allen, and G. Burghardt (Cambridge, MA: MIT Press), 77–87.

Harvey, P. J., Lui, X., Li, Y., and Bennett, D. J. (2006). 5-HT2 receptor activation facilitates a persistent sodium current and repetitive firing in spinal motoneurons of rats with and without chronic spinal cord injury. *J. Neurophysiol.* 96, 1158–1170. doi: 10.1152/jn.01088.2005

Hayes, H. B., Chang, Y.-H., and Hochman, S. (2008). An *in vitro* spinal cord-hindlimb preparation for studying behaviorally relevant rat locomotor function. *J. Neurophysiol.* 101, 1114–1122. doi: 10.1152/jn.90523.2008

Hayes, H. B., Chang, Y.-H., and Hochman, S. (2012). Stance-phase force on the opposite limb dictates swing-phase afferent presynaptic inhibition during locomotion. *J. Neurophysiol.* 107, 3168–3180. doi: 10.1152/jn.01134.2011

Institutes of Laboratory Animal Resources. (2011). *Guide for the Care and Use of Laboratory Animals*. Washington, DC: National Academy Press.

Jankowska, E., Maxwell, D. J., Dolk, S., and Dahlstrom, A. (1999). A confocal and electron microscopic study of contacts between 5-HT fibers and feline dorsal horn interneurons in pathways from muscle afferents. *J. Comp.Neurol.* 387, 430–438.

Jankowska, E., Maxwell, D. J., Dolk, S., Krutki, P., Belichenko, P. V., and Dahlstrom, A. (1995). Contact between serotoninergic fibres and dorsal horn spinocerebellar tract neurons in the cat and rat: a confocal microscopic study. *Neuroscience* 67, 477–487. doi: 10.1016/0306-4522(95)00059-R

Kao, T., Shumsky, J. S., Jacob-Vadakot, S., Himes, B. T., Murray, M., and Moxon, K. A. (2006). Role of the 5-HT2C receptor in improving weight-supported stepping in adult rats spinalized as neonates. *J. Brain Res.* 1112, 159–168. doi: 10.1016/j.brainres.2006.07.020

Kao, T., Shumsky, J. S., Knudsen, E. B., Murray, M., and Moxon, K. A. (2011). Functional role of exercise-induced cortical organization of sensorimotor cortex after spinal transection. *J. Neurophysiol.* 106, 2662–2674. doi: 10.1152/jn.01017.2010

Kong, X. Y., Wienecke, J., Chen, M., Hultborn, H., and Zhang, M. (2011). The time course of serotonin 2A receptor expression after spinal transection of rats: an immunohistochemical study. *Neuroscience* 177, 114–126. doi: 10.1016/j.neuroscience.2010.12.062

Kong, X. Y., Wienecke, J., Hultborn, H., and Zhang, M. (2010). Robust upregulation of serotonin 2A receptors after chronic spinal transection of rats: an immunohistochemical study. *Brain Res.* 1320, 60–68. doi: 10.1016/j.brainres.2010.01.030

Lapointe, N. P., and Guertin, P. A. (2008). Synergistic effects of D1/5 and 5-HT1A/7 receptor agonists on locomotor movement induction in complete spinal cord-transected mice. *J. Neurophysiol.* 100, 160–160. doi: 10.1152/jn.90339.2008

Liu, J., and Jordan, L. M. (2005). Stimulation of the parapyramidal region of the neonatal rat brain stem produces locomotor-like activity involving spinal 5-HT7 and 5-HT2A receptors. *J. Neurophysiol.* 94, 1391–1404. doi: 10.1152/jn.00136.2005

McEwen, M. L., Van Hartesveldt, C., and Stehouwer, D. J. (1997). L-DOPA and quipazine elicit air-stepping in neonatal rats with spinal cord transections. *Behav. Neurosci.* 111, 825–833. doi: 10.1037/0735-7044.111.4.825

Myoga, H., Nonaka, S., Matsuyama, K., and Mori, S. (1995). Postnatal development of locomotor movements in normal and para-chlorophenylalanine-treated newborn rats. *Neurosci. Res.* 21, 211–221. doi: 10.1016/0168-0102(94)00857-C

Nakajima, K., Matsuyama, K., and Mori, S. (1998). Prenatal administration of para-chlorophenylalanine results in suppression of serotonergic system and disturbance of swimming movements in newborn rats. *Neurosci. Res.* 31, 155–169. doi: 10.1016/S0168-0102(98)00034-0

Noga, B. R., Johnson, D. M. G., Riesgo, M. I., and Pinzon, A. (2009). Locomotor-activated neurons of the cat. I. Serotonergic innervation and co-localization of 5-HT7, 5-HT 2A, and 5-HT1A receptors in the thoraco-lumbar spinal cord. *J. Neurophysiol.* 102, 1560–1576. doi: 10.1152/jn.91179.2008

Norreel, J.-C., Pflieger, J.-F., Pearlstein, E., Simeoni-Alias, J., Clarac, F., and Vinay, L. (2003). Reversible disorganization of the locomotor pattern after neonatal spinal cord transection in the rat. *J. Neurosci.* 23, 1924–1932.

Pang, M. Y. C., Lam, T., and Yang, J. F. (2003). Infants adapt their stepping to repeated trip-inducing stimuli. *J. Neurophysiol.* 90, 2731–2740. doi: 10.1152/jn.00407.2003

Pearlstein, E., Ben Mabrouk, F., Pflieger, J.-F., and Vinay, L. (2005). Serotonin refines the locomotor-related alternations in the *in vitro* neonatal rat spinal cord. *Eur.J. Neurosci.* 21, 1338–1346. doi: 10.1111/j.1460-9568.2005.03971.x

Pepin, A., Ladoucer, M., and Barbeau, H. (2003). Treadmill walking in incomplete spinal-cord-injured subjects: 2. Factors limiting the maximal speed. *Spinal Cord* 41, 271–279. doi: 10.1038/sj.sc.3101452

Pflieger, J.-F., Clarac, F., and Vinay, L. (2002). Postural modifications and neuronal excitability changes induced by a short-term serotonin depletion during neonatal development in the rat. *J. Neurosci.* 22, 5108–5117.

Robinson, S. R. (2005). Conjugate limb coordination after experience with an interlimb yoke: evidence for motor learning in the rat fetus. *Dev. Psychobiol.* 47, 328–344. doi: 10.1002/dev.20103

Robinson, S. R., Kleven, G. A., and Brumley, M. R. (2008). Prenatal development of interlimb motor learning in the rat fetus. *Infancy* 13, 204–228. doi: 10.1080/15250000802004288

Slawinska, U., Majczynski, H., Dai, Y., and Jordan, L. M. (2012). The upright posture improves plantar stepping and alters responses to serotonergic drugs in spinal rats. *J. Physiol.* 590, 1721–1736. doi: 10.1113/jphysiol.2011.224931

Stelzner, D. J., Weber, E. D., and BryzGornia, W. F. (1986). "Sparing of function in developing spinal cord: anatomical substrate," in *Development and Plasticity of the Mammalian Spinal Cord*, Vol. 3, eds M. E. Goldstein, A. Gorio, and M. Murray (Padova: Liviana Press), 81–99.

Teulier, C., Smith, B. A., Kubo, M., Chang, C. L., Moerchen, V., Murazko, K., et al. (2009). Stepping responses of infants with myelomeningocele when supported on a motorized treadmill. *Phys. Ther.* 89, 60–72. doi: 10.2522/ptj.20080120

Ung, R. V., Landry, E. S., Rouleau, P., Lapointe, N. P., Rouillard, C., and Guertin, P.A. (2008). Role of spinal 5-HT2 receptor subtypes in quipazine-induced hindlimb movements after a low-thoracic spinal cord transection. *Eur. J. Neurosci.* 28, 2231–2242. doi: 10.1111/j.1460-9568.2008.06508

Viala, D., Viala, G., and Fayein, N. (1986). "Plasticity of locomotor organization in infant rabbits spinalized shortly after birth," in *Development and Plasticity of the Mammalian Spinal Cord*, Vol. 3, eds M. E. Goldstein, A. Gorio, and M. Murray (Padova: Liviana Press), 301–310

Vinay, L., Ben-Mabrouk, F., Brocard, F., Clarac, F., Jean-Xavier, C., Pearlstein, E., et al. (2005). Perinatal development of the motor systems involved in postural control. *Neural Plast.* 12, 131–139. doi: 10.1155/NP.2005.131

Wang, X., Duffy, P., McGee, A. W., Hasan, O., Gould, G., Tu, N., et al. (2011). Recovery from chronic spinal cord contusion after Nogo receptor intervention. *Ann. Neurol.* 70, 805–821. doi: 10.1002/ana.22527

Weber, E. D., and Stelzner, D. J. (1980). Synaptogenesis in the intermediate gray region of the lumbar spinal cord in the postnatal rat. *Brain Res.* 185, 17–37. doi: 10.1016/0006-8993(80)90667-8

Wolpaw, J. R. (2006). "The education and re-education of the spinal cord," in *Progression in Brain Research*, Vol. 157, ed A. R. Miller (New York, NY: Elsevier), 261–280.

Wolpaw, J. R. (2007). Spinal cord plasticity in acquisition and maintenance of the motor skills. *Acta Physiol.* 189, 155–169. doi: 10.1111/j.1748-1716.2006.01656.x

Zhong, H., Roy, R. R., Nakada, K. K., Zdunowski, S., Khalili, N., de Leon, R. D., et al. (2012). Accommodation of the spinal cat to a tripping perturbation. *Front. Physiol.* 3:112. doi: 10.3389/fphys.2012.00112

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Gain control mechanisms in spinal motoneurons

Michael D. Johnson[1]* and Charles J. Heckman[1,2]

[1] *Department of Physiology, Feinberg School of Medicine, Northwestern University, Chicago, IL, USA*
[2] *Department of Physical Medicine and Rehabilitation and Department of Physical Therapy and Human Movement Sciences, Feinberg School of Medicine, Northwestern University, Chicago, IL, USA*

Edited by:
Shawn Hochman, Emory University, USA

Reviewed by:
Tim C. Cope, Wright State University, USA
David Bennett, University of Alberta, Canada

****Correspondence:***
Michael D. Johnson, Department of Physiology, Feinberg School of Medicine, Northwestern University, 303 E Chicago Ave., W5-295, Chicago, IL 60611, USA
e-mail: m-johnson16@northwestern.edu

Motoneurons provide the only conduit for motor commands to reach muscles. For many years, motoneurons were in fact considered to be little more than passive "wires". Systematic studies in the past 25 years however have clearly demonstrated that the intrinsic electrical properties of motoneurons are under strong neuromodulatory control via multiple sources. The discovery of potent neuromodulation from the brainstem and its ability to change the gain of motoneurons shows that the "passive" view of the motor output stage is no longer tenable. A mechanism for gain control at the motor output stage makes good functional sense considering our capability of generating an enormous range of forces, from very delicate (e.g., putting in a contact lens) to highly forceful (emergency reactions). Just as sensory systems need gain control to deal with a wide dynamic range of inputs, so to might motor output need gain control to deal with the wide dynamic range of the normal movement repertoire. Two problems emerge from the potential use of the brainstem monoaminergic projection to motoneurons for gain control. First, the projection is highly diffuse anatomically, so that independent control of the gains of different motor pools is not feasible. In fact, the system is so diffuse that gain for all the motor pools in a limb likely increases in concert. Second, if there is a system that increases gain, probably a system to reduce gain is also needed. In this review, we summarize recent studies that show local inhibitory circuits within the spinal cord, especially reciprocal and recurrent inhibition, have the potential to solve both of these problems as well as constitute another source of gain modulation.

Keywords: gain, serotonin, motoneuron, spinal cord, spinal cord injury

NEUROMODULATION, A RHEOSTAT FOR MN EXCITABILITY

It is approaching 40 years since the discovery of the powerful effects of persistent inward currents (PICs) and their ability to transform spinal motoneurons from passive conduits to active processors of incoming signals. PICs are depolarizing currents, mediated by sodium and calcium channels, primarily in the dendrites (Eckert and Lux, 1976; Schwindt and Crill, 1977; Hounsgaard and Kiehn, 1993). PICs amplify (Lee and Heckman, 2000; Hultborn et al., 2003) and prolong (Heckman et al., 2008) the effects of ionotropic synaptic inputs by producing plateau potentials and self-sustained firing and regulate the overall excitability of the cell.

PICs depend on the presence of the monoamines serotonin and norepinephrine which are produced in brainstem cells of the raphe nucleus and locus coeruleus (Hultborn et al., 2004). These cell groups send axons down to diffusely innervate all laminae and segments of the spinal cord (Björklund and Skagerberg, 1982). These so called neuromodulators act intracellularly via G-protein coupled second messengers to confer persistent behavior to dendritic calcium and sodium channels (Simon et al., 2003; Ballou et al., 2006) creating an inward depolarizing current (Carlin et al., 2000). In addition to their effects on PICs, both serotonin and norepinephrine have potent effects on the threshold of the motoneuron (Power et al., 2010). There likely also exist many other neuromodulators that influence motoneurons. Local spinal circuits can reduce the motoneuron spike afterhyperpolarization (AHP; Miles et al., 2007) whereas 5HT and NE have very little AHP effect in the adult (Li et al., 2007). Much further work is needed on neuromodulation of motoneurons; for the present, this review focuses on the effects of serotonin and norepinephrine on the PIC, which has remarkably potent effects on input-output gain of motoneurons, as explained next.

Input amplification by PICs is readily seen in electrophysiological recordings and is manifest as an increase in depolarizing current or membrane potential elicited by an excitatory synaptic input that, in the absence of PICs, would be much reduced. PIC amplification can be as great as 5-fold (Lee and Heckman, 2000). This is an essential feature for motor outputs, being one of the key mechanisms that allows spinal motoneurons to achieve firing frequencies sufficient to produce maximum voluntary muscle contractions (Binder et al., 2002). But this powerful control of intrinsic excitability of motoneurons is potentially gradable. Brainstem output patterns correlate with arousal state via the noradrenergic system and with the intensity of motor output via the serotonergic system (Rasmussen et al., 1986; Rajkowski et al., 1994, 1998). Thus by varying output from these brainstem

neuromodulatory centers, motor commands can very PIC amplitude and thus vary the input-output gain of motoneurons.

It should be emphasized however this gain control has not yet been clearly demonstrated in either intact animals or in human subjects and, in fact, further experiments are needed in animal preparations to understand how much a given change in PIC amplitude increases the overall gain of the motor pool as a whole system. Nonetheless controlling gain at the motor pool makes good functional sense for the motor system as a whole. Motor output has to vary over a huge range from delicate (e.g., putting in a contact lens) to high force (moving heavy weights, high speed escapes). Varying the gain at the motor output stage allows input neurons to employ their full range of rate modulation across a wide range of motor tasks. Consistent with this possibility, chronic recordings of motor cortex neurons show a range of rate modulation that is similar at high and low forces outputs (Maier et al., 1993; Andrykiewicz et al., 2007).

PIC induced input prolongation is clearly seen in electrophysiological recordings as tail currents, plateau potentials and self sustained firing (Schwindt and Crill, 1980, 1981; Hounsgaard et al., 1988; Simon et al., 2003; Moritz et al., 2007), all present immediately after the termination of an excitatory input. Input prolongation may serve useful in the maintenance of posture, allowing brief descending commands to postural muscles in the limbs and trunk to produce persistent motor outputs ("bistable" behavior). Therefore their effect is analogous to changing the behavior of the cell to a positive integrator, for the time that the PIC is active it can sum its brief inputs to create a long lasting output. Moreover, this bistable behavior is strongest in low threshold type S motoneurons, which are heavily involved in posture. It seems reasonable to suppose that bistable behavior is routinely used for postural control, but, as for the gain control discussed above, definite data on this speculation are not yet available.

THE PHYSICAL PLANT, A NEURAL BIOMECHANICAL LINK

INHIBITION PROVIDES SPECIFIC CONTROL OF PICs

The descending monoaminergic input to spinal MNs is diffuse and non-specific and operates through both synaptic and extra synaptic transmission (Agnati et al., 2010). Therefore judging by anatomy alone PIC effects would presumably also be broad and non-specific. Furthermore even though PIC amplitude can be globally controlled by the brainstem, the dynamics of this control are slow (Raymond et al., 2001; Hentall et al., 2006) and, since these descending tracts have no apparent somatotopy, they are not motor pool specific. Under such slow control, PIC effects such as input prolongation, which is primarily seen in low input conductance MNs and may benefit postural behaviors, could seriously interfere with the MNs ability to rapidly respond to dynamic motor commands. It has been shown that inhibition from electrical stimulation of antagonist nerves increases the threshold for plateau potentials and presumable the PICs that underlie them (Bennett et al., 1998). We have recently revealed that the Ia sensory system activated during muscle stretch associated with changes in joint angle provides a key control mechanism that confers rapid and specific modulation of PIC amplitude and effects. PICs are excellent amplifiers of excitatory synaptic inputs but can be rapidly "turned off" with synaptic inhibition (Hultborn et al., 2003; Kuo et al., 2003b; Hyngstrom et al., 2007). The Ia reciprocal inhibition system of agonist/antagonist muscle pairs is ideally constructed to provide a motor-pool-specific inhibitory control mechanism. Passively changing the joint angle in one direction (extension) stretches antagonist muscles and provides inhibition to agonist MNs (**Figure 1**). Changing joint angle in the opposite direction (flexion) decreases inhibition to the MN. In the case of ankle extensor MNs, antagonist muscle stretch (via ankle extension) decreases PIC amplitude by about 29% while ankle flexion, which results in a net reduction in reciprocal inhibition to extensor MNs, increases PIC amplitude the same amount (**Figure 2**) (more on this below). The synaptic inhibitory component of these joint rotations reduces PIC amplitude and grades their effects on MN outputs. Inhibition also modulates the electrical properties of MNs facilitating transitions between high and low excitability states. In this way the neuro-muscular physical plant provides a biomechanical control system that allows MNs to take advantage of PIC effects, which are beneficial to MN activation and output, while minimizing the potentially detrimental aspects these effects would have on motor task that involve rapidly alternating activation of muscles with opposing action.

In considering the functional effect of excitation and inhibition on the PIC, it is important to realize that the electrode is at the soma and that much of the dendritic tree is not clamped. As a result, both inhibitory and excitatory synaptic currents are more effective in changing activation of the PIC than current injected at the soma (Bennett et al., 1998; Lee et al., 2003). Previous studies (Kuo et al., 2003a; Bui et al., 2008a) and computer simulations (Bui et al., 2008b; Powers et al., 2012) suggest that the reduction in PIC amplitude by inhibition is due to both its hyperpolarizing and shunting effects. These changes likely account for the differences in PIC activation shown in **Figure 2A**. In other words, changes in PIC activation measured by voltage clamp at the soma are distorted by lack of space clamp of the dendritic tree. Yet, from a functional perspective, clamp at the soma is entirely appropriate and the "distortions" directly affect motor output. When a motoneuron is functioning normally, the AHP after each spike maintains the average membrane potential at a reasonably steady level—that is to say, the AHP approximately "clamps" the soma to this level (about −50 mV). Thus the effect of excitatory and inhibitory synaptic inputs on the PIC provide a reasonable estimate of how naturally evoked firing will be generated, with the important caveat that the net current to be considered should be in the voltage range for average membrane potential during repetitive firing (i.e., ∼−50 mV). This functional relevance of voltage clamp current at firing level is not just an assumption. We have shown that the clamp current at firing level induced by muscle stretch (which strongly activates the PIC) provides a good prediction of the firing rate and pattern induced by an identical stretch in the unclamped state in the same cell (Lee et al., 2003). Thus it is appropriate to assess the functional effect of excitation and inhibition on PICs using voltage clamp at the soma. Nonetheless, the interaction between inputs and PICs is complicated and further work is warranted, Cutaneous, joint and muscle afferents are all activated to varying degrees during joint

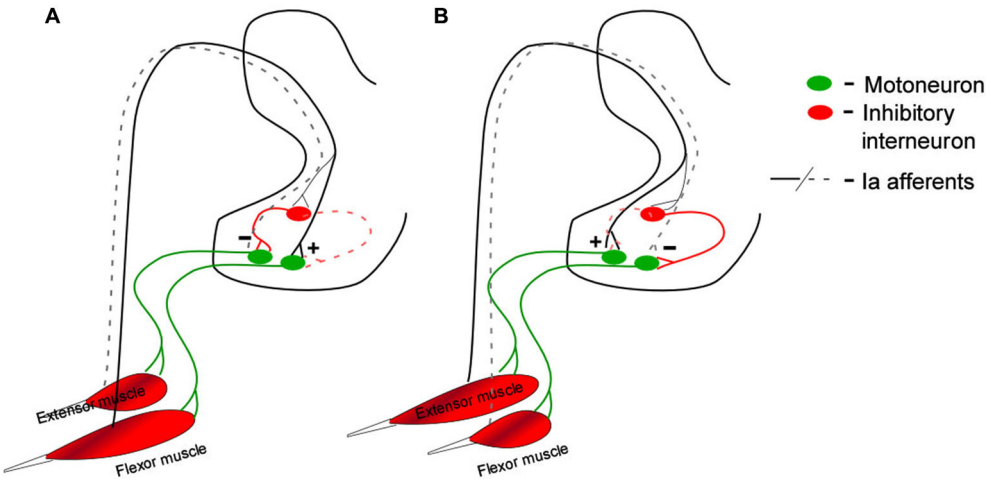

FIGURE 1 | Ia reciprocal connections to spinal motoneurons by antagonist muscle pairs. Rotating the joint to stretch flexor muscles provides disynaptic inhibition to extensor motoneurons and direct activation of the flexor motoneuron (**A**, solid lines). The opposite rotation stretches extensor muscles providing disynaptic inhibition to flexor motoneurons and direct activation of the extensor motoneuron (**B**, solid lines). The plus and minus signs in this figure are meant to indicate net depolarization (+) and net hyperpolarization (−). During these rotations the opposite conductance change occurs at each motoneuron type: extensor muscle shortening disfacilitates extensor motoneurons (**A**, black dashed lines) contributing to their hyperpolarization, and disinhibits flexor motoneurons (**A**, red dashed lines), contributing to their depolarization. Flexor muscle shortening disfacilitates flexor motoneurons (**B**, black dashed lines, hyperplolarization) and disinhibits extensor motoneurons (**B**, red dashed lines, depolarization).

rotations and could potentially contribute to PIC modulation, but reciprocal inhibition by primary spindle Ia afferents dominates. This system is very sensitive to muscle length change, and the modulation of PIC amplitude by joint angle is exactly what would be predicted by Ia reciprocal inhibitory effects. We have demonstrated that PIC reduction does not occur when the tendons to antagonist muscles are cut prior to imposing joint angle changes. In fact PICs tended to be larger in the extended, flexed and midpoint joint positions in these experiments (Hyngstrom et al., 2007). The lack of PIC reduction as well as the increase in PIC amplitude in the absence of Ia reciprocal inhibition illustrates the importance of inhibitory proprioceptive inputs for modulating this intrinsic property. Finally when denervation was performed to eliminate cutaneous afferents in these experiments, results were similar to the non-deafferented condition i.e., PIC amplitudes were clearly modulated by joint angle in the absence of cutaneous inputs.

These experiments demonstrate the importance of focused reciprocal inhibition and the considerable degree of flexibility imparted by reciprocal inhibition, exerting temporally specific control over the diffuse descending neuromodulatory system. Descending brainstem inputs modulate PIC amplitude globally across all motor pools, increasing and decreasing general excitability throughout the motor system. The tightly focused inhibition from the Ia system allows specific MN behaviors to be sculpted from a slowly changing background monoaminergic state.

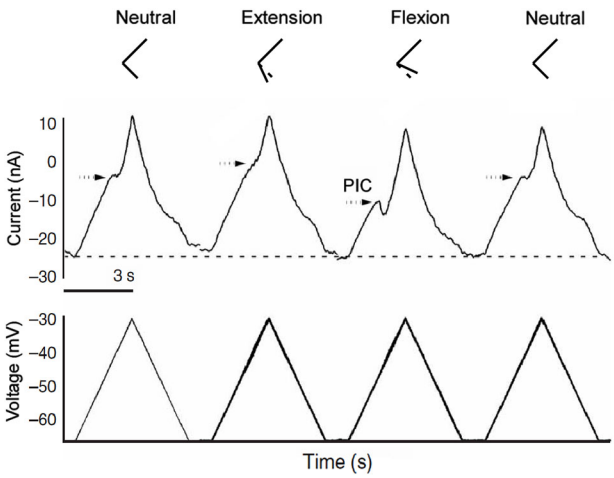

FIGURE 2 | Joint angle effects on PIC amplitude. Joint angle positions are represented by the stick figures on top. In the midpoint position the PIC is clearly present as a downward deflection (indicating an inward depolarizing current) in the current trace. During extension, inhibition from antagonist muscle stretch greatly reduces PIC amplitude in the recorded extensor motoneuron. During flexion, antagonist muscles are shorter than in the midpoint position, providing disinhibition to extensor motoneurons revealing a large PIC.

PUSH-PULL: INTERACTIONS BETWEEN INHIBITION AND EXCITATION

Passive joint rotation provides alternating stretch of agonist and antagonist muscles. In each rotational direction Ia afferents provide direct monosynaptic excitation to homonymous MNs and indirect inhibition to antagonist MNs (**Figure 1**). We have shown that reciprocal inhibition changes PIC amplitude by at least 50% (Hyngstrom et al., 2007). But there is a complementary component to both inhibition and excitation: disinhibition and disfacillitation. Synaptic inputs can interact in a number of ways. In one scheme excitation and inhibition occurs concomitantly,

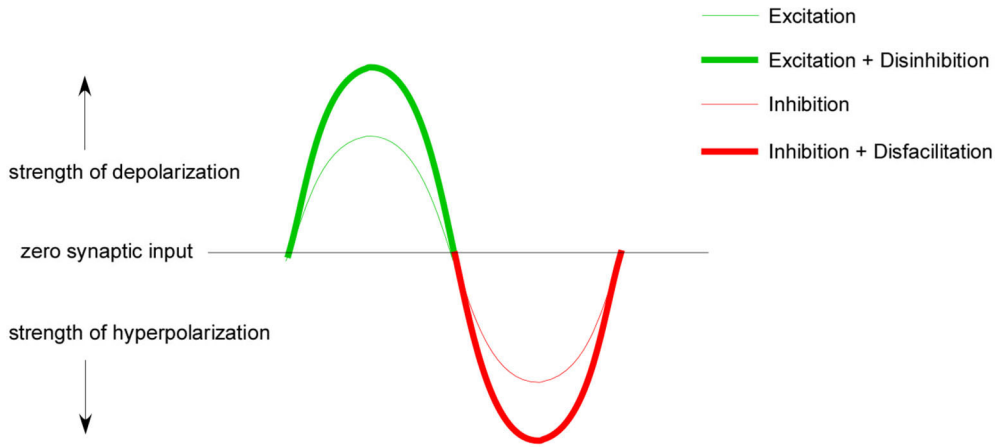

FIGURE 3 | Push-pull conceptual model. A tonic background of excitation and inhibition is required for push-pull control. When this background is present excitation can temporally couple with disinhibition to increase excitatory gain (thick green line) and inhibition can couple with disfacillitation to increase inhibitory gain (thick red line). This conductance coupling can theoretically lead to a greater range of excitability modulation than excitation and inhibition alone (thin green/red line).

with one being slightly larger than the other, in what are known as "balanced networks" (Berg et al., 2007). Though metabolically expensive, balanced networks are thought to be common in the CNS. They are thought to be involved with the control of breathing (Parkis et al., 1999; de Almeida and Kirkwood, 2010) acoustic signal processing (Magnusson et al., 2008) and most notably sensory processing in the neocortex (Borg-Graham et al., 1998; Shu et al., 2003; Haider et al., 2006). Once in a balanced state, excitation and inhibition can change out of phase, creating a larger driving force for de- and hyperpolarizations, in a so called push-pull arrangement (Ferster, 1988; Grande et al., 2010; Johnson et al., 2012). Push-pull requires the presence of a tonic background of excitatory and inhibitory conductances and occurs when excitation is temporally coupled with disinhibition or when inhibition is coupled with disfacillitation (**Figure 3**).

Push-pull conductance changes are reinforcing (Ferster, 1988; Conway and Livingstone, 2006) and should produce larger effective synaptic currents and larger depolarizations, greater firing frequencies and larger muscle forces than excitation alone. When opposite sign conductances modulate in phase, their effect on the neuron should cancel each other out. Combinations of excitation and inhibition and their impact on muscle force production are illustrated in **Figure 4**. These predictions assume purely linear interactions, which is not likely to be the case. Nonetheless our work in cat spinal MNs supports this same general pattern: coupling disinhibition with excitation produces larger excitatory currents in MNs measured in voltage clamp, larger depolarizations and higher firing frequencies in current clamp as well as greater muscle forces in unparalyzed animal experiments (**Figure 5**). In these same experiments we also ran trials with the inhibitory component removed by cutting the antagonist muscle tendons, effectively removing the input that provides both inhibition and disinhibition. In this altered state where excitatory inputs were modulated exclusively, MN currents, firing frequencies and muscle forces were all dramatically reduced, suggesting that Ia reciprocal inputs are superimposed on a tonic base of excitation and inhibition (Johnson et al., 2012; **Figure 5**).

The reciprocal organization of Ia afferents from agonist/antagonist muscle pairs is ideally suited to operate in a push-pull fashion. Push-pull is another effective strategy to increase MN input-output gain ultimately translating to increased muscle force production. Under this arrangement PIC effects are nicely regulated as well. Inhibition and excitation are smoothly modulated throughout the range of joint rotation and, from the perspective of a single MN, reverse in sign in concert with reciprocal inhibition. This allows greater depolarization in the excitatory phase, where excitation is biomechanically coupled with disinhibition, as well as strong hyperpolarizations, and therefore control of MN PICs, in the inhibitory phase where inhibition is coupled with disfacillitation. The disinhibition provided by push-pull will enhance the force output of the agonist, but if co-contraction is needed, reciprocal inhibition presumably has to be reduced and thus this mechanism will no longer be operative. Our studies involve only ankle and knee rotations, but the diverse set of descending inputs to Ia inhibitory interneurons (Baldissera et al., 1981; Jankowska, 2001) make it possible to modulate reciprocal inhibition to allow push-pull control in a wide variety of motor behaviors. Further study is required to see if push-pull control occurs at the hip or within the forelimb. The strength of push-pull effects on MN gain can be controlled by altering the background levels of each conductance.

SPINAL INJURY

Spinal cord injury (SCI) not only impairs motor commands but also damages descending control of spinal excitability. Paralysis, impairment and loss of function following SCI arises from loss of inputs from supraspinal structures, including those from descending neuromodulatory systems (Frigon and Rossignol, 2006). In the decerebrate cat preparation, spinal transection eliminates brainstem monoaminergic pathways as well as all

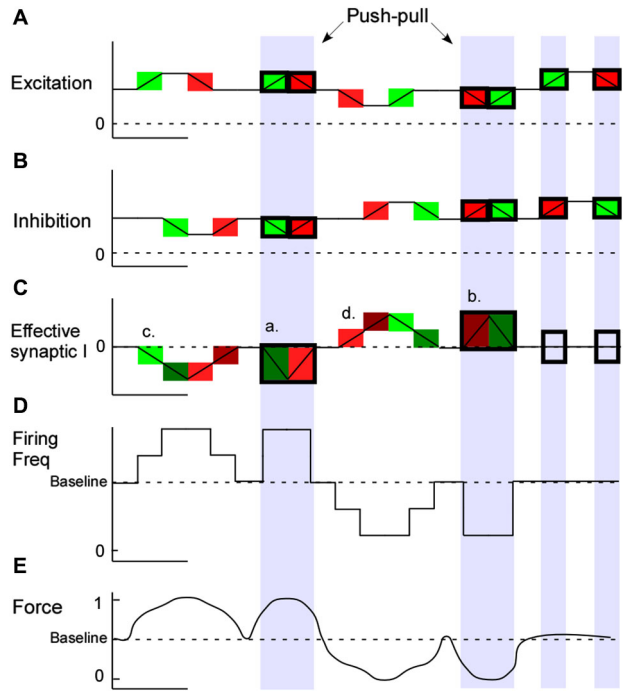

FIGURE 4 | Push-pull combinations and outcomes. Modulating excitation and inhibition from a tonic background of each conductance should allow for greater output gain. Combinations of excitatory and inhibitory input coupling and the predicted impact on motoneuron firing and muscle force production are illustrated. In this basic model as excitatory **(A)** and inhibitory **(B)** condudtances occur in the dendrites the effective synaptic current at the motoneuron soma is illustrated in green (net depolarizing) and red (net hyperpolarizing) **(C)**. Motoneuron firing frequency changes are illustrated in **(D)** and muscle force production **(E)**. During concomitant conductance changes, push-pull effects are seen (shaded blue).

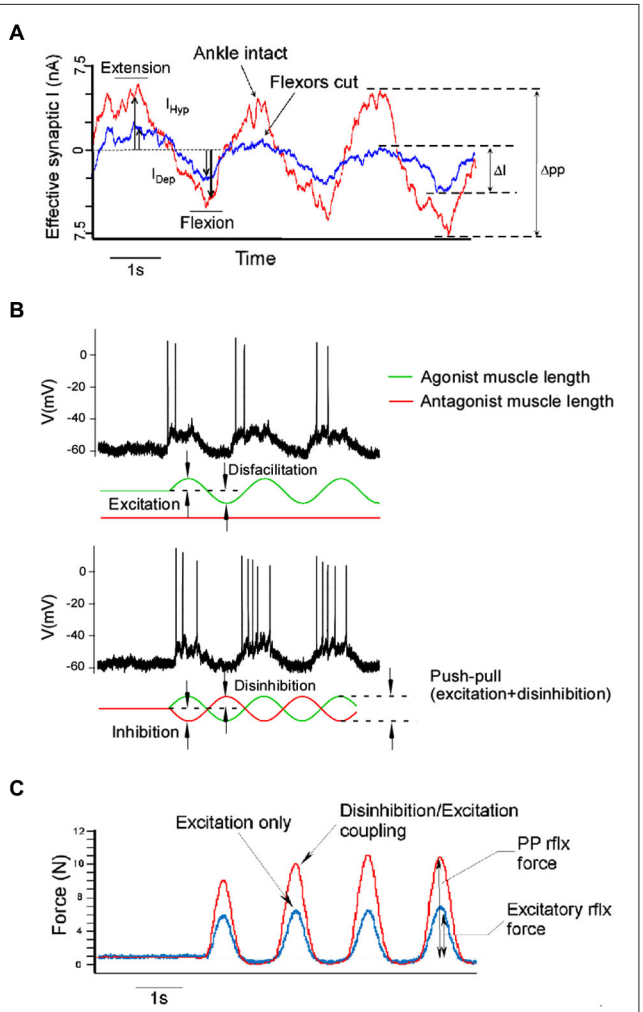

FIGURE 5 | Push-pull effects *in vivo*. Intracellular currents measured at the voltage clamped motoneuron as linear motors connected to the distal tendon of it's agonist and antagonist muscles alternately stretch and shorten the muscles show that the push-pull configuration (tendons intact, red trace) results in greater peak to peak current amplitude than in the non-push-pull configuration (flexor tendons cut, blue trace) **(A)**. The push-pull configuration, where muscles are stretched and shortened in an alternating pattern (lower panel in **B**), also results in greater motoneuron firing rates than modulating excitation alone (upper panel in **B**). Push-pull effects manifest at the system level as well. Greater muscle force is produced when excitation is coupled with disinhibition **(C)**.

other remaining descending inputs to the spinal cord caudal to the injury and thus eliminates PICs and their effects (Lee and Heckman, 2000). This leads to perhaps the most profound immediate result of spinal injury: a state of complete spinal shock where no amount of natural synaptic stimulation can bring MNs to firing threshold. In this scenario motor commands from spared pathways in incomplete spinal injury may still produce depolarizing currents in recipient MNs, but without the amplifying effect of PICs, muscle activation cannot occur. The gradual return of spinal excitability, and emergence of muscle spasms that sometimes follows, we now know, matches the time course of the re-emergence of MN PICs in animal experiments (Bennett et al., 2001a,b).

In the non-injured state spinal processing of sensory inputs produces motor outputs that are focused, reciprocal and consistent. The tightly focused Ia system that dominates MN sensory processing radically changes following SCI. Normally MNs have movement related receptive fields (MRRFs) that are joint specific. For example, in voltage clamp experiments ankle extensor MNs show strong synaptic currents during passive ankle rotation, while rotations of the hip are largely ignored. Immediately (minutes-hours) following spinal transection their MRRFs broaden and these same MNs are now strongly depolarized by rotations of the hip (Hyngstrom et al., 2008a). In this altered state the reciprocal arrangement of inputs from myotactic agonist/antagonist muscle pairs no longer dominates spinal MN behavior, in fact inputs from far away joints unrelated to the MNs muscle evoke the strongest synaptic currents. This disruption in MRRF somatotopy has, at its core, disruption of effective synaptic strengths and has the potential to interfere with reciprocal inhibitions ability to modulate MN PICs, which in the weeks following spinal injury, re-emerges.

The most likely source of this receptive field widening is acute loss of descending monoaminergic drive causing a disinhibition of polysynaptic excitatory pathways on to recipient MNs.

PICs amplify both excitatory and inhibitory inputs individually in a linear fashion. But in active networks with tonic levels of excitatory and inhibitory conductances, their combined effects display a non-linear relationship as membrane potential changes from hyper- to depolarized (Hyngstrom et al., 2008b). This non-linear relationship is actually sub linear for excitation and supra-linear for inhibition. That is, the amplifying effects of the PIC on these two separate sources of simultaneous input, which for inhibition grows stronger as the cell is more depolarized, was greater and resulted in more net inhibition in this depolarized range than what would be predicted if the inputs were applied separately and summed. The supra-linear inhibitory amplification underscores the importance inhibition plays in controlling PICs. Hence another consequence for loss of neuromodulation is disruption of the balance between the effects of excitatory and inhibitory conductances due to PIC interactions.

Though PICs and the input-output gain enhancement they impart on spinal MNs are lost in the acute stages of spinal injury, it has been shown that PICs recover within 1–5 months following complete spinal transection. This is primarily due to the emergence of constitutive activity in serotonin receptors (Murray et al., 2010). This recovery includes the plateau potentials that impart input prolongation to MNs as well as input amplification (Bennett et al., 2001a; Johnson et al., 2013). However we have shown that MNs do not recover input specificity, so they continue to have the wide MRRFs seen in acute spinal transection. As a result joint rotations not associated with their function can cause strong activations in the form of depolarizing currents (Johnson et al., 2013). These aberrant receptive fields interacting with a nearly fully recovered PIC elicit broad activation of muscles throughout the entire limb constituting, we believe, a substrate for multi-joint spasticity in the sub-acute stages of spinal injury. Our ongoing studies are focused on monitoring the changes at both the cellular, via intracellular recordings of spinal MNs, and system level, via multiple motor-unit recordings in muscles, that occur as symptoms progress through the chronic stages of spinal injury.

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REFERENCES

Agnati, L. F., Guidolin, D., Guescini, M., Genedani, S., and Fuxe, K. (2010). Understanding wiring and volume transmission. *Brain Res. Rev.* 64, 137–159. doi: 10.1016/j.brainresrev.2010.03.003

Andrykiewicz, A., Patino, L., Naranjo, J. R., Witte, M., Hepp-Reymond, M. C., and Kristeva, R. (2007). Corticomuscular synchronization with small and large dynamic force output. *BMC Neurosci.* 8:101. doi: 10.1186/1471-2202-8-101

Baldissera, F., Hultborn, H., and Illert, M. (1981). "Integration in spinal neuronal systems," in *Handbook of Physiology. The Nervous System. Motor Control*, ed V. B. Brooks (Bethesda: American Physiological Society), 509–595.

Ballou, E. W., Smith, W. B., Anelli, R., and Heckman, C. J. (2006). Measuring dendritic distribution of membrane proteins. *J. Neurosci. Methods* 156, 257–266. doi: 10.1016/j.jneumeth.2006.03.014

Bennett, D. J., Hultborn, H., Fedirchuk, B., and Gorassini, M. (1998). Synaptic activation of plateaus in hindlimb motoneurons of decerebrate cats. *J. Neurophysiol.* 80, 2023–2037.

Bennett, D. J., Li, Y., Harvey, P. J., and Gorassini, M. (2001a). Evidence for plateau potentials in tail motoneurons of awake chronic spinal rats with spasticity. *J. Neurophysiol.* 86, 1972–1982.

Bennett, D. J., Li, Y., and Siu, M. (2001b). Plateau potentials in sacrocaudal motoneurons of chronic spinal rats, recorded in vitro. *J. Neurophysiol.* 86, 1955–1971.

Berg, R. W., Alaburda, A., and Hounsgaard, J. (2007). Balanced inhibition and excitation drive spike activity in spinal half-centers. *Science* 315, 390–393. doi: 10.1126/science.1134960

Binder, M. D., Heckman, C. J., and Powers, R. K. (2002). Relative strengths and distributions of different sources of synaptic input to the motoneurone pool: implications for motor unit recruitment. *Adv. Exp. Med. Biol.* 508, 207–212. doi: 10.1007/978-1-4615-0713-0_25

Björklund, A., and Skagerberg, G. (1982). "Descending monoaminergic projections to the spinal cord," in *Brain Stem Control of Spinal Mechanisms*, eds B. Sjolund and A. Bjorklund (Amsterdam: Elsevier Biomedical Press), 55–88.

Borg-Graham, L. J., Monier, C., and Fregnac, Y. (1998). Visual input evokes transient and strong shunting inhibition in visual cortical neurons. *Nature* 393, 369–373. doi: 10.1038/30735

Bui, T. V., Grande, G., and Rose, P. K. (2008a). Multiple modes of amplification of synaptic inhibition to motoneurons by persistent inward currents. *J. Neurophysiol.* 99, 571–582. doi: 10.1152/jn.00717.2007

Bui, T. V., Grande, G., and Rose, P. K. (2008b). Relative location of inhibitory synapses and persistent inward currents determines the magnitude and mode of synaptic amplification in motoneurons. *J. Neurophysiol.* 99, 583–594. doi: 10.1152/jn.00718.2007

Carlin, K. P., Jones, K. E., Jiang, Z., Jordan, L. M., and Brownstone, R. M. (2000). Dendritic L-type calcium currents in mouse spinal motoneurons: implications for bistability. *Eur. J. Neurosci.* 12, 1635–1646. doi: 10.1046/j.1460-9568.2000.00055.x

Conway, B. R., and Livingstone, M. S. (2006). Spatial and temporal properties of cone signals in alert macaque primary visual cortex. *J. Neurosci.* 26, 10826–10846. doi: 10.1523/jneurosci.2091-06.2006

de Almeida, A. T., and Kirkwood, P. A. (2010). Multiple phases of excitation and inhibition in central respiratory drive potentials of thoracic motoneurones in the rat. *J. Physiol.* 588, 2731–2744. doi: 10.1113/jphysiol.2009.186346

Eckert, R., and Lux, H. D. (1976). A voltage-sensitive persistent calcium conductance in neuronal somata of Helix. *J. Physiol.* 254, 129–151.

Ferster, D. (1988). Spatially opponent excitation and inhibition in simple cells of the cat visual cortex. *J. Neurosci.* 8, 1172–1180.

Frigon, A., and Rossignol, S. (2006). Functional plasticity following spinal cord lesions. *Prog. Brain Res.* 157, 231–260. doi: 10.1016/s0079-6123(06)57016-5

Grande, G., Bui, T. V., and Rose, P. K. (2010). Distribution of vestibulospinal contacts on the dendrites of ipsilateral splenius motoneurons: an anatomical substrate for push-pull interactions during vestibulocollic reflexes. *Brain Res.* 1333, 9–27. doi: 10.1016/j.brainres.2010.03.065

Haider, B., Duque, A., Hasenstaub, A. R., and McCormick, D. A. (2006). Neocortical network activity in vivo is generated through a dynamic balance of excitation and inhibition. *J. Neurosci.* 26, 4535–4545. doi: 10.1523/jneurosci.5297-05.2006

Heckman, C. J., Hyngstrom, A. S., and Johnson, M. D. (2008). Active properties of motoneurone dendrites: diffuse descending neuromodulation, focused local inhibition. *J. Physiol.* 586, 1225–1231. doi: 10.1113/jphysiol.2007.145078

Hentall, I. D., Pinzon, A., and Noga, B. R. (2006). Spatial and temporal patterns of serotonin release in the rat's lumbar spinal cord following electrical stimulation of the nucleus raphe magnus. *Neuroscience* 142, 893–903. doi: 10.1016/j.neuroscience.2006.06.038

Hounsgaard, J., Hultborn, H., Jespersen, B., and Kiehn, O. (1988). Bistability of alpha-motoneurones in the decerebrate cat and in the acute spinal cat after intravenous 5-hydroxytryptophan. *J. Physiol.* 405, 345–367.

Hounsgaard, J., and Kiehn, O. (1993). Calcium spikes and calcium plateaux evoked by differential polarization in dendrites of turtle motoneurones in vitro. *J. Physiol.* 468, 245–259.

Hultborn, H., Brownstone, R. B., Toth, T. I., and Gossard, J. P. (2004). Key mechanisms for setting the input-output gain across the motoneuron pool. *Prog. Brain Res.* 143, 77–95. doi: 10.1016/s0079-6123(03)43008-2

Hultborn, H., Denton, M. E., Wienecke, J., and Nielsen, J. B. (2003). Variable amplification of synaptic input to cat spinal motoneurones by dendritic persistent inward current. *J. Physiol.* 552, 945–952. doi: 10.1113/jphysiol.2003.050971

Hyngstrom, A., Johnson, M., Schuster, J., and Heckman, C. J. (2008a). Movement-related receptive fields of spinal motoneurones with active dendrites. *J. Physiol.* 586, 1581–1593. doi: 10.1113/jphysiol.2007.149146

Hyngstrom, A. S., Johnson, M. D., and Heckman, C. J. (2008b). Summation of excitatory and inhibitory synaptic inputs by motoneurons with highly active dendrites. *J. Neurophysiol.* 99, 1643–1652. doi: 10.1152/jn.01253.2007

Hyngstrom, A. S., Johnson, M. D., Miller, J. F., and Heckman, C. J. (2007). Intrinsic electrical properties of spinal motoneurons vary with joint angle. *Nat. Neurosci.* 10, 363–369. doi: 10.1038/nn1852

Jankowska, E. (2001). Spinal interneuronal systems: identification, multifunctional character and reconfigurations in mammals. *J. Physiol.* 533, 31–40. doi: 10.1111/j.1469-7793.2001.0031b.x

Johnson, M. D., Hyngstrom, A. S., Manuel, M., and Heckman, C. J. (2012). Push-pull control of motor output. *J. Neurosci.* 32, 4592–4599. doi: 10.1523/JNEUROSCI.4709-11.2012

Johnson, M. D., Kajtaz, E., Cain, C. M., and Heckman, C. J. (2013). Motoneuron intrinsic properties, but not their receptive fields, recover in chronic spinal injury. *J. Neurosci.* 33, 18806–18813. doi: 10.1523/JNEUROSCI.2609-13.2013

Kuo, J. J., Lee, R. H., Johnson, M. D., Heckman, H. M., and Heckman, C. J. (2003a). Active dendritic integration of inhibitory synaptic inputs in vivo. *J. Neurophysiol.* 90, 3617–3624. doi: 10.1152/jn.00521.2003

Kuo, J. J., Lee, R. H., Johnson, M. D., Heckman, H. M., and Heckman, C. J. (2003b). Active dendritic integration of inhibitory synaptic inputs in vivo. *J. Neurophysiol.* 90, 3617–3624. doi: 10.1152/jn.00521.2003

Lee, R. H., and Heckman, C. J. (2000). Adjustable amplification of synaptic input in the dendrites of spinal motoneurons in vivo. *J. Neurosci.* 20, 6734–6740.

Lee, R. H., Kuo, J. J., Jiang, M. C., and Heckman, C. J. (2003). Influence of active dendritic currents on input-output processing in spinal motoneurons in vivo. *J. Neurophysiol.* 89, 27–39. doi: 10.1152/jn.00137.2002

Li, X., Murray, K., Harvey, P. J., Ballou, E. W., and Bennett, D. J. (2007). Serotonin facilitates a persistent calcium current in motoneurons of rats with and without chronic spinal cord injury. *J. Neurophysiol.* 97, 1236–1246. doi: 10.1152/jn.00995.2006

Magnusson, A. K., Park, T. J., Pecka, M., Grothe, B., and Koch, U. (2008). Retrograde GABA signaling adjusts sound localization by balancing excitation and inhibition in the brainstem. *Neuron* 59, 125–137. doi: 10.1016/j.neuron.2008.05.011

Maier, M. A., Bennett, K. M., Hepp-Reymond, M. C., and Lemon, R. N. (1993). Contribution of the monkey corticomotoneuronal system to the control of force in precision grip. *J. Neurophysiol.* 69, 772–785.

Miles, G. B., Hartley, R., Todd, A. J., and Brownstone, R. M. (2007). Spinal cholinergic interneurons regulate the excitability of motoneurons during locomotion. *Proc. Natl. Acad. Sci. U S A* 104, 2448–2453. doi: 10.1073/pnas.0611134104

Moritz, A. T., Newkirk, G., Powers, R. K., and Binder, M. D. (2007). Facilitation of somatic calcium channels can evoke prolonged tail currents in rat hypoglossal motoneurons. *J. Neurophysiol.* 98, 1042–1047. doi: 10.1152/jn.01294.2006

Murray, K. C., Nakae, A., Stephens, M. J., Rank, M., D'Amico, J., Harvey, P. J., et al. (2010). Recovery of motoneuron and locomotor function after spinal cord injury depends on constitutive activity in 5-HT2C receptors. *Nat. Med.* 16, 694–700. doi: 10.1038/nm.2160

Parkis, M. A., Dong, X., Feldman, J. L., and Funk, G. D. (1999). Concurrent inhibition and excitation of phrenic motoneurons during inspiration: phase-specific control of excitability. *J. Neurosci.* 19, 2368–2380.

Power, K. E., McCrea, D. A., and Fedirchuk, B. (2010). Intraspinally mediated state-dependent enhancement of motoneurone excitability during fictive scratch in the adult decerebrate cat. *J. Physiol.* 588, 2839–2857. doi: 10.1113/jphysiol.2010.188722

Powers, R. K., Elbasiouny, S. M., Rymer, W. Z., and Heckman, C. J. (2012). Contribution of intrinsic properties and synaptic inputs to motoneuron discharge patterns: a simulation study. *J. Neurophysiol.* 107, 808–823. doi: 10.1152/jn.00510.2011

Rajkowski, J., Kubiak, P., and Aston-Jones, G. (1994). Locus coeruleus activity in monkey: phasic and tonic changes are associated with altered vigilance. *Brain Res. Bull.* 35, 607–616. doi: 10.1016/0361-9230(94)90175-9

Rajkowski, J., Kubiak, P., Ivanova, S., and Aston-Jones, G. (1998). State-related activity, reactivity of locus ceruleus neurons in behaving monkeys. *Adv. Pharmacol.* 42, 740–744. doi: 10.1016/s1054-3589(08)60854-6

Rasmussen, K., Morilak, D. A., and Jacobs, B. L. (1986). Single unit activity of locus coeruleus neurons in the freely moving cat. I. During naturalistic behaviors and in response to simple and complex stimuli. *Brain Res.* 371, 324–334. doi: 10.1016/0006-8993(86)90371-9

Raymond, J. R., Mukhin, Y. V., Gelasco, A., Turner, J., Collinsworth, G., Gettys, T. W., et al. (2001). Multiplicity of mechanisms of serotonin receptor signal transduction. *Pharmacol. Ther.* 92, 179–212. doi: 10.1016/s0163-7258(01)00169-3

Schwindt, P., and Crill, W. E. (1977). A persistent negative resistance in cat lumbar motoneurons. *Brain Res.* 120, 173–178. doi: 10.1016/0006-8993(77)90510-8

Schwindt, P. C., and Crill, W. E. (1980). Properties of a persistent inward current in normal and TEA-injected motoneurons. *J. Neurophysiol.* 43, 1700–1724.

Schwindt, P. C., and Crill, W. E. (1981). Negative slope conductance at large depolarizations in cat spinal motoneurons. *Brain Res.* 207, 471–475. doi: 10.1016/0006-8993(81)90381-4

Shu, Y., Hasenstaub, A., and McCormick, D. A. (2003). Turning on and off recurrent balanced cortical activity. *Nature* 423, 288–293. doi: 10.1038/nature01616

Simon, M., Perrier, J. F., and Hounsgaard, J. (2003). Subcellular distribution of L-type Ca2+ channels responsible for plateau potentials in motoneurons from the lumbar spinal cord of the turtle. *Eur. J. Neurosci.* 18, 258–266. doi: 10.1046/j.1460-9568.2003.02783.x

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Multiple monoaminergic modulation of posturo-locomotor network activity in the newborn rat spinal cord

Lauriane Beliez, Gregory Barrière, Sandrine S. Bertrand and Jean-René Cazalets*

CNRS UMR 5287, Institut de Neurosciences Cognitives et Intégratives d'Aquitaine, Université de Bordeaux, Bordeaux, France

Edited by:
Shawn Hochman, Emory University, USA

Reviewed by:
Deborah Baro, Georgia State University, USA
Wolfgang Stein, Illinois State University, USA
Aharon Lev-Tov, Hebrew University Medical School, Israel

***Correspondence:**
Jean-René Cazalets, CNRS UMR 5287, Institut de Neurosciences Cognitives et Intégratives d'Aquitaine, Université de Bordeaux, Zone nord, Bat 2 2e étage, 146 rue Leo Saignat, 33076 Bordeaux cedex, France
e-mail: jean-rene.cazalets@u-bordeaux.fr

Studies devoted to understanding locomotor control have mainly addressed the functioning of the neural circuits controlling leg movements and relatively little is known of the operation of networks that activate trunk muscles in coordination with limb movements. The aim of the present work was (1) to identify the exogenous neurotransmitter cocktail that most strongly activates postural thoracic circuitry; (2) to investigate how the biogenic amines serotonin (5-HT), dopamine (DA), and noradrenaline (NA) modulate the coordination between limb and axial motor networks. Experiments were carried out on *in vitro* isolated spinal cord preparations from newborn rats. We recorded from ventral roots to monitor hindlimb locomotor and axial postural network activity. Each combination of the three amines with excitatory amino acids (EAAs) elicited coordinated rhythmic motor activity at all segmental levels with specific characteristics. The variability in cycle period was similar with 5-HT and DA while it was significantly higher with NA. DA elicited motor bursts of smaller amplitude in thoracic segments compared to 5-HT and NA, while both DA and NA elicited motor bursts of higher amplitude than 5-HT in the lumbar and sacral segments. The amines modulated the phase relationships of bursts in various segments with respect to the reference lumbar segment. At the thoracic level there was a phase lag between all recorded segments in the presence of 5-HT, while DA and NA elicited synchronous bursting. At the sacral level, 5-HT and DA induced an intersegmental phase shift while relationships became phase-locked with NA. Various combinations of EAAs with two or even all three amines elicited rhythmic motor output that was more variable than with one amine alone. Our results provide new data on the coordinating processes between spinal cord networks, demonstrating that each amine has a characteristic "signature" regarding its specific effect on intersegmental phase relationships.

Keywords: dopamine, serotonin, noradrenaline, locomotion, posture, spinal cord, neuromodulation

INTRODUCTION

The achievement of efficient locomotory movements necessary for survival in a demanding external environment requires the integrated functioning and synergistic action of many muscle groups in order to ensure the appropriate positioning of all body regions during self-motion (Cazalets, 2000; Falgairolle et al., 2006). However, most studies devoted to understanding locomotor control in humans and quadrupeds (Rossignol, 1996; Duysens and Van de Crommert, 1998) have only addressed the functioning of the neural circuits controlling leg movements, and relatively little is known about the functioning of neuronal networks that activate trunk muscles in coordination with limb movements (Thorstensson et al., 1982; Koehler et al., 1984; Zomlefer et al., 1984; Gramsbergen et al., 1999; De Sèze et al., 2008; Rauscent et al., 2009; Combes et al., 2012). Similarly, the role of the forelimbs during locomotion remains poorly understood (Akazawa et al., 1982; Duenas et al., 1990; Ballion et al., 2001; Zehr and Duysens, 2004). Indeed all these motor networks do not operate in isolation but interact with each other, and it is only recently that increasing attention has been paid to the interactive functioning of various spinal segments in quadrupedal mammals and humans during locomotion (Cazalets, 2000; Wannier et al., 2001; Dietz, 2002; Falgairolle et al., 2006, 2013; Maclellan et al., 2011; Nakajima et al., 2013).

Several seminal reviews (Harris-Warrick, 2011; Miles and Sillar, 2011) have recently highlighted the role of neuromodulators in governing the functional flexibility of spinal circuitry. The motor networks responsible for locomotor activity and which are the potential targets of neuromodulators are intrinsic to the spinal cord and can generate rhythmic motor activity in the absence of sensory feedback. The cervical and lumbar enlargements contain the neural machinery necessary to operate the fore- and hind-limbs, respectively, while thoracic and sacral segments control the axial systems. As underlined by Harris-Warrick (2011), neuromodulatory actions are required for the normal function of these networks. In using the *in vitro* isolated spinal cord from new born rat, the neuromodulatory impact of the three biogenic amines serotonin, dopamine (DA), and noradrenaline (NA) on locomotion has been extensively studied. Their effect had been previously established for the motor activity generated by the lumbar segments and subsequent studies further investigated aspects of their pharmacological features (Cazalets et al., 1992; Kiehn and Kjaerulff, 1996; Kiehn et al.,

1999; Sqalli-Houssaini and Cazalets, 2000; Barriere et al., 2004; Madriaga et al., 2004; Pearlstein et al., 2005; Gordon and Whelan, 2006; Liu et al., 2009). It has been shown that they may differentially modulate a motor pattern according to the specific receptor subtypes they activate (for review, see Miles and Sillar, 2011). 5-HT exerts an excitatory (permissive) effect through an activation of 5-HT2 and 5-HT7 receptors (Madriaga et al., 2004; Pearlstein et al., 2005; Liu et al., 2009) or an inhibitory (suppressive) action through 5-HT1 receptor activation (Beato and Nistri, 1998) on motoneurons or interneurons (Zhong et al., 2006a). Similarly, NA (Kiehn et al., 1999; Sqalli-Houssaini and Cazalets, 2000), exerts a multi-modal control of lumbar locomotor networks through an α1 receptor mediated activation and an α2 receptors mediated inhibition (Sqalli-Houssaini and Cazalets, 2000; Gabbay et al., 2002; Gordon and Whelan, 2006). The dopaminergic modulation of lumbar locomotor patterns (Kiehn and Kjaerulff, 1996; Whelan et al., 2000; Madriaga et al., 2004) is mediated via D1 and D2 receptor activation leading to an increase in motoneuron and Hb-9 interneuron excitability (Han et al., 2007). Most of the effects reported for these drugs were also found to occur in spinally transected adult rodents (Landry et al., 2006; Lapointe and Guertin, 2008; Lapointe et al., 2008, 2009). Surprisingly, however, despite the numerous studies that have investigated the individual action of each amine alone or in combination with excitatory amino acids (EAAs), there has not been a study that has systematically compared the action of the three amines, alone or in various combinations, on locomotor pattern genesis.

The aim of the present study therefore was (1) to identify the neuromodulator mixture that most strongly activates spinal motor networks involved in hindlimb and axial muscle control; (2) to investigate how the biogenic amines serotonin (5-HT), DA, and NA, shape the operation of and the interactions between the limb and axial motor networks during ongoing locomotor-like rhythmogenesis initiated by excitatory amino-acid receptor activation.

MATERIALS AND METHODS

Experiments were performed on the *in vitro* isolated spinal cord of newborn Sprague–Dawley rats aged between 1 and 5 post-natal days (P1–P5, $n = 25$ preparations). All procedures were conducted in accordance with the local ethics committee of the University of Bordeaux and the European Committee Council Directive. All efforts were made to minimize animal suffering and to reduce the number of animals.

IN VITRO ISOLATED SPINAL CORD

Rat pups were anesthetized using isofluorane until no reflex could be elicited in response to tail or toe pinching. Animals were decapitated, the skin of the back removed, and the preparations were placed ventral side up in a dissecting chamber. A laminectomy was performed to expose the spinal cord that was then dissected out using fine forceps and microscissors under binocular microscope control. Dissections and recording procedures were performed under the continuous perfusion of an artificial cerebrospinal fluid (aCSF) equilibrated with 95% O_2–5% CO_2, pH7.4 at room temperature (24–26°C) and containing (mM): NaCl 130, KCl 3, $CaCl_2$ 2.5, $MgSo_4$ 1.3, NaH_2PO_4 0.58, $NaHCO_3$ 25 and glucose 10. Spinal cords were sectioned at the T2 level at the beginning of the experiment.

ELECTROPHYSIOLOGICAL RECORDINGS AND ANALYSIS

Extracellular motor activities were recorded from various spinal cord ventral roots using Vaseline-insulated, stainless steel pin electrodes at the lumbar level and glass suction electrodes for the shorter thoracic ventral roots. Recorded activities were amplified using custom-made amplifiers then digitized (Digidata 1322A, Molecular Device, CA, USA) using an interface driven by Axograph software (Axograph, AU). The raw signals were high-passed (50 Hz), rectified and integrated before analysis. Burst amplitudes (trough to peak) and locomotor pattern cycle periods were calculated using a program developed in Matlab (Mathworks) based on burst onset and offset detection. Since it is not possible to compare absolute amplitude values of extracellular data between experiments, we calculated only the relative change in amplitude from a control condition. Such amplitude changes could be either due to changes in firing frequency of already active motor units or to a recruitment of previously silent units. Due to the difficulty in establishing stable and persistent rhythmic locomotor-like activity with bath-application of EAAs alone, the combination of EAAs and 5-HT that is the most commonly used cocktail to induce locomotor-related activity *in vitro* (Sqalli-Houssaini et al., 1993) was taken as the reference condition to compare the actions of the amines.

We used recordings at L2 as the reference trace because it invariably exhibited the best signal-to-noise ratio. Pair-wise phase relationships between bursting activities recorded simultaneously from different ventral roots were calculated using wavelet transform analysis (for an extensive description of the method see Grinsted et al., 2004; Mor and Lev-Tov, 2007; Torrence and Compo, 2010). For this purpose, we used custom-made software based on the MatLab wavelet coherence package provided by Aslak Grinsted (http://noc.ac.uk/using-science/crosswavelet-wavelet-coherence). For each pair-wise recording we performed both a cross wavelet transform and a wavelet coherence determination. The results obtained with both algorithms were combined into a single time-frequency map so as to extract phase relationships from delineated coherent common high power frequency regions. Phase values were plotted as a circular representation (0–1 rad), with the mean phase being indicated by the direction of the vector, and its length (range from 0 to 1) indicating the strength of the mean.

PHARMACOLOGY

Episodes of locomotor-like activity were elicited by the exogenous application of a mixture of 15 μM N-methyl-D,L-aspartate (NMA, Sigma) and serotonin (5-HT, Sigma) and/or DA (Sigma), and/or NA (Sigma). DA and NA were freshly prepared and protected from light exposure during bath superfusion in order to prevent photodegradation. Sodium metabisulfite (0.1%; Sigma) was also added to the DA supply in order to prevent oxidation (Barriere et al., 2004). Drugs were bath-applied using a peristaltic pump (flow rate 4 ml/min; recording chamber volume 4 ml). The effects of the drugs were monitored from 5 to 10 min after reaching the Petri

dish (the time required for total replacement of the normal saline and diffusion into the tissue).

Different drug combinations were tested on the same preparation in a random order except for DA. Since we previously demonstrated that high DA concentrations (500 μM) may exert long-lasting effects on the *in vitro* newborn rat spinal cord (Barriere et al., 2004), this amine was always tested after the other amines in a given experiment and at a 10 times lower concentration than that found to exert prolonged actions (i.e., 50 μM instead of 500 μM). When different drug concentrations were tested in the same preparation, we always began with the lowest concentration. Total drug application lasted 20 min. During an episode of locomotor-like activity, cycle periods were initially longer and then shortened progressively until stable rhythmicity was reached within 5 min (Sqalli-Houssaini et al., 1993; Cazalets et al., 1999). All measurements were performed on 30 consecutive cycles during this steady-state condition. Each drug bath-application was followed by a prolonged wash out with normal saline for at least 30 min.

STATISTICAL ANALYSIS

Data were tested for normality, and in its absence, non-parametric analysis was applied. Statistical analyses were performed using Prism software (Graphpad software, CA, USA). The coefficient of variation (COV), known as "relative variability," was determined by the SD divided by the mean and expressed as a percent. In each experiment it was calculated from the same recording sequence as the motor period and amplitude. The degree of variability (or stability) of the motor pattern cycle period was assessed by computing the COV. Comparisons between conditions were performed using Student's *t*-test, one-way or two-way ANOVA with Tukey's test *post hoc* analysis. The equality of group variance was tested using the Bartlett's test or the *F* test.

Statistical analyses of circular data were performed on raw data by descriptive statistics of circular distribution using IgoPro software (Wavemetrics, OR, USA) and Oriana software (KCS, UK). The Rayleigh test was used to determine the coupling strength. The Watson–Williams test (a circular analog of the one-factor ANOVA, Berens, 2009) and a non-parametric second-order two-sample test (which is less sensitive to departure from normality) consisting of pre-processing where the grand mean is subtracted from the two inputs followed by application of Watson's $U2$-test (IgoPro software, Wavemetrics, OR, USA) were used.

The significance threshold was taken to be $p < 0.05$ unless otherwise specified. All data values in the text are means ± SEM.

RESULTS

Since no direct comparison of the neuromodulatory action of the three monoamines 5-HT, NA and DA has been so far reported, we first established how they each modulate the basic rhythmic motor activity elicited by EAAs. For this purpose we recorded ipsi- and contra-lateral L2 and L5 lumbar ventral roots to respectively monitor the flexor and extensor phases of the motor pattern cycle (**Figures 1A1–A3**). From a given experiment we selected representative episodes of locomotor-like activity with comparable cycle periods elicited by the conjoint bath-application of the EAA agonist NMA and one amine in order to highlight possible differences between other parameters (such as COV, phase relationship, and motor burst amplitude). Several parameters that characterize the ongoing locomotor patterned activity were then calculated, namely the cycle period (the time that separates the onsets of two successive bursts of activity, **Figure 1B**), the COV of cycle period which is an index of the temporal regularity of the motor pattern (**Figure 1C**) and the burst amplitude (**Figure 1D**). As previously found (Sqalli-Houssaini and Cazalets, 2000; Barriere et al., 2004), there was a significant dose-dependent action for DA on cycle period (one-way ANOVA, $F = 6.06$, $p = 0.0016$) but not for NA (one-way ANOVA, $F = 0.8$, $p = 0.5$) and 5-HT (Student's *t*-test, $t = 1.53$, $p = 0.14$), over the range of concentrations tested. In the present study, subsequent experiments were performed using DA at 50 μM, an intermediate concentration in the dose-dependent range. When comparing the variance of cycle period in the presence of the three amines at the concentrations used in **Figure 1A** (and throughout the study), we found that values were significantly different (Bartlett's test for equal variance, Bartlett's statistics = 10.5, $p = 0.005$). This variability in cycle period revealed by the COV calculation (**Figure 1C**), was comparable for NMA/5-HT and NMA/DA (9.3 ± 1.3% and 11 ± 2%, respectively) while it was significantly higher for NMA/NA (20.7 ± 2.8%, one-way ANOVA, $F = 10.5$, $p = 0.0002$). For each amine, there was no evident dose-dependency for period variability. **Figure 1D** shows the relative changes in motor burst amplitude. For each trial, the mean burst amplitude of activity in an individual ventral root under NMA/5-HT was considered as the control condition and measurements under other pharmacological conditions were normalized to this value. Both DA and NA significantly increased the amplitude of flexor (L2) and extensor (L5) motor bursts. Altogether, these results confirm the previously reported individual effects of the amines on EAA-induced locomotor-related rhythmicity, although our comparative assessment indicates that each amine exerts a specific action on hindlimb motor network activity.

In a next step, we tested which amine most strongly activates the axial and hindlimb motor networks. **Figure 2A** presents typical simultaneous recordings from thoracic (T6 and T11), lumbar (L2) and sacral (S1, S3) ipsilateral ventral roots obtained from the same experiment in the presence of NMA and successively 5-HT (A1), DA (A2), and NA (A3) at amine concentrations that elicited motor rhythms with similar cycle periods. Each amine elicited coordinated motor burst at all segmental levels, although with qualitative differences. Visual trace inspection suggests that the most striking changes took place in the sacral burst amplitude in the presence of DA and especially NA. In the thoracic region, motor bursts were more distinguishable in the presence of 5-HT than either DA or NA. **Figure 2B** indicates the proportion of experiments in which motor bursts were recorded from thoracic and sacral segments when sustained lumbar locomotor-like activity was elicited as defined in **Figure 1**. In this respect, 5-HT and NA were noticeably more effective than DA. Moreover, relative amplitude calculations indicate that burst amplitudes in thoracic segments were significantly decreased by DA (**Figure 2C**; paired Student's *t*-test, $t = 5.4$, $p = 0.0002$), while burst amplitude in the sacral segments was significantly increased by DA (**Figure 2C**; paired Student's *t*-test,

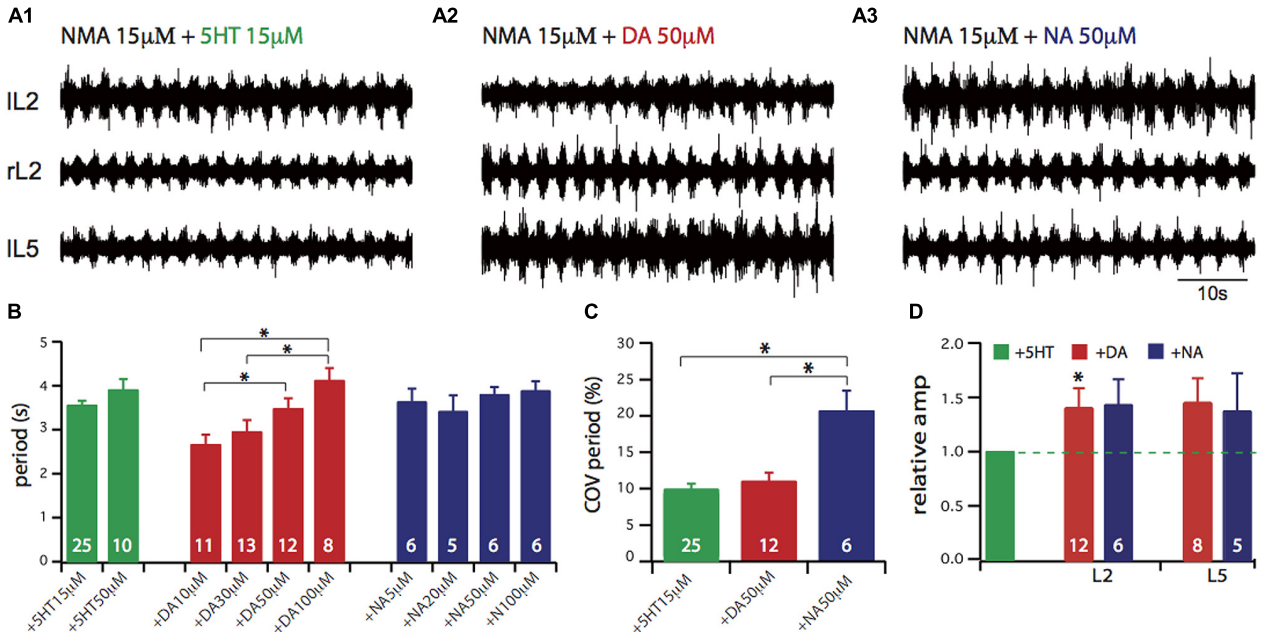

FIGURE 1 | Comparison of lumbar locomotor network activation *in vitro* by 5-HT, or DA or NA. (A) Each amine was added to the bathing saline containing the NMDA excitatory amino acid receptor agonist NMA (15 µM), while extracellular recordings were made from the second left and right lumbar ventral root (lL2, rL2) and the fifth left lumbar ventral root (lL5) of the isolated spinal cord. **(B)** Plots of mean cycle period value obtained for different amine concentrations. A dose-dependent effect is only observable for DA. **(C)** Plots of the coefficient of variation (COV) of cycle period for each amine (NMA 15 µM, 5-HT 15 µM, DA 50 µM, NA 50 µM), indicating that NA leads to a more unstable motor activity. **(D)** Plots of mean relative amplitude changes normalized to the amplitude observed in the NMA/5-HT condition (green bar). DA and NA elicit larger burst amplitude in the lumbar ventral roots. In this and in subsequent figures, the number on each bar histogram indicates the number of experiments. $*p < 0.05$.

$t = 2.8$, $p = 0.03$) and NA (**Figure 2C**; Student's t-test, $t = 4.5$, $p = 0.006$).

Since the combination of NMA and the amines elicited coordinated rhythmic motor activity in the various spinal cord areas, we examined whether, in addition to their effects on cycle period and burst amplitude, they were able to shape locomotor-related activity by modulating the phase relationships between the various segmental levels. Phase relationships were calculated by taking the ipsilateral L2 ventral root recording as the reference trace (see Materials and Methods). **Figure 3A** presents averaged individual cycles replicated in a continuous sequence to reveal the rhythmic nature of the activity (see Figure 6A in Falgairolle and Cazalets, 2007), in the presence of each of the three amines. Each time a burst was detected in the reference L2 trace, it was sampled with the other associated recorded traces. Individual cycles were superimposed and then averaged. This process allowed switching from a continuous mode of recording to an episodic one (**Figures 3A** and **4B**) so as to increase the signal-to-noise ratio in order to obtain a more accurate detection of burst peaks (Falgairolle and Cazalets, 2007). None of the amines elicited significantly different phase relationships in the lumbar left/right alternating pattern, the flexor and extensor coordination nor burst durations, as indicated by the strict superimposition of L2 and L5 traces under all three amine conditions. In contrast, each amine established a distinct phase relationship between the thoracic, sacral and lumbar segments. **Figure 3B** illustrates the phase relationships between each recording location and reference L2. Data were pooled for all concentrations tested as there was no dose-dependent effect of concentration for any amine and any ventral root (multisample Watson–Williams test, $p > 0.1$). Each point in the polar plots represents the mean phase value obtained in a single experiment, with the vector direction representing the mean phase value and its length the coupling strength. Inspection of the polar plots reveals that with 5-HT and DA, the phase values were more variable between experiments in the sacral region than in the lumbar or thoracic segments. In the presence of 5-HT there was a phase lag between lumbar and thoracic segments that was previously shown to increase significantly with the intervening distance (Falgairolle and Cazalets, 2007), while almost synchronous bursting occurred in the recorded thoracic segments with no phase lag increase with segmental distance in the presence of DA (mean vector value 0.93 in T6 and 0.88 in T11; Mardia's two sample U 0.08, $p > 0.2$, **Figure 3**) and NA (mean vector value 0.94 in T6 and 0.89 in T11; Mardia's two sample U 0.07, $p > 0.2$, **Figure 3**). At the sacral level, a significant progressive phase shift relative to L2 occurred both in the presence of 5-HT (mean vector value 0.61 in S1 and 0.52 in S3; Mardia's two sample U 0.24, $p < 0.02$, **Figure 3**), and DA (mean vector value 0.91 in S1, 0.61 in S3; Mardia's two sample U 0.3, $p < 0.005$, **Figure 3**). In contrast, NA elicited a phase-locked motor pattern with a similar phase relationship at all sacral levels (mean vector value 0.97 in S1 and 0.93 in S3; Mardia's two sample U 0.18, $p > 0.05$, **Figure 3**). These results therefore demonstrate that each amine possesses a specific instructive "signature" regarding

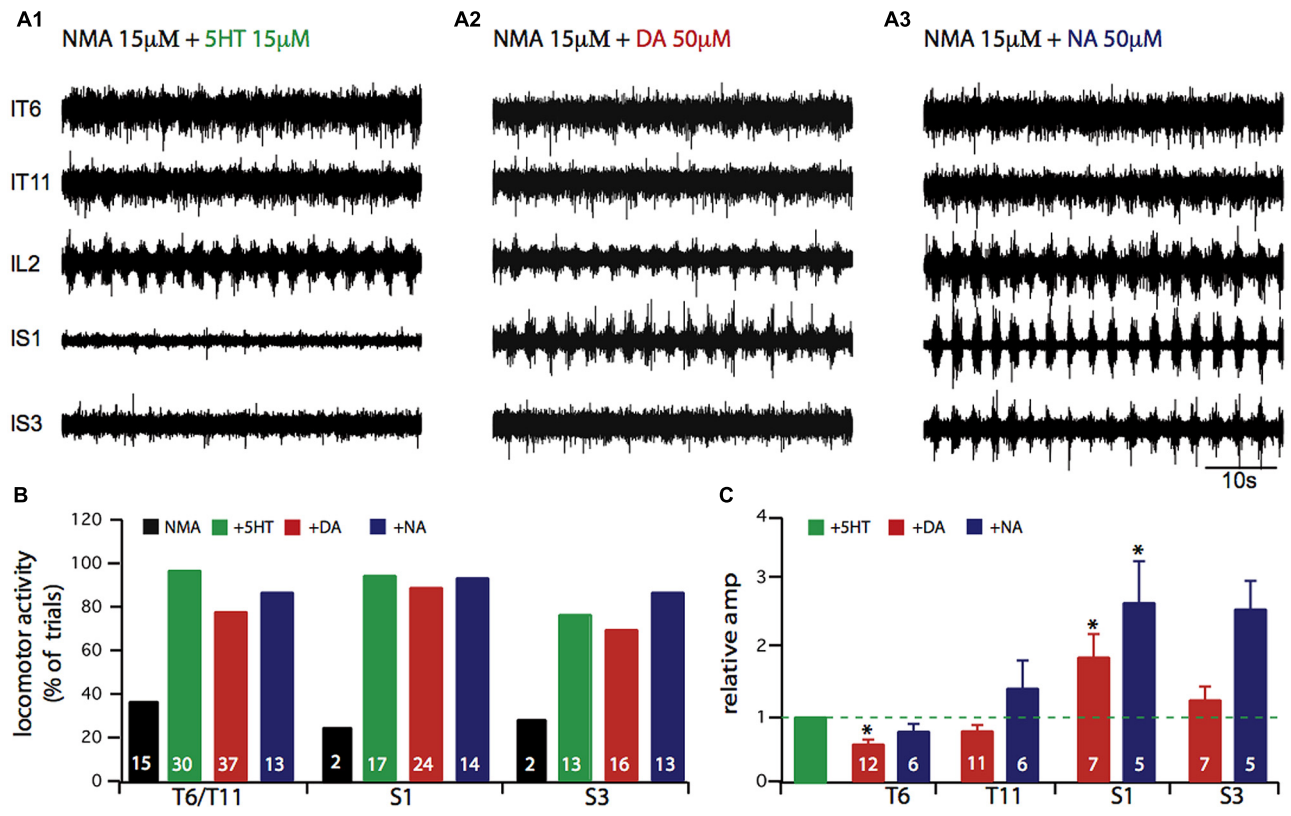

FIGURE 2 | Comparison of axial locomotor network activation *in vitro* by 5-HT, or DA or NA. (A) Each amine was added to 15 μM NMA-containing saline during recordings from the sixth and eleventh left thoracic ventral roots (IT6, IT11), the second left lumbar ventral root (IL2), and the first and third left sacral ventral roots (IS1, IS3). **(B)** Plots indicating the percentage of experiments in which rhythmic motor activity was observed at a given segmental level. In these experiments, lumbar locomotor activity was always expressed. **(C)** Plots of mean relative amplitude changes normalized to the amplitude observed in the NMA/5-HT condition (green bar). Note the higher noradrenergic sensitivity of the more distal segments. $*p < 0.05$.

its modulatory affect on the distributed temporal aspects of the motor pattern.

Finally, we investigated different neuromodulatory combinations by mixing EAA receptor agonists with two or even the three amines (**Figure 4**). A typical experiment in which the four drugs were added to the bath saline is shown in **Figure 4A**. This neuromodulatory cocktail still elicited rhythmic motor output that, at a first glance, did not differ markedly from the condition with one amine alone. However, rhythmic bursting was less frequently observed (67% of experiments, **Table 1**), in the thoracic compartment under the multiple amine condition. Moreover, the amines in combination elicited motor rhythms with significantly longer cycle periods (**Figure 4B**; unpaired Student's *t*-test; 5-HT/DA, $t = 2.96$, $p = 0.0057$; 5-HT/DA/NA, $t = 6.82$, $p = 0.0001$) and increased burst amplitude in the most caudal (L5 and sacral) segments (**Figure 4C**; paired Student's *t*-test; 5-HT/NA in L2, $t = 2.5$, $p = 0.04$; 5-HT/NA in L5, $t = 2.5$, $p = 0.04$; 5-HT/NA in S1, $t = 4.8$, $p = 0.002$; 5-HT/NA/DA in S1, $t = 3.5$, $p = 0.017$). The rhythm stability, as indicated by the COV was within the same range as values with the mixture of NMA/5-HT or NMA/DA (see **Figure 1C**), i.e. 10.4 ± 1.2% for NMA/5-HT/DA, 13 ± 2% for NMA/5-HT/NA and 12.6 ± 3.4% for NMA/5-HT/DA/NA. Moreover, phase analysis (**Figure 4D**), revealed that the flexor/extensor relationship remained extremely stable under all conditions (see also **Figure 3B**). In contrast, and especially for the sacral segments, the phase relationships generally became more variable with a very small vector length. Interestingly, however, in the mid-thoracic segment (T6), the phase relationships observed in the presence of all three amines or 5-HT/DA or 5-HT/NA was close to that observed in the presence of 5-HT alone (i.e., at about 0.19; see **Table 2**).

DISCUSSION

COMBINATORY ACTION OF DRUGS ON MOTOR RHYTHM GENERATION AND STABILITY

As pointed out by Guertin (2009) there is a need in the field of spinal cord injury and recovery for elaborating therapeutic strategies based on drug-induced CPG activation. Hence the identification of candidate molecules that could become first-in-class treatments for spinal cord injured patients in animal models becomes a prerequisite. The first objective of the present work was therefore to determine which neuromodulator cocktail most effectively activates spinal motor networks involved in locomotion. In pioneering studies (Kudo and Yamada, 1987; Smith and Feldman, 1987), the EAA receptor agonist NMDA was reported

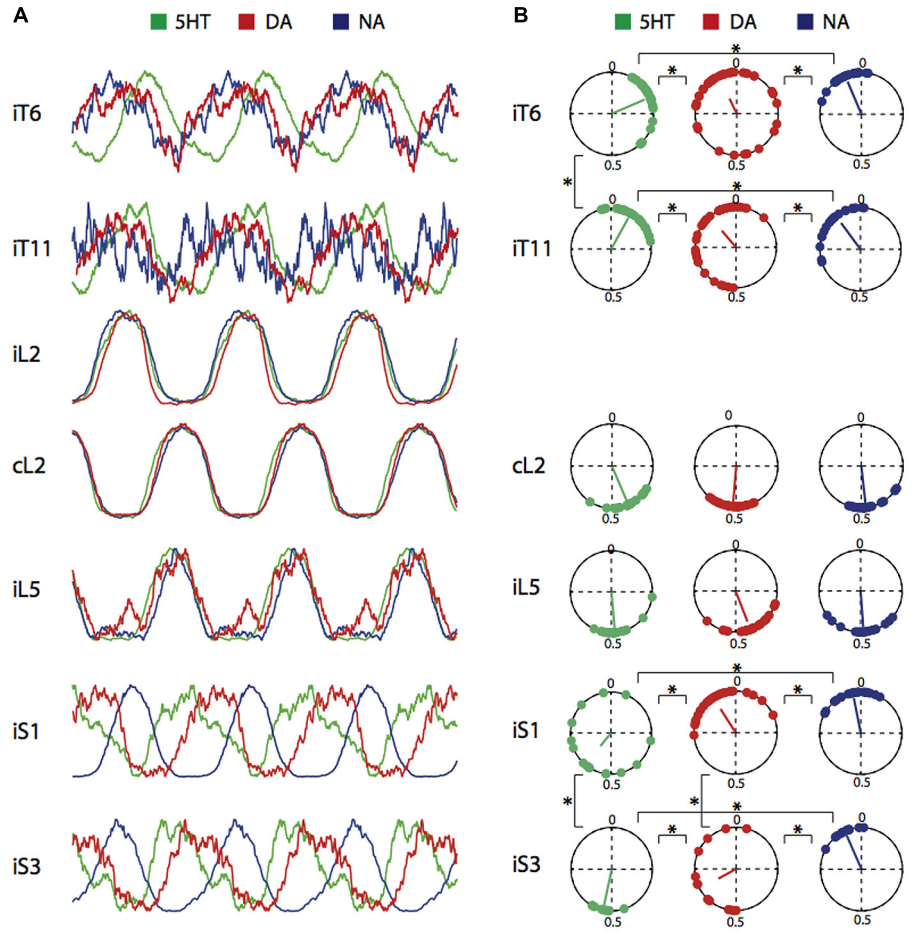

FIGURE 3 | Control of phase relationships by 5-HT, DA, or NA. **(A)** Averaged and replicated cycle sequences during locomotor-like activity induced by a combination of NMA (15 μM) and one of the three amines (5-HT, 15 μM; DA, 50 μM; NA, 50 μM). Bursts were normalized for the amplitude. Note phase differences relative to reference L2 bursts shift occurring in the thoracic and sacral segments. **(B)** Circular plots showing the mean phase values for burst activity in the six ventral roots recorded in A relative to the L2 ventral root in the presence of each three amines. Each point is the phase value measured from one experiment. The vector direction represents the grand mean phase value and its length is a function of the coupling strength. Data were pooled from all concentrations tested. i; ipsilateral; c, contralateral. $^*p < 0.05$.

as the first molecule to evoke locomotor-like episodes in the isolated neonatal rat spinal cord. Later on, we established 5-HT as one of the main actors in locomotor network activation in rodents (Cazalets et al., 1990, 1992), and discovered that the combined bath-application of 5-HT and EAA agonists was a very effective way to reliably elicit sustained episodes of locomotor-like activity (Sqalli-Houssaini et al., 1991, 1993). Since then, it has become the most widely used method to eliciting locomotor-like activity in this preparation, and hundreds of reports have been released based on this seminal observation. In contrast, only few studies have documented the combined activating action of EAA/DA or EAA/DA/5-HT (Kiehn and Kjaerulff, 1996; Jiang et al., 1999; Whelan et al., 2000; Juvin et al., 2005; Zhong et al., 2007; Talpalar and Kiehn, 2010), or EAA/NA (Kiehn et al., 1999; Sqalli-Houssaini and Cazalets, 2000), and in each case there was no clear experimental rational for employing this combined EAA/amine approach. Surprisingly, despite the outstanding opportunity provided by the use of an *in vitro* preparation, no study has systematically addressed the issue of combined drug actions and no comparative study of permissive neuromodulator interactions is available. Although some idea of the influences of these amines together can be gleaned indirectly from the literature, the disparity in protocols used (saline composition, temperature, rat strain,. . .) has prevented a direct comparison to be made. Furthermore in most studies, motor output from only the lumbar cord region was investigated.

An important prerequisite is to clearly define the limits and the terms of what can be considered as the "optimal" form of locomotor output *in vitro*. There is a general consensus in the field that spinally generated rhythmic motor activity is considered as "locomotor-like" when alternating flexor and extensor bursts are recorded extracellularly from the L2 and L5 ventral roots (Cazalets et al., 1992; Kiehn and Kjaerulff, 1996) and when the pattern cycle period falls within a range similar to that observed in the intact animal at the same age (1s; Cazalets et al., 1990; Jamon and Clarac, 1998). However, caveats in the respect include the

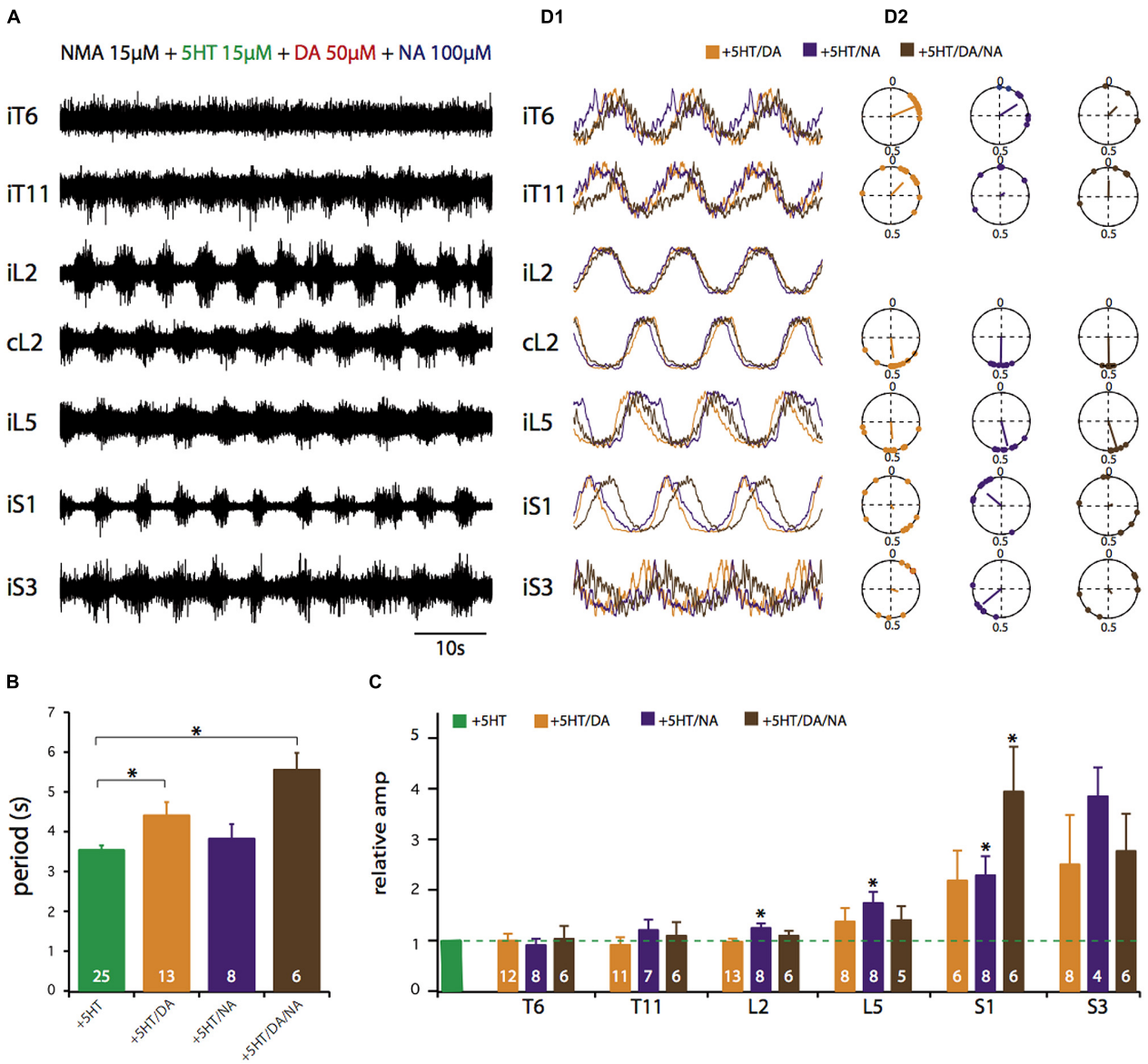

FIGURE 4 | Control of activity phase relationships by various combinations of NMA and 5-HT/DA/NA. (A) Simultaneous recordings of seven ventral roots in the presence of all four drugs. **(B)** Plots of mean cycle periods under various drug combinations. **(C)** Plots of mean relative amplitude changes normalized to the amplitude observed in the NMA/5-HT condition (green bar). **(D1)** Averaged and replicated cycles during locomotor-like activity induced by a combination of NMA (15 µM) and the three amines in various combinations. **(D2)** Circular plots calculated from the recordings in **D1** showing the mean phase values for the six ventral roots relative to L2 in the presence of NMA and the different amine cocktails. All data presented were obtained at the following drug concentrations: NMA, 15 µM; 5-HT, 15 µM; DA, 50 µM; NA, 50 µM. $*p < 0.05$.

effect of temperature on the *in vitro* rhythm frequency, since from 25°C (the typical mean room temperature used in most *in vitro* studies) to 35°C (the usual pup body temperature and the temperature at which *in vivo* experiments are performed (Cazalets et al., 1990), the cycle period decreases by more than 50% (Sqalli-Houssaini et al., 1991). A similar observation has been reported for saline K^+ concentration that is frequently twice the normal concentration (3 mM; Somjen, 1979) measured in the intact animal. As such, the "best" *in vitro* motor output pattern would have a cycle period of ∼2–3 s (when taking into account the slowing down of the motor period resulting from the isolated CNS conditions), with fictive locomotor episodes lasting for the duration of drug bath-application (i.e., without receptor desensitization or any other processes that would prematurely curtail rhythmogenesis) and ventral root bursts that, in a given drug condition, occur as regularly as possible and with little variation both in their amplitude and inter-root phase relationships. Based on these premises, **Table 1** summarizes the data collected in the present and previous studies. It includes data of thoracic, lumbar and sacral motor output and under exposure to various drug

Table 1 | Summary of the effects of 5-HT, DA and NA.

	Episode duration	Period (s)	Period variability (%)	Relative Amplitude			Presence of motor bursts (%)		
				Thoracic	Lumbar	Sacral	Thoracic	Lumbar	Sacral
NMA		3.04 ± 0.5							
NMA/5-HT	All bath-application	3.55 ± 0.10	9.30 ± 1.34	1	1	1	98	100	76
NMA/DA 50μM	All bath-application	3.47 ± 0.24	11.04 ± 1.17	0.66 ± 0.06	1.40 ± 0.18	1.54 ± 0.21	89	100	79
NMA/NA 50μM	All bath-application	3.8 ± 0.16	20.68 ± 2.76	1.18 ± 0.29	1.22 ± 0.17	2.68 ± 0.45	90	100	100
NMA/5-HT/DA	All bath-application	4.41 ± 0.32	10.38 ± 1.79	0.96 ± 0.10	0.97 ± 0.06	2.35 ± 0.57	85	100	78
NMA/5-HT/NA	All bath-application	3.82 ± 0.36	13.03 ± 1.00	1.04 ± 0.11	1.24 ± 0.09	2.81 ± 0.37	81	100	83
NMA/5-HT/DA/NA	All bath-application	5.56 ± 0.42	12.65 ± 3.40	1.06 ± 0.17	1.09 ± 0.09	3.35 ± 0.57	67	100	92

Note that the category "episode duration" indicates that a given neuromodulator cocktail induced rhythmic motor activity throughout bath-application.

Table 2 | Phase and mean vector length (*r*) value for burst activity in the six ventral roots (Figure 4D2) relative to the L2 ventral root in the presence of various combinations of NMA and 5-HT/DA/NA.

	iT6	iT11	cL2	iL5	iS1	iS3
	Phase (*n*) R	Phase R	Phase R	Phase R	Phase R	Phase R
NMA/5-HT	0.19 (31) 0.86	0.08 (31) 0.88	0.43 (28) 0.93	0.49 (18) 0.91	0.61 (15) 0.41	0.53 (9) 0.92
NMA/DA	0.93 (39) 0.40	0.89 (45) 0.54	0.51 (47) 0.94	0.44 (38) 0.80	0.91 (25) 0.67	0.67 (13) 0.49
NMA/NA	0.94 (21) 0.85	0.90 (23) 0.794	0.48 (26) 0.88	0.49 (23) 0.87	0.97 (21) 0.86	0.93 (13) 0.89
NMA/5-HT/DA	0.19 (11) 0.90	0.12 (12) 0.63	0.48 (12) 0.71	0.49 (10) 0.61	0.41 (8) 0.18	0.30 (6) 0.27
NMA/5-HT/NA	0.16 (5) 0.74	0.16 (8) 0.19	0.50 (8) 0.92	0.46 (8) 0.89	0.86 (8) 0.66	0.64 (5) 0.84
NMA/5-HT/DA/NA	0.12 (3) 0.42	0.00 (5) 0.56	0.50 (6) 0.97	0.45 (5) 0.95	0.19 (6) 0.16	0.40 (6) 0.21

combinations. A comparison of the compiled values indicates that there is no optimal way to elicit locomotor-like activity *in vitro* since several neurotransmitter combinations are able to elicit stable rhythmicity with *in vitro* locomotor compatible periods and phase-relationships. For example, motor burst amplitude is evidently more sensitive to NA, whereas rhythm stability is provided by DA or 5-HT. Presumably such differences indicate that the various parameters of the spinal motor system can be separately adjusted according to changing behavioral requirements. Nevertheless, it remains apparent that the combination of EAA/5-HT constitutes a good compromise in terms of rhythm stability, burst amplitude, and the recruitment of the different spinal compartments. However, specifically for sacral activity, it would be perhaps more interesting to use NA or DA which elicit higher burst amplitudes. To date it is not possible to determine the location at which the neuromodulatory processes described here may occur. With increasing age, there is a decrease in the motor period variability in the intact animal (Cazalets et al., 1990). Both the bath-application technique used here and the developmental changes undergone by neuromodulatory pathways themselves do not allow us to draw any conclusion about the precise functional role played by each amine in the intact adult animal.

Rhythmic motor activity elicited by the conjoint bath-application of EAA agonists and the three amines separately or in combination always led to locomotor pattern genesis (**Figures 1** and **4**). Our previous studies have already described the pharmacological action of the three amines and how they modulate NMA-induced locomotor-like activity (Sqalli-Houssaini et al., 1993; Sqalli-Houssaini and Cazalets, 2000; Barriere et al., 2004). In the present study, the same NMA concentration was the common parameter for all conditions, thus allowing direct comparison to be made between the different amine actions. Of the three amines, however, only 5-HT triggered activity that corresponded most closely to actual locomotion in terms of cycle period and inter-segmental phase relationships. In contrast, neither NA nor DA alone evokes locomotor-compatible rhythms, the latter in terms of cycle periods (Kiehn and Kjaerulff, 1996; Barriere et al., 2004) and the former in terms of cycle periods and flexor/extensor phase relationships (Kiehn et al., 1999; Sqalli-Houssaini and Cazalets, 2000; Gabbay and Lev-Tov, 2004). On this basis, therefore, it could be that the locomotor signature of spinal output rhythmicity is provided mainly by EAAs since the activity they elicit possess the closest parameters to the cycle periods and flexor and extensor alternation observed *in vivo* (Kudo and Yamada, 1987; Cazalets et al., 1992). In such a framework, EAAs could establish basal temporal features of the motor rhythm with a cycle period that varies according to the amount of endogenous EAA released (Kudo and Yamada, 1987; Cazalets et al., 1992; Beato et al., 1997; Talpalar and Kiehn, 2010). During real CPG operation, the main task of the amines would

be to modulate this "primary" EAA-induced pattern via their combinatorial release.

To date, the cellular mechanisms by which the amines exert their effects remain unclear, although undoubtedly they will be multiple and complex (Harris-Warrick, 2011). It is likely that each parameter set by an amine – motor burst period, amplitude and stability – involves various cellular targets. For example, all three amines strengthen burst activity, i.e. they increase the amplitude and regularity of bursting by increasing neuronal excitability at the motoneuronal and/or premotoneuronal levels (Berger and Takahashi, 1990; Sqalli-Houssaini and Cazalets, 2000; Kjaerulff and Kiehn, 2001; Gabbay et al., 2002; Zhong et al., 2006b; Han et al., 2007).

Their dose-dependent action on rhythm cycle period (Cazalets et al., 1992; Sqalli-Houssaini and Cazalets, 2000) suggests that the amines directly access components of the spinal CPG itself. Moreover, since EAAs and amines individually increase neuronal excitability, it could be expected that the period of the motor rhythm induced by their combined presence would be even shorter than when each compound is acting separately. This was not the case, and indeed the cycle period was found to be set at an intermediate value between that evoked by EAA or the amine alone (**Figure 1**; **Table 1**). Pharmacological data provide a partial explanation for the aminergic effect on cycle period, since as mentioned previously, both NA and 5-HT slow down the locomotor rhythm through an inhibitory action via α2 receptors (Sqalli-Houssaini and Cazalets, 2000; Gabbay and Lev-Tov, 2004), and 5-HT1 (Beato and Nistri, 1998), while α1, 5-HT7, and 5-HT2 receptor agonists have an activatory action on pattern generation (Cazalets et al., 1992; Sqalli-Houssaini and Cazalets, 2000; Madriaga et al., 2004; Landry et al., 2006; Liu et al., 2009). Surprisingly, however, DA which has been reported solely to have activatory effects also slows down the EAA-induced spinal motor rhythm (**Figure 1**; Barriere et al., 2004). Therefore, it is likely that several underlying processes are operating simultaneously, as reported in other preparations (Harris-Warrick, 2011; Miles and Sillar, 2011; Clemens et al., 2012). For example, in the lamprey the serotonin-induced prolongation of motor burst duration and cycle period involves at least two mechanisms, presynaptic inhibition of glutamate release on CPG neurons (Schwartz et al., 2005) and post-synaptic blockade of sAHP (Wallen et al., 1989; El Manira et al., 1994).

AMINERGIC SHAPING OF THE SPINAL MOTOR PATTERN

The physiology of locomotion in quadrupeds relies mostly on a simplified dichotomy between dynamic locomotor activity in which only hindlimb movements are considered and postural activity during static tasks. The main characteristics of locomotor-like rhythmic activity recorded in the *in vitro* spinal cord preparation essentially consist of burst cycle period, alternating left/right and flexor/extensor phase relationships, and burst duration. We found here that these parameters were amazingly constant whatever the neuromodulatory condition (**Figures 1** and **4**; **Table 2**). This lack of flexibility may reflect a real functional situation since during ongoing locomotion there is an absolute requirement for fundamental parameters such as right/left movement alternation to be strictly maintained on order preserve balance and speed. Alternatively, the apparent lack of modulator-induced flexibility may be attributable to an *in vitro* bias. In the isolated spinal cord preparation, simultaneous ventral root recordings provide only a global view of various temporally overlapping motor activities and, for example, the slightly differing temporal relationships that exist between various muscle groups, as has been observed with electromyographic recordings in the intact animal (Leblond et al., 2003), may not be evident.

In several systems, interactions between distinct rhythmically active networks have been also described, as for example between walking and swimmeret movements in crustaceans (Cattaert and Clarac, 1983; Chrachri and Neil, 1993), gastric and pyloric motor rhythms in the crustacean stomatogastric system (Bartos et al., 1999; Faumont et al., 2005), trunk and hindlimb activities in Xenopus (Beyeler et al., 2008; Rauscent et al., 2009), locomotion and respiration (Kawahara et al., 1989; Morin and Viala, 2002), scratching and locomotion (Hao et al., 2011), respiration and swallowing (Gestreau et al., 2000). In the newt, axial neuronal networks switch from sequential motor burst propagation to stationary phase-locked activation of the same axial muscles when the animal switches from swimming to quadrupedal locomotion (Delvolve et al., 1997). To date, however, the link between the systems level and the underlying cellular mechanisms has only been established in simpler invertebrate systems in which identified neurons can be individually activated and recorded (Cang and Friesen, 2002; Smarandache et al., 2009). Indeed the pharmacological approach that we have used in the present study also has some limitations, especially when considering the temporal aspects of neuromodulation. During functioning in the intact animal it is likely that descending aminergic pathways are momentarily active whereas the pharmacological protocol used here necessarily consists of tonic, continuous bath-application that in turn leads to the disappearance of the temporal dimension of neuromodulation. A second limitation is the state dependence of the system when several bath-applications are performed on the same spinal cord. Indeed, for the NMA/5-HT combination, we observed in a previous study that successive applications elicited sequences of locomotor-like activity with the same characteristics (Cazalets et al., 1999). Exogenous DA has been shown in other systems to lead to persistent changes in ionic conductances (Rodgers et al., 2011; Krenz et al., 2014), and we also reported in the isolated spinal cord preparation (see Materials and Methods, Barriere et al., 2004) that it may exert long-lasting effects. It is for this reason that DA was always the last drug tested in our experiments and at a much lower concentration than that at which these long-lasting effects were previously observed. There is no evidence that NA exerts such persistent actions.

When considering the integrated functioning of hindlimb and axial networks involved in dynamic posture, a different perspective is required since as we demonstrate, this «metanetwork» can be configured into various functional states according to the neuromodulatory environment. As shown in **Figures 1** and **2**, although the amines and EAAs have a qualitatively cumulative effect on rhythm stability and burst amplitude, each amine has its own characteristic imprint. DA and NA specifically enhance sacral motor bursts (**Figure 2**) while 5-HT and DA better stabilize the temporal parameters of the motor pattern (**Figure 1**). Each amine can also specify distinct phase relationships between the various spinal

segments (**Figure 3**). In a previous study, in which only the action of a mixture of NMA/5-HT on the interconnected spinal networks was investigated, we found a constant lag between lumbar and thoracic segments, with a significant tendency for caudo-rostral propagation (Falgairolle and Cazalets, 2007). Here, we find that DA and NA, in contrast to 5-HT's action, substantially decreases the phase lags along the cord so that almost synchronous bursting occurs throughout the spinal cord. Changes from rostro-caudal to caudo-rostral propagation are also observed in the lamprey, when switching from forward to backward swimming occurs (Islam et al., 2006). Although, the cellular targets have not been identified, spinal commissural interneurons could be one possible target as they are known to be modulated by 5-HT (Zhong et al., 2006a), and since we previously established in the neonatal rat that a partial or complete hemicord section suppressed the phase delays between sacral segments (Falgairolle and Cazalets, 2007).

In conclusion, the present paper provides new data on the coordinating processes between spinal cord networks. We demonstrate that each of three amines 5-HT, DA, and NA, when associated with EAA receptor agonists elicits a specific locomotor pattern whose temporal characteristics, motor burst amplitude and intersegmental phase relationships have a distinct signature. These differences are likely to be related to changing behavioral requirements. Furthermore, we find here that the various amines acting in combination do not elicit more robust locomotor-like activity than when bath-applied alone (i.e., more is not better!). The aminergic neuromodulators thus represent an important potential source of adaptive flexibility at the level of the underlying central neuronal networks and the motor output patterns they produce.

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REFERENCES

Akazawa, K., Aldridge, J. W., Steeves, J. D., and Stein, R. B. (1982). Modulation of stretch reflexes during locomotion in the mesencephalic cat. *J. Physiol.* 329, 553–567.

Ballion, B., Morin, D., and Viala, D. (2001). Forelimb locomotor generators and quadrupedal locomotion in the neonatal rat. *Eur. J. Neurosci.* 14, 1727–1738. doi: 10.1046/j.0953-816x.2001.01794.x

Barriere, G., Mellen, N., and Cazalets, J.-R. (2004). Neuromodulation of the locomotor network by dopamine in the isolated spinal cord of newborn rat. *Eur. J. Neurosci.* 19, 1325–1335. doi: 10.1111/j.1460-9568.2004.03210.x

Bartos, M., Manor, Y., Nadim, F., Marder, E., and Nusbaum, M. P. (1999). Coordination of fast and slow rhythmic neuronal circuits. *J. Neurosci.* 19, 6650–6660.

Beato, M., Bracci, E., and Nistri, A. (1997). Contribution of NMDA and non-NMDA glutamate receptors to locomotor pattern generation in the neonatal rat spinal cord. *Proc. Biol. Sci.* 264, 877–884. doi: 10.1098/rspb.1997.0122

Beato, M., and Nistri, A. (1998). Serotonin-induced inhibition of locomotor rhythm of the rat isolated spinal cord is mediated by the 5-HT1 receptor class. *Proc. Biol. Sci.* 265, 2073–2080. doi: 10.1098/rspb.1998.0542

Berens, P. (2009). CircStat: a MATLAB toolbox for circular statistics. *J. Stat. Softw.* 31, 1–21.

Berger, A. J., and Takahashi, T. (1990). Serotonin enhances a low-voltage-activated calcium current in rat spinal motoneurons. *J. Neurosci.* 10, 1922–1928.

Beyeler, A., Metais, C., Combes, D., Simmers, J., and Le Ray, D. (2008). Metamorphosis-induced changes in the coupling of spinal thoraco-lumbar motor outputs during swimming in *Xenopus laevis*. *J. Neurophysiol.* 100, 1372–1383. doi: 10.1152/jn.00023.2008

Cang, J., and Friesen, W. O. (2002). Model for intersegmental coordination of leech swimming: central and sensory mechanisms. *J. Neurophysiol.* 87, 2760–2769.

Cattaert, D., and Clarac, F. (1983). Influence of walking on swimmeret beating in the lobster *Homarus gammarus*. *J. Neurobiol.* 14, 421–439. doi: 10.1002/neu.480140603

Cazalets, J. R. (2000). "Organization of the spinal locomotor network in neonatal rat," in *Neurobiology of Spinal Cord Injury*, eds R. Kalb, and S. M. Stritmatter (Totowa, NJ: Humana Press Inc.), 89–111. doi: 10.1007/978-1-59259-200-5_4

Cazalets, J. R., Grillner, P., Menard, I., Cremieux, J., and Clarac, F. (1990). Two types of motor rhythm induced by NMDA and amines in an in vitro spinal cord preparation of neonatal rat. *Neurosci. Lett.* 111, 116–121. doi: 10.1016/0304-3940(90)90354-C

Cazalets, J. R., Sqalli-Houssaini, Y., and Clarac, F. (1992). Activation of the central pattern generators for locomotion by serotonin and excitatory amino acids in neonatal rat. *J. Physiol.* 455, 187–204.

Cazalets, J. R., Sqalli-Houssaini, Y., and Magoul, R. (1999). Differential effects of potassium channel blockers on the activity of the locomotor network in neonatal rat. *Brain Res.* 827, 185–197. doi: 10.1016/S0006-8993(99)01342-6

Chrachri, A., and Neil, D. M. (1993). Interaction and synchronization between two abdominal motor systems in crayfish. *J. Neurophysiol.* 69, 1373–1383.

Clemens, S., Belin-Rauscent, A., Simmers, J., and Combes, D. (2012). Opposing modulatory effects of D1- and D2-like receptor activation on a spinal central pattern generator. *J. Neurophysiol.* 107, 2250–2259. doi: 10.1152/jn.00366.2011

Combes, D., Sillar, K. T., and Simmers, J. (2012). A switch in aminergic modulation of locomotor CPG output during amphibian metamorphosis. *Front. Biosci. (Schol. Ed.)* 4:1364–1374.

Delvolve, I., Bem, T., and Cabelguen, J. M. (1997). Epaxial and limb muscle activity during swimming and terrestrial stepping in the adult newt, *Pleurodeles waltl*. *J. Neurophysiol.* 78, 638–650.

De Sèze, M., Falgairolle, M., Viel, S., Assaiante, C., and Cazalets, J.-R. (2008). Sequential activation of axial muscles during different forms of rhythmic behavior in man. *Exp. Brain Res.* 185, 237–247. doi: 10.1007/s00221-007-1146-2

Dietz, V. (2002). Do human bipeds use quadrupedal coordination? *Trends Neurosci.* 25, 462–467. doi: 10.1016/S0166-2236(02)02229-4

Duenas, S. H., Loeb, G. E., and Marks, W. B. (1990). Monosynaptic and dorsal root reflexes during locomotion in normal and thalamic cats. *J. Neurophysiol.* 63, 1467–1476.

Duysens, J., and Van de Crommert, H. W. (1998). Neural control of locomotion: the central pattern generator from cats to humans. *Gait Posture* 7, 131–141. doi: 10.1016/S0966-6362(97)00042-8

El Manira, A., Tegner, J., and Grillner, S. (1994). Calcium-dependent potassium channels play a critical role for burst termination in the locomotor network in lamprey. *J. Neurophysiol.* 72, 1852–1861.

Falgairolle, M., and Cazalets, J. R. (2007). Metachronal coupling between spinal neuronal networks during locomotor activity in newborn rat. *J. Physiol.* 580, 87–102. doi: 10.1113/jphysiol.2006.115709

Falgairolle, M., Ceccato, J.-C., Seze, M. D., Herbin, M., and Cazalets, J.-R. (2013). Metachronal propagation of motor activity. *Front. Biosci. (Landmark Ed.)* 18:820–837.

Falgairolle, M., De Seze, M., Juvin, L., Morin, D., and Cazalets, J. R. (2006). Coordinated network functioning in the spinal cord: an evolutionary perspective. *J. Physiol. Paris* 100, 304–316. doi: 10.1016/j.jphysparis.2007.05.003

Faumont, S., Combes, D., Meyrand, P., and Simmers, J. (2005). Reconfiguration of multiple motor networks by short- and long-term actions of an identified modulatory neuron. *Eur. J. Neurosci.* 22, 2489–2502. doi: 10.1111/j.1460-9568.2005.04442.x

Gabbay, H., Delvolvé, I., and Lev-Tov, A. (2002). Pattern generation in caudal-lumbar and sacrococcygeal segments of the neonatal rat spinal cord. *J. Neurophysiol.* 88, 732–739.

Gabbay, H., and Lev-Tov, A. (2004). Alpha-1 adrenoceptor agonists generate a "fast" NMDA receptor-independent motor rhythm in the neonatal rat spinal cord. *J. Neurophysiol.* 92, 997–1010. doi: 10.1152/jn.00205.2004

Gestreau, C., Grelot, L., and Bianchi, A. L. (2000). Activity of respiratory laryngeal motoneurons during fictive coughing and swallowing. *Exp. Brain Res.* 130, 27–34. doi: 10.1007/s002210050003

Gordon, I. T., and Whelan, P. J. (2006). Monoaminergic control of cauda-equina-evoked locomotion in the neonatal mouse spinal cord. *J. Neurophysiol.* 96, 3122–3129. doi: 10.1152/jn.00606.2006

Gramsbergen, A., Geisler, H. C., Taekema, H., and Van Eykern, L. A. (1999). The activation of back muscles during locomotion in the developing rat. *Brain Res. Dev. Brain Res.* 112, 217–228. doi: 10.1016/S0165-3806(98)00184-9

Grinsted, A., Moore, J. C., and Jevrejeva, S. (2004). NPG – Abstract – application of the cross wavelet transform and wavelet coherence to geophysical time series. *Nonlinear Process. Geophys.* 11, 561–566. doi: 10.5194/npg-11-561-2004

Guertin, P. A. (2009). Recovery of locomotor function with combinatory drug treatments designed to synergistically activate specific neuronal networks. *Curr. Med. Chem.* 16, 1366–1371. doi: 10.2174/092986709787846541

Han, P., Nakanishi, S. T., Tran, M. A., and Whelan, P. J. (2007). Dopaminergic modulation of spinal neuronal excitability. *J. Neurosci.* 27, 13192–13204. doi: 10.1523/JNEUROSCI.1279-07.2007

Hao, Z. Z., Spardy, L. E., Nguyen, E. B., Rubin, J. E., and Berkowitz, A. (2011). Strong interactions between spinal cord networks for locomotion and scratching. *J. Neurophysiol.* 106, 1766–1781. doi: 10.1152/jn.00460.2011

Harris-Warrick, R. M. (2011). Neuromodulation and flexibility in central pattern generator networks. *Curr. Opin. Neurobiol.* 21, 685–692. doi: 10.1016/j.conb.2011.05.011

Islam, S. S., Zelenin, P. V., Orlovsky, G. N., Grillner, S., and Deliagina, T. G. (2006). Pattern of motor coordination underlying backward swimming in the lamprey. *J. Neurophysiol.* 96, 451–460. doi: 10.1152/jn.01277.2005

Jamon, M., and Clarac, F. (1998). Early walking in the neonatal rat: a kinematic study. *Behav. Neurosci.* 112, 1218–1228. doi: 10.1037/0735-7044.112.5.1218

Jiang, Z., Carlin, K. P., and Brownstone, R. M. (1999). An in vitro functionally mature mouse spinal cord preparation for the study of spinal motor networks. *Brain Res.* 816, 493–499. doi: 10.1016/S0006-8993(98)01199-8

Juvin, L., Simmers, J., and Morin, D. (2005). Propriospinal circuitry underlying interlimb coordination in mammalian quadrupedal locomotion. *J. Neurosci.* 25, 6025–6035. doi: 10.1523/JNEUROSCI.0696-05.2005

Kawahara, K., Nakazono, Y., Yamauchi, Y., and Miyamoto, Y. (1989). Coupling between respiratory and locomotor rhythms during fictive locomotion in decerebrate cats. *Neurosci. Lett.* 103, 326–330. doi: 10.1016/0304-3940(89)90121-3

Kiehn, O., and Kjaerulff, O. (1996). Spatiotemporal characteristics of 5-HT and dopamine-induced rhythmic hindlimb activity in the in vitro neonatal rat. *J. Neurophysiol.* 75, 1472–1482.

Kiehn, O., Sillar, K. T., Kjaerulff, O., and Mcdearmid, J. R. (1999). Effects of noradrenaline on locomotor rhythm-generating networks in the isolated neonatal rat spinal cord. *J. Neurophysiol.* 82, 741–746.

Kjaerulff, O., and Kiehn, O. (2001). 5-HT modulation of multiple inward rectifiers in motoneurons in intact preparations of the neonatal rat spinal cord. *J. Neurophysiol.* 85, 580–593.

Koehler, W. J., Schomburg, E. D., and Steffens, H. (1984). Phasic modulation of trunk muscle efferents during fictive spinal locomotion in cats. *J. Physiol.* 353, 187–197.

Krenz, W. D., Parker, A. R., Rodgers, E. W., and Baro, D. J. (2014). Dopaminergic tone persistently regulates voltage-gated ion current densities through the D1R-PKA axis, RNA polymerase II transcription, RNAi, mTORC1, and translation. *Front. Cell. Neurosci.* 8:39. doi: 10.3389/fncel.2014.00039

Kudo, N., and Yamada, T. (1987). N-methyl-D,L-aspartate-induced locomotor activity in a spinal cord-hindlimb muscles preparation of the newborn rat studied in vitro. *Neurosci. Lett.* 75, 43–48. doi: 10.1016/0304-3940(87)90072-3

Landry, E. S., Lapointe, N. P., Rouillard, C., Levesque, D., Hedlund, P. B., and Guertin, P. A. (2006). Contribution of spinal 5-HT1A and 5-HT7 receptors to locomotor-like movement induced by 8-OH-DPAT in spinal cord-transected mice. *Eur. J. Neurosci.* 24, 535–546. doi: 10.1111/j.1460-9568.2006.04917.x

Lapointe, N. P., and Guertin, P. A. (2008). Synergistic effects of D1/5 and 5-HT1A/7 receptor agonists on locomotor movement induction in complete spinal cord-transected mice. *J. Neurophysiol.* 100, 160–168. doi: 10.1152/jn.90339.2008

Lapointe, N. P., Rouleau, P., Ung, R. V., and Guertin, P. A. (2009). Specific role of dopamine D1 receptors in spinal network activation and rhythmic movement induction in vertebrates. *J. Physiol.* 587, 1499–1511. doi: 10.1113/jphysiol.2008.166314

Lapointe, N. P., Ung, R. V., Rouleau, P., and Guertin, P. A. (2008). Effects of spinal alpha(2)-adrenoceptor and I(1)-imidazoline receptor activation on hindlimb movement induction in spinal cord-injured mice. *J. Pharmacol. Exp. Ther.* 325, 994–1006. doi: 10.1124/jpet.107.134874

Leblond, H., L'esperance, M., Orsal, D., and Rossignol, S. (2003). Treadmill locomotion in the intact and spinal mouse. *J. Neurosci.* 23, 11411–11419.

Liu, J., Akay, T., Hedlund, P. B., Pearson, K. G., and Jordan, L. M. (2009). Spinal 5-HT7 receptors are critical for alternating activity during locomotion: in vitro neonatal and in vivo adult studies using 5-HT7 receptor knockout mice. *J. Neurophysiol.* 102, 337–348. doi: 10.1152/jn.91239.2008

Maclellan, M. J., Ivanenko, Y. P., Cappellini, G., Sylos Labini, F., and Lacquaniti, F. (2011). Features of hand-foot crawling behavior in human adults. *J. Neurophysiol.* 107, 114–125. doi: 10.1152/jn.00693.2011

Madriaga, M. A., Mcphee, L. C., Chersa, T., Christie, K. J., and Whelan, P. J. (2004). Modulation of locomotor activity by multiple 5-HT and dopaminergic receptor subtypes in the neonatal mouse spinal cord. *J. Neurophysiol.* 92, 1566–1576. doi: 10.1152/jn.01181.2003

Miles, G. B., and Sillar, K. T. (2011). Neuromodulation of vertebrate locomotor control networks. *Physiology (Bethesda)* 26, 393–411. doi: 10.1152/physiol.00013.2011

Mor, Y., and Lev-Tov, A. (2007). Analysis of rhythmic patterns produced by spinal neural networks. *J. Neurophysiol.* 98, 2807–2817. doi: 10.1152/jn.00740.2007

Morin, D., and Viala, D. (2002). Coordinations of locomotor and respiratory rhythms in vitro are critically dependent on hindlimb sensory inputs. *J. Neurosci.* 22, 4756–4765.

Nakajima, T., Mezzarane, R. A., Klarner, T., Barss, T. S., Hundza, S. R., Komiyama, T., et al. (2013). Neural mechanisms influencing interlimb coordination during locomotion in humans: presynaptic modulation of forearm H-reflexes during leg cycling. *PLoS ONE* 8:e76313. doi: 10.1371/journal.pone.0076313

Pearlstein, E., Ben Mabrouk, F., Pflieger, J. F., and Vinay, L. (2005). Serotonin refines the locomotor-related alternations in the in vitro neonatal rat spinal cord. *Eur. J. Neurosci.* 21, 1338–1346. doi: 10.1111/j.1460-9568.2005.03971.x

Rauscent, A., Einum, J., Le Ray, D., Simmers, J., and Combes, D. (2009). Opposing aminergic modulation of distinct spinal locomotor circuits and their functional coupling during amphibian metamorphosis. *J. Neurosci.* 29, 1163–1174. doi: 10.1523/JNEUROSCI.5255-08.2009

Rodgers, E. W., Krenz, W. D., and Baro, D. J. (2011). Tonic dopamine induces persistent changes in the transient potassium current through translational regulation. *J. Neurosci.* 31, 13046–13056. doi: 10.1523/JNEUROSCI.2194-11.2011

Rossignol, S. (1996). "Neural control of stereotypic limb movements," in *Handbook of Physiology, Section 12. Exercise: Regulation and Integration of Multiple Systems*, eds B. Rowell and J. T. Sheperd (Bethesda, MD: American Physiological Society), 173–216.

Schwartz, E. J., Gerachshenko, T., and Alford, S. (2005). 5-HT prolongs ventral root bursting via presynaptic inhibition of synaptic activity during fictive locomotion in lamprey. *J. Neurophysiol.* 93, 980–988. doi: 10.1152/jn.00669.2004

Smarandache, C., Hall, W. M., and Mulloney, B. (2009). Coordination of rhythmic motor activity by gradients of synaptic strength in a neural circuit that couples modular neural oscillators. *J. Neurosci.* 29, 9351–9360. doi: 10.1523/JNEUROSCI.1744-09.2009

Smith, J. C., and Feldman, J. L. (1987). In vitro brainstem-spinal cord preparations for study of motor systems for mammalian respiration and locomotion. *J. Neurosci. Methods* 21, 321–333. doi: 10.1016/0165-0270(87)90126-9

Somjen, G. G. (1979). Extracellular potassium in the mammalian central nervous system. *Annu. Rev. Physiol.* 41, 159–177. doi: 10.1146/annurev.ph.41.030179.001111

Sqalli-Houssaini, Y., and Cazalets, J. R. (2000). Noradrenergic control of locomotor networks in the in vitro spinal cord of the neonatal rat. *Brain Res.* 852, 100–109. doi: 10.1016/S0006-8993(99)02219-2

Sqalli-Houssaini, Y., Cazalets, J. R., and Clarac, F. (1993). Oscillatory properties of the central pattern generator for locomotion in neonatal rats. *J. Neurophysiol.* 70, 803–813.

Sqalli-Houssaini, Y., Cazalets, J. R., Fabre, J. C., and Clarac, F. (1991). A cooling/heating system for use with in vitro preparations: study of temperature effects on newborn rat rhythmic activities. *J. Neurosci. Methods* 39, 131–139. doi: 10.1016/0165-0270(91)90079-F

Talpalar, A. E., and Kiehn, O. (2010). Glutamatergic mechanisms for speed control and network operation in the rodent locomotor CPG. *Front. Neural Circuits* 4:19. doi: 10.3389/fncir.2010.00019

Thorstensson, A., Carlson, H., Zomlefer, M. R., and Nilsson, J. (1982). Lumbar back muscle activity in relation to trunk movements during locomotion in man. *Acta Physiol. Scand.* 116, 13–20. doi: 10.1111/j.1748-1716.1982.tb10593.x

Torrence, C., and Compo, G. P. (2010). A practical guide to wavelet analysis. *Bull. Am. Meteorol. Soc.* 79, 61–78. doi: 10.1175/1520-0477(1998)079<0061:APGTWA>2.0.CO;2

Wallen, P., Christenson, J., Brodin, L., Hill, R., Lansner, A., and Grillner, S. (1989). Mechanisms underlying the serotonergic modulation of the spinal circuitry for locomotion in lamprey. *Prog. Brain Res.* 80, 321–327; discussion 315–329. doi: 10.1016/S0079-6123(08)62227-X

Wannier, T., Bastiaanse, C., Colombo, G., and Dietz, V. (2001). Arm to leg coordination in humans during walking, creeping and swimming activities. *Exp. Brain Res.* 141, 375–379. doi: 10.1007/s002210100875

Whelan, P., Bonnot, A., and O'donovan, M. J. (2000). Properties of rhythmic activity generated by the isolated spinal cord of the neonatal mouse. *J. Neurophysiol.* 84, 2821–2833.

Zehr, E. P., and Duysens, J. (2004). Regulation of arm and leg movement during human locomotion. *Neuroscientist* 10, 347–361. doi: 10.1177/1073858404264680

Zhong, G., Diaz-Rios, M., and Harris-Warrick, R. M. (2006a). Intrinsic and functional differences among commissural interneurons during fictive locomotion and serotonergic modulation in the neonatal mouse. *J. Neurosci.* 26, 6509–6517. doi: 10.1523/JNEUROSCI.1410-06.2006

Zhong, G., Diaz-Rios, M., and Harris-Warrick, R. M. (2006b). Serotonin modulates the properties of ascending commissural interneurons in the neonatal mouse spinal cord. *J. Neurophysiol.* 95, 1545–1555. doi: 10.1152/jn.01103.2005

Zhong, G., Masino, M. A., and Harris-Warrick, R. M. (2007). Persistent sodium currents participate in fictive locomotion generation in neonatal mouse spinal cord. *J. Neurosci.* 27, 4507–4518. doi: 10.1523/JNEUROSCI.0124-07.2007

Zomlefer, M. R., Provencher, J., Blanchette, G., and Rossignol, S. (1984). Electromyographic study of lumbar back muscles during locomotion in acute high decerebrate and in low spinal cats. *Brain Res.* 290, 249–260. doi: 10.1016/0006-8993(84)90942-9

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5-HT_2 and 5-HT_7 receptor agonists facilitate plantar stepping in chronic spinal rats through actions on different populations of spinal neurons

Urszula Sławińska[1]*, Krzysztof Miazga[1] and Larry M. Jordan[2]*

[1] *Department of Neurophysiology, Nencki Institute of Experimental Biology PAS, Warsaw, Poland*
[2] *Department of Physiology, Spinal Cord Research Centre, University of Manitoba, Winnipeg, MB, Canada*

Edited by:
Shawn Hochman, Emory University, USA

Reviewed by:
Patrick John Whelan, University of Calgary, Canada
Heather Hayes, Emory University, USA

****Correspondence:***
Urszula Sławińska, Department of Neurophysiology, Nencki Institute of Experimental Biology PAS, 3 Pasteur Str., 02-093 Warsaw, Poland
e-mail: u.slawinska@nencki.gov.pl;
Larry M. Jordan, Department of Physiology, Spinal Cord Research Centre, University of Manitoba, 745 Bannatyne Avenue, Room No. 425, Basic Medical Sciences Building, Winnipeg, MB R3E 0J9, Canada
e-mail: larry@scrc.umanitoba.ca

There is considerable evidence from research in neonatal and adult rat and mouse preparations to warrant the conclusion that activation of 5-HT_2 and 5-$HT_{1A/7}$ receptors leads to activation of the spinal cord circuitry for locomotion. These receptors are involved in control of locomotor movements, but it is not clear how they are implicated in the responses to 5-HT agonists observed after spinal cord injury. Here we used agonists that are efficient in promoting locomotor recovery in paraplegic rats, 8-hydroxy-2-(di-n-propylamino)-tetralin (8-OHDPAT) (acting on 5-$HT_{1A/7}$ receptors) and quipazine (acting on 5-HT_2 receptors), to examine this issue. Analysis of intra- and interlimb coordination confirmed that the locomotor performance was significantly improved by either drug, but the data revealed marked differences in their mode of action. Interlimb coordination was significantly better after 8-OHDPAT application, and the activity of the extensor soleus muscle was significantly longer during the stance phase of locomotor movements enhanced by quipazine. Our results show that activation of both receptors facilitates locomotion, but their effects are likely exerted on different populations of spinal neurons. Activation of 5-HT_2 receptors facilitates the output stage of the locomotor system, in part by directly activating motoneurons, and also through activation of interneurons of the locomotor central pattern generator (CPG). Activation of 5-$HT_{7/1A}$ receptors facilitates the activity of the locomotor CPG, without direct actions on the output components of the locomotor system, including motoneurons. Although our findings show that the combined use of these two drugs results in production of well-coordinated weight supported locomotion with a reduced need for exteroceptive stimulation, they also indicate that there might be some limitations to the utility of combined treatment. Sensory feedback and some intraspinal circuitry recruited by the drugs can conflict with the locomotor activation.

Keywords: locomotion, recovery, spinal cord, total transection, serotonin

INTRODUCTION

During the last few decades the potential role of 5-HT in locomotor system activation, modulation and functional recovery after spinal cord lesions has received considerable attention and continues to be an area of major interest and investigation (reviewed in Hochman et al., 2001; Jordan and Schmidt, 2002; Orsal et al., 2002; Jordan and Sławińska, 2011). Using the *in vitro* neonatal rat spinal cord preparation it was demonstrated that 5-HT application induces locomotor-like discharge of lumbar ventral roots (Kudo and Yamada, 1987; Cazalets et al., 1990, 1992; Sqalli-Houssaini et al., 1993; Cowley and Schmidt, 1994; Kiehn and Kjaerulff, 1996), the frequency of which was concentration-dependent (Cazalets et al., 1992; Beato et al., 1997). The selective application of 5-HT on different spinal cord levels in this preparation using the bath partitioning method confirmed that the 5-HT-sensitive oscillatory network, capable of producing a locomotor-like pattern of activity, is diffusely distributed throughout the supralumbar spinal cord and mediates descending rhythmic drive to lumbar motor centers (Cowley and Schmidt, 1994, 1997; Schmidt and Jordan, 2000). The lumbosacral region in isolation is responsible for inducing only the tonic discharge that, at least in part, could be related to the direct excitatory effect of 5-HT on lumbar motoneurons. There are further data suggesting a regional differentiation of certain receptors in different rostro-caudal regions (Jordan and Schmidt, 2002; Liu and Jordan, 2005; Liu et al., 2009) that are in line with the differential effects of 5-HT on supralumbar *vs.* lumbar regions. There is increasing evidence that activation of specific serotonin receptors in the spinal cord is effective for the production of locomotor activity (Antri et al., 2005; Sławińska et al., 2012b). A number of serotonergic receptors are found in the spinal cord, but 5-HT_{2A}, 5-HT_{2C} and 5-HT_7

are the major ones implicated in the control of locomotion (Schmidt and Jordan, 2000; Hochman et al., 2001; Liu and Jordan, 2005; Landry et al., 2006; Liu et al., 2009; Dunbar et al., 2010; Fouad et al., 2010; Bos et al., 2013). Attempts to restore locomotion after spinal cord injury using systemic drug applications, combined with other interventions such as training and electrical stimulation of the spinal cord, have included the use of agents that target these receptors (Courtine et al., 2009; Musienko et al., 2011; van den Brand et al., 2012). The most commonly used agonists that are effective when applied systemically are quipazine, which has high affinity for both 5-HT_{2A} and 5-HT_{2C} receptors, and 8-hydroxy-2-(di-n-propylamino)-tetralin (8-OHDPAT), which binds selectively to 5-HT_7 and 5-HT_{1A} receptors (Antri et al., 2003; Landry et al., 2006; Sławińska et al., 2012b).

Because of the strong evidence suggesting that serotonergic innervation that descends from brainstem raphe nuclei to the spinal cord is involved in activation and modulation of central pattern generator (CPG) activity (Schmidt and Jordan, 2000; Jordan and Schmidt, 2002; Liu and Jordan, 2005; Liu et al., 2009), increased attention to the use of intraspinal grafting of 5-HT neurons to enhance restoration of locomotion is warranted (Orsal et al., 2002; Sławińska et al., 2014). It was demonstrated that in paraplegic rats with the serotonergic innervation in the spinal cord destroyed by total transection, sublesional transplantation of embryonic brainstem tissue containing 5-HT neurons is able to enhance the recovery of locomotor hindlimb movements (Feraboli-Lohnherr et al., 1997; Ribotta et al., 2000; Sławińska et al., 2000, 2013; Majczyński et al., 2005). In our recent paper we demonstrated for the first time that restored coordinated hindlimb locomotion by this grafting technique is mediated by 5-HT_2 and 5-HT_7 receptors (Sławińska et al., 2013). Moreover we demonstrated that it acts through different populations of spinal locomotor neurons. Specifically, 5-HT_2 receptors control CPG activation as well as motoneuron output, while 5-HT_7 receptors contribute primarily to activity of the locomotor CPG. These results are consistent with the roles for these receptors during locomotion in intact rodents and in rodent brainstem-spinal cord *in vitro* preparations (Madriaga et al., 2004; Liu and Jordan, 2005; Pearlstein et al., 2005; Liu et al., 2009; Dunbar et al., 2010).

Because the activation of these receptors is currently a prominent feature in the translational strategies that are now being undertaken to restore locomotion after spinal cord injury (Antri et al., 2005; Courtine et al., 2009; van den Brand et al., 2012), it is important to determine the mechanisms of action of these drugs on the spinal locomotor CPG and the output elements of the locomotor system, including motoneurons. At this point, there is little known about which neurons in the spinal locomotor networks and what functional components of the locomotor system are influenced by these drugs. Here we investigated these features of the actions of these drugs in rats with a chronic complete spinal cord injury. Motor performance on a treadmill was tested before and after i.p. drug application. Exteroceptive stimulation was used to trigger hindlimb movements that were monitored using video recordings synchronized with simultaneous electromyographic (EMG) recordings from the soleus and tibialis anterior muscles of both hindlimbs. In our previous paper using grafts of 5-HT neurons and serotonergic antagonists (Sławińska et al., 2013), we suggested the hypothesis that transplant-mediated activation of 5-HT_2 receptors likely governs the function of both CPG neurons and motoneurons, whereas the 5-HT_7 receptor activation may be more restricted to CPG neurons. Here we compare the effects of agonists to these receptors to test this hypothesis, using EMG recordings to determine the effects of these drugs on motoneuron activity and on inter- and intralimb coordination in chronic spinal rats.

A preliminary report of these results has been presented in an abstract form (Sławińska et al., 2011).

MATERIALS AND METHODS

Experiments were performed on WAG (Wistar Albino Glaxo) female rats (n = 11) 3-months-old at the time of spinal cord injury. All surgical and experimental procedures were conducted with care to minimize pain and suffering of animals in accordance with the guidelines of the First Local Ethics Committee in Poland, according to the principles of experimental conditions and laboratory animal care of European Union, the Polish Law on Animal Protection, and of the University of Manitoba Animal Care Committee, in accordance with the guidelines of the Canadian Council on Animal Care.

SPINAL CORD TRANSECTION PROCEDURE

Complete spinal cord transection (SCI) was performed under deep anesthesia (Isofluorane, 2% and Butomidor, 0.05 mg/kg b.w.). Under aseptic conditions a mid-dorsal skin incision was performed at the rat back over Th8-11 vertebrae, and the back muscles were separated from the vertebral column. Then after laminectomy the spinal cord was completely transected between Th9/Th10 spinal cord level. A 2–3 mm piece of spinal cord tissue was cut using scissors and gently aspirated as previously described (Sławińska et al., 2000, 2012b; Majczyński et al., 2005). The muscles and fascia overlying the paravertebral muscles were closed in layers using sterile sutures, and the skin was closed with stainless-steel surgical clips. After surgery, the animals received a non-steroidal anti-inflammatory and analgesic treatment (Tolfedine 4 mg/kg s.c.), and during the following days the animals were given antibiotics (5 days Baytril 5 mg/kg s.c. and 8 days Gentamicin 2 mg/kg s.c.). The bladder was emptied manually twice a day until the voiding reflex was re-established (about 7 days). Female rats were chosen for these experiments because of the relative ease with which the manual bladder emptying can be achieved.

IMPLANTATION OF EMG RECORDING ELECTRODES

Two months after spinal cord transection the animals were anesthetized with Equithesin (3.5 ml/kg i.p., for the mixture details see Sławińska et al., 2000) and bipolar EMG recording electrodes were implanted in the extensor soleus (Sol) and flexor tibialis anterior (TA) muscles of both hindlimbs as previously described (Sławińska et al., 2000, 2012b; Majczyński et al., 2005). The electrodes were made of Teflon-coated stainless-steel wire (0.24 mm in diameter: AS633, Cooner Wire, Chastworth, CA, USA). The hook electrodes (1.5 mm of the insulation removed)

were inserted into investigated muscles and secured by a suture. The distance between the tips of electrodes was 1–2 mm. The ground electrode was placed under the skin on the back of the animal. After electrode implantation animals received a single dose of antibiotic (Baytril 5 mg/kg s.c.).

VIDEO AND ELECTROMYOGRAPHIC RECORDINGS

Video and EMG recording started 3–5 days after electrode implantation. One minute recordings at speeds of 5 cm/s or 10 cm/s were taken before and at a few time points after drug application as described previously (Sławińska et al., 2000, 2012b; Majczyński et al., 2005). EMG recordings during locomotor-like hindlimbs activity of the SCI rats before and after drug application were filtered (0.1–1 kHz band pass), digitized and stored on computer (3 kHz sampling frequency) using the Winnipeg Spinal Cord Research Centre capture system. The EMG activity was simultaneously recorded and synchronized with video recordings, thus enabling the further off-line analysis of portions of EMG activity related to the best locomotor performance in every animal.

EVALUATION OF HINDLIMB LOCOMOTION

The quality of plantar stepping was established based upon EMG analysis, as described previously (Sławińska et al., 2012b, 2013, 2014). The rats were suspended above a treadmill with the forelimbs and thorax placed on a platform above the moving belt, and tail pinch was used to elicit hindlimb movements. Stimulation of tail or perineal area afferents has been used for eliciting locomotion in cases of complete spinal cord transection (Meisel and Rakerd, 1982; Pearson and Rossignol, 1991; Rossignol et al., 2006; Etlin et al., 2010; Lev-Tov et al., 2010; Sławińska et al., 2012b), and has been used in all prior attempts to reveal locomotor recovery after 5-HT transplants (Feraboli-Lohnherr et al., 1997; Ribotta et al., 2000; Sławińska et al., 2000, 2013; Majczyński et al., 2005). The tail stimulus was adjusted by the experimenter to maximize the quality of plantar stepping. The salient features of plantar stepping as defined here were: (1) sustained soleus activity throughout the stance phase; (2) soleus burst duration related to step cycle duration; (3) brief TA activity of consistent duration; and (4) consistent intra- and interlimb coordination (Sławińska et al., 2014).

DRUG TEST

To examine the effects of 5-HT agonists on the hindlimb locomotor-like movements we started from evaluation of the pre-drug baseline performance. Then the evaluation of hindlimbs movements was carried out 15–30 min after drug application. Quipazine (0.25 mg/kg dissolved in saline with 10% of propylene glycol) and (±)-8-hydroxy-dipropylaminotetralin hydrobromide (8-OHDPAT) (0.2–0.4 mg/kg dissolved in saline) were used to activate 5-HT_2 and 5-HT_1/5-HT_7 receptors respectively. During the combined treatment, both drugs were applied with the dose 0.1 mg/kg within a 20 min delay in between (the quipazine i.p. injection was followed by the 8-OHDPAT i.p. applications). Quipazine was always first, because the effect of 8-OHDPAT is usually shorter than that of quipazine. To avoid any training or drug accumulation effects the locomotor testing was carried out not more often than twice a week with at least 3–4 days break in between. Doses were determined on the basis of previous experiments using these drugs (Antri et al., 2003; Majczyński et al., 2005; Musienko et al., 2011; Sławińska et al., 2012b).

STATISTICAL ANALYSIS

The EMG pattern related to the best locomotor movement in each experimental condition was analyzed using custom software.[1] The coordination between left and right TA (interlimb coordination) and between right Sol and right TA (intralimb coordination) was analyzed using polar plots as previously described (Zar, 1974; Batschelet, 1981; Kjaerulff and Kiehn, 1996; Cowley et al., 2005; Sławińska et al., 2013). In the polar plots the position of the vector at 0 or 360° reflects synchrony of analyzed EMG burst onsets, whereas 180° is equivalent to alternation. The length of the vector (***r***, ranging from 0 to 1) indicates the concentration of phase values around the mean, and the strength of coordination between muscle burst onsets. Rayleigh's circular statistical test was used to determine whether the inter- and intra-limb coordination ***r***-values were concentrated, suggesting coupling of burst activity, or dispersed, indicating loss of hindlimb coordination. The relationships between the EMG burst duration and step cycle duration were determined using the regression line method. Values are reported here as mean ± SD (Standard Deviation). The normal distribution of the data was confirmed using a Shapiro-Wilk test. For comparison of two groups of results (after the high dose of quipazine and 8-OHDPAT alone) Student's *t*-Test was used. For comparison of the pre-drug data with the results obtained in two experimental conditions (after the low dose of quipazine, then after a subsequent low dose of 8-OHDPAT) one-way repeated measures ANOVA followed by Tukey's *post-hoc* test for multiple comparison was used (Prism, GraphPad Software, La Jolla, CA).

RESULTS

Spinal cord total transection induced impairment of hindlimb movements in adult rats as described below. When inspected in their home cages the rats were typically paraplegic, and they used the forelimbs to move around with the hindlimbs dragging on the floor behind the body. Before drug application locomotor hindlimb movement of spinal rats was strongly impaired (**Figures 1A,B** upper panel). During locomotor trials on the treadmill the hindlimbs were kept extended behind the rat body and their movements were limited only to dragging of the paws with dorsal surfaces touching the moving treadmill belt. Tail stimulation induced some muscle contractions related to rather limited hindlimb movements, which were accompanied by uncoordinated EMG activity in antagonist Sol (antigravity extensor) and TA (dorsiflexor of the ankle joint) muscles, lack of rhythm consistency, and loss of sustained Sol muscle activity through the stance phase of the cycle. Moreover, the bursts of Sol muscle activity very often overlapped with ipsilateral TA muscle activity, often resulting in failure to initiate a normal swing phase of the step cycle. The details of locomotion induced by tail stimulation in spinal rats (**Figure 1**) have been described in

[1] http://www.scrc.umanitoba.ca/doc/

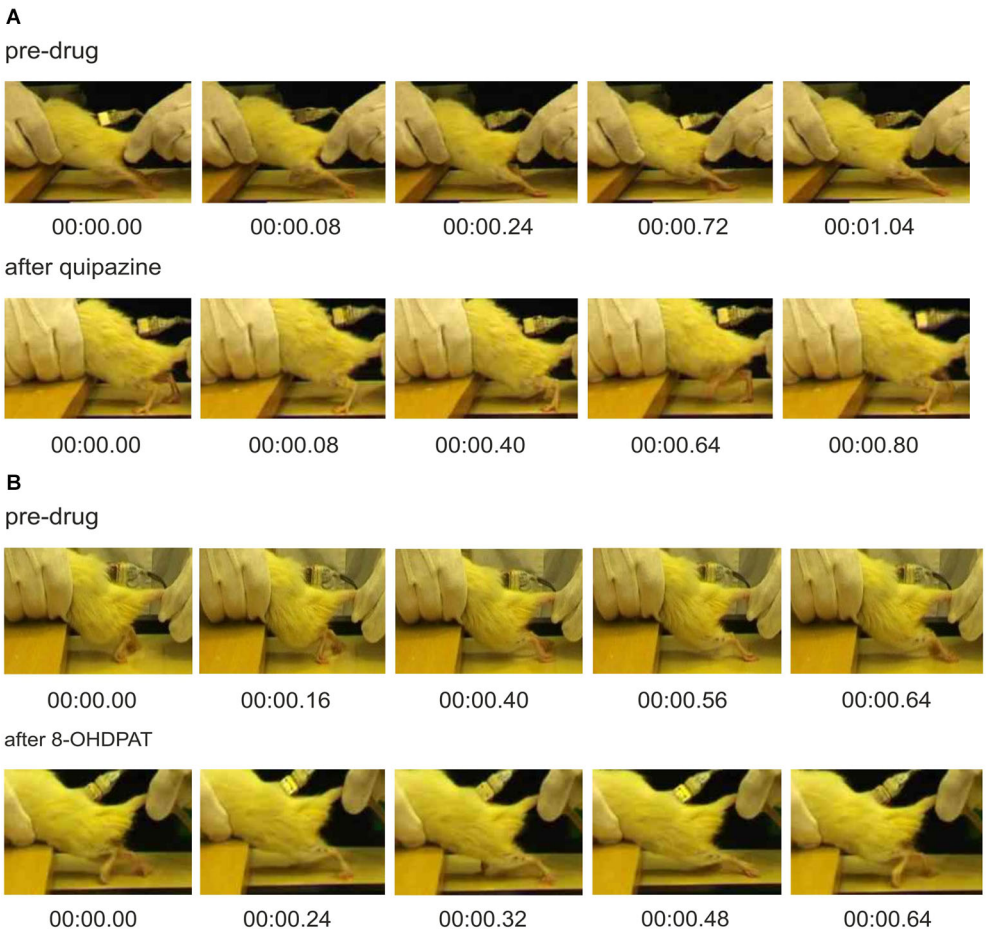

FIGURE 1 | Video frames showing consecutive phases of locomotor-like hindlimb movements in spinal rats before and after drug application (A) quipazine (0.25 mg/kg, i.p.) or (B) 8-OHDPAT (0.4 mg/kg, i.p.). Plantar paw placement and weight supported stepping are evident after application of either drug. However, after quipazine application, spinal rats present better body weight support (**A**, lower panel) in comparison to that after 8-OHDPAT (**B**, lower panel) where the hindlimbs remain extended behind the body.

our previous publications (Jordan and Sławińska, 2011; Sławińska et al., 2012b).

We have also previously described improvement in locomotion produced by quipazine and 8-OHDPAT (Jordan and Sławińska, 2011; Sławińska et al., 2012b), but here we provide a comparison of the effects of these two agonists, and show how these data reveal aspects of the organization of the locomotor system that are influenced by serotonin receptor activation that have not previously been appreciated. A single dose of serotonin receptor agonists, quipazine or 8-OHDPAT, resulted in improvement of locomotor hindlimb movements in both cases (**Figures 1A** or **1B** respectively). After i.p. injection of 0.25 mg/kg of quipazine, or 0.4 mg/kg of 8-OHDPAT, spinal rats could be induced by tail pinching to present regular hindlimb movements associated with plantar stepping. However, hindlimb locomotor movements observed after application of these two drugs were markedly different. Hindlimb movements were more regular after 8-OHDPAT than after quipazine application (confirmed by smaller Standard Deviation of the mean cycle duration; 536 ± 73 ms *vs.* 630 ± 210 ms). Moreover, after quipazine application, spinal animals presented better body weight support and extensor muscle activity in comparison to that after 8-OHDPAT. Improved locomotor hindlimb movements observed after application of both drugs were accompanied by sustained regular EMG recordings with alternating burst activity of extensor and flexor muscles. EMG activity was more regular than before drug application. In both cases EMG bursts of Sol muscle were robust during the period between ipsilateral flexor EMG bursts, which did not overlap with TA muscle bursts. EMG bursts of Sol and TA muscles were more regular and consistent after 8-OHDPAT than after quipazine application, but the excitation of Sol was more likely to be sustained throughout the stance phase after quipazine alone (**Figures 2A,B**, upper and lower panels). This is consistent with the absence of effective stance just before the onset of flexion observed in **Figure 1B**. Moreover, the burst duration of the Sol muscle was shorter during locomotion induced by 8-OHDPAT than after quipazine (duty cycle for soleus burst duration: 56 ± 10% *vs.* 72 ± 10%; Student's *t*-Test, $P < 0.05$).

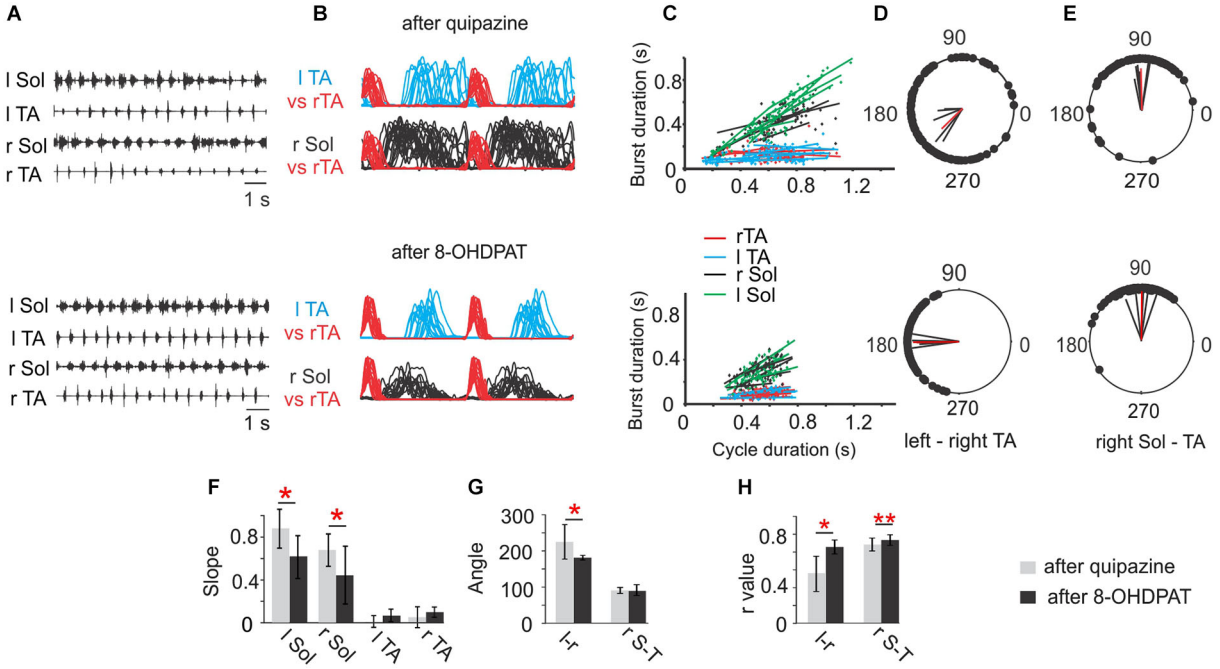

FIGURE 2 | Comparison of Soleus (Sol) and tibialis anterior (TA) EMG activity patterns during locomotor hindlimb movements enhanced by quipazine (upper panel) or 8-OHDPAT (lower panel) application. **(A)** EMG activity during hindlimb movements in two experimental conditions (upper traces after quipazine; lower traces after 8-OHDPAT). **(B)** Envelops of EMG burst activity established from raw records rectified, filtered, and normalized to the step cycle (onset of activity in the right TA considered the onset of the cycle). Note longer burst activity of the soleus muscle during locomotor movements enhanced by quipazine application (upper panel) than after 8-OHDPAT (lower panel). **(C)** Regression lines illustrating the relationships between the step cycle durations and burst durations for the left and right TA and Sol muscles; **(D)** and **(E)** polar plots showing the relationships between the onset of right TA (r TA) activity and either the contralateral TA or the ipsilateral extensor (r Sol). The 0 position on the polar plot corresponds to the onset of activity in the right TA muscle, and the positions of the filled black circles indicate the times of onset of activity in the left TA—interlimb coordination (**D**, lower and upper panels) or the onset of activity in the right Sol—intralimb coordination (**E**, lower and upper panels), the black lines each represent results of analysis established for one animal and the red line represents the average results across animals in the analyzed group. **(F)** Bar diagrams showing the mean slopes (±SD) for the regression lines between step cycle durations and burst durations for the left and right TA and Sol muscles in rats in locomotor hindlimb movement enhanced by either drug. **(G)** Bar diagrams showing the means (±SD) of the angle of the left–right TA (l–r) and of the right Sol–right TA (r S–T) relationships (relative timing of the onsets for the two muscles) represented in each polar plot in locomotor movements enhanced by application of quipazine and 8-OHDPAT in spinal rats. **(H)** Bar diagrams showing the means (±SD) of ***r***-value between the times of onset of activity in both left-right and flexor-extensor EMG established for spinal rats treated either by quipazine or by 8-OHDPAT. Statistical significance by Student's *t*-Test is indicated by red stars for ($n = 6$) spinal rats tested during locomotor movement enhanced by either drug: * $P < 0.05$; ** $P < 0.005$. The details of the comparison between pre-drug and each agonist alone were presented in Figures 2, 6 and 7 of Sławińska et al. (2012b).

Investigation of the relationship between the EMG burst duration and step cycle duration using analysis of regression lines for left and right Sol and TA muscles show that after application of either 8-OHDPAT or quipazine, regression lines for Sol muscle burst duration plotted against cycle duration demonstrated a positive relationship (**Figure 2C**, upper and lower panels) that is not seen in control pre-drug analysis (for comparison see Sławińska et al., 2012b). It is important to note that after quipazine application the slopes of regression lines were significantly higher than after 8-OHDPAT administration (statistically significant, $P < 0.05$) suggesting that quipazine application leads to increased excitability of motoneurons or last-order interneurons (**Figure 2F**).

Using polar-plot analysis we investigated the inter- (left-right TA; **Figure 2D**) and intra- (right Sol-right TA) limb coordination (**Figure 2E**). The bar graphs presenting the mean polar-plot angles (**Figure 2G**) and ***r***-values (**Figure 2H**) for quipazine and 8-OHDPAT show that the mean ***r***-values for intra- and interlimb coordination were significantly higher after 8-OHDPAT than after quipazine applications, suggesting that the 5-HT_7 receptors participate more significantly in rhythm generation.

As we demonstrated above, activation of either 5-HT_2 or 5-$HT_{7/1A}$ receptors alone facilitates hindlimb locomotion in spinal rats, but their effects are significantly different, probably due to activation of different populations of spinal neurons constituting different elements of the spinal cord circuitry that control locomotor movements. Given these differences and our preliminary observation that the combined use of these two drugs results in more robust locomotion without the need for tail stimulation (Sławińska et al., 2012b), we decided to investigate in detail the effects of combined treatment of low doses of 5-HT receptor agonists: quipazine and 8-OHDPAT. As is shown in **Figures 3A,B** the low dose of quipazine alone significantly prolonged the extensor bursts during the hindlimb movements: however the

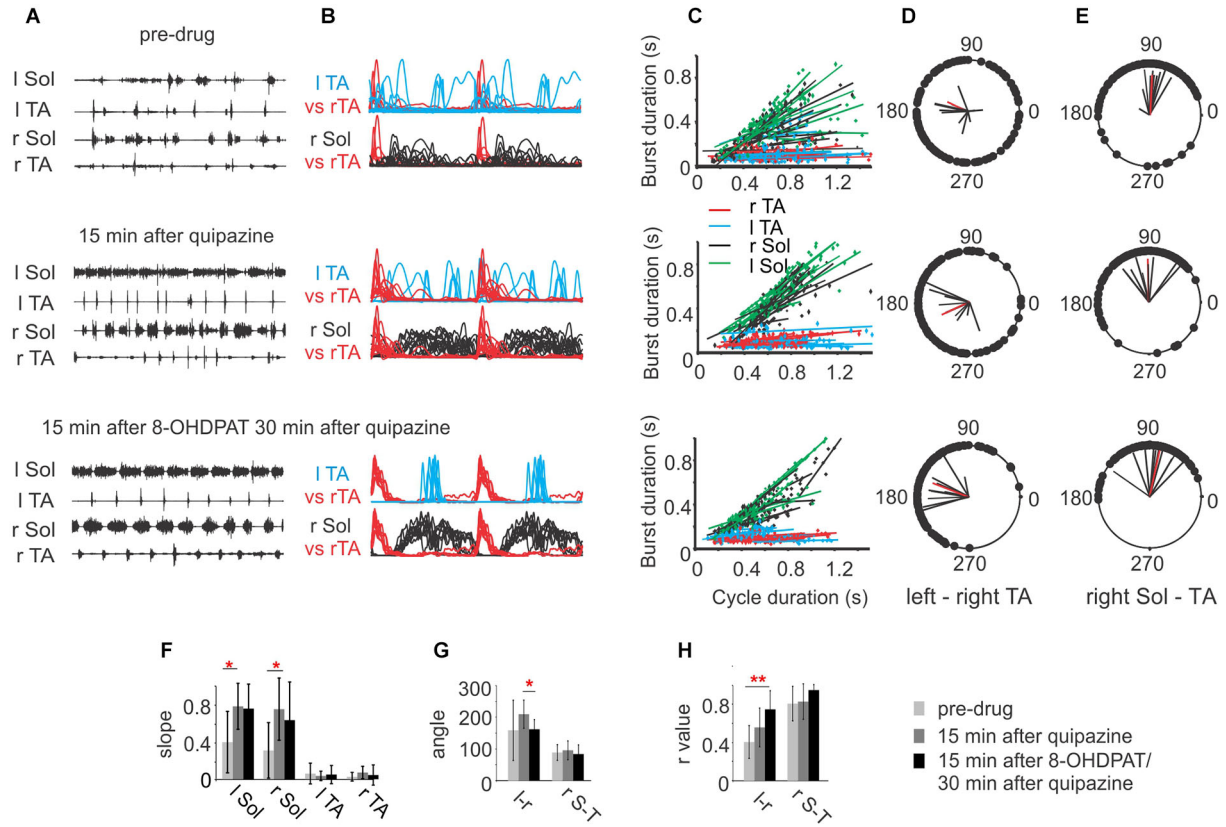

FIGURE 3 | (A) EMG activity during hindlimb movements in three sequential experimental conditions: before (upper panel), after a low dose (0.1 mg/kg, i.p.) of quipazine (middle panel) and after a subsequent low dose (0.1 mg/kg, i.p.) of 8-OHDPAT (lower panel). Note the longer but rather irregular burst activity of Sol muscle during locomotor movements after quipazine application and improved regularity after a subsequent dose of 8-OHDPAT. **(B)** Envelopes of EMG burst activity established from raw records rectified, filtered, and normalized to the step cycle (onset of activity in the right TA considered the onset of the cycle); **(C)** regression lines illustrating the relationships between the step cycle durations and burst durations for the left and right TA and Sol muscles for the three conditions; **(D)** and **(E)** polar plots showing the relationships between the onset of r TA activity and either the contralateral TA **(D)** or the ipsilateral r Sol **(E)**. The 0 position on the polar plot corresponds to the onset of activity in the right TA muscle, and the positions of the filled black circles indicate the times of onset of activity in the left TA (interlimb coordination in **D**) or the onset of activity in the right Sol (intralimb coordination in **E**), the black lines each represent results of analysis established for one animal and the red line represents the average results across animals in the analyzed group. **(F)** Bar diagrams showing the mean slopes (±SD) for the regression lines between step cycle durations and burst durations for the left and right TA and Sol muscles in rats in locomotor hindlimb movements in the three experimental conditions. **(G)** Bar diagrams showing the means (±SD) of the angle of the left–right TA (l–r) and of the right Sol–right TA (r S–T) relationships (relative timing of the onsets for the two muscles) represented in each polar plot in the three experimental conditions. **(H)** Bar diagrams showing the means (±SD) of ***r***-value between the times of onset of activity in both left-right and flexor-extensor EMG established for spinal rats ($n = 5$) in the three experimental conditions. Each rat was tested twice with at least a week's delay between tests. * $P < 0.05$, ** $P < 0.01$, statistical significance established by one-way repeated measures ANOVA followed by Tukey's *post-hoc* test.

locomotor pattern remained rather irregular. Addition of 8-OHDPAT improved coordination significantly. An analysis of the relationship between the EMG burst duration and step cycle duration shows significantly increased slopes of regression lines established for the Sol EMG burst activity during a hindlimb locomotor pattern facilitated by quipazine (one-way ANOVA for ***r*** Sol $F_{(2,27)} = 4.026$, $P = 0.048$, for l Sol $F_{(2,27)} = 5.317$, $P = 0.0157$; multiple comparison by Tukey's *post-hoc* test $P < 0.05$). The slopes were slightly reduced (not significantly) after the subsequent 8-OHDPAT application (**Figures 3C,F**). Quipazine at a low dose significantly facilitated locomotor hindlimb movements with prolonged extensor activity and plantar stepping; however interlimb coordination (**Figures 3D,H**) was not significantly improved (before quipazine application $\boldsymbol{r} = 0.401 \pm 0.17$, while after the drug was applied $\boldsymbol{r} = 0.55 \pm 0.20$). The difference was not significant (one-way ANOVA $F_{(2,27)} = 5.931$, $P = 0.019$; multiple comparison by Tukey's *post-hoc* test, $P > 0.05$). Addition of 8-OHDPAT improved interlimb coordination: $\boldsymbol{r} = 0.739 \pm 0.19$. Although the changes in coordination after addition of 8-OHDPAT were not statistically significant in comparison to the preceding quipazine application alone, when compared with the pre-drug situation interlimb coordination significantly improved (multiple comparison by Tukey's *post-hoc* test, $P < 0.01$). The need for tail pinching was greatly reduced or eliminated in such cases, consistent with our previous preliminary observation (Sławińska et al., 2012b) after combined drug treatment. Thus,

the combined application of both drugs at lower doses produces a markedly improved locomotor outcome. However, the coordination was not improved by the combination of the two drugs in comparison to either drug alone after high dose applications. This result should be taken into account when designing strategies for restoration of locomotion using serotonergic agonists. It should also be noted that 5-HT agonist applications combined with other interventions that promote plantar stepping reveal that there can be an additive effect that degrades locomotion, so the proper balance between drug effect and other effects is necessary (Sławińska et al., 2012b).

The presence of a period of inhibition of the soleus burst that coincided with the presence of a burst in the contralateral flexor muscle was consistently observed during the combined drug treatment (**Figure 3B** third panel, **Figure 4**). This was confirmed in 5/5 animals. These observations are consistent with excitatory inputs to neurons responsible for production of synchronous movements of the hindlimb, as in gallop, where ipsilateral flexor activity could be associated with inactivation of contralateral extensor activity to some advantage. When the animal is walking on a treadmill with alternating activity in the hindlimbs, this inhibition breaks through and reduces the amplitude of the contralateral extensor burst. We interpret this to be the extensor inhibitory component of a synchrony microcircuit. Examples in **Figure 4** taken from one rat demonstrate the presence of a period of inhibition of the r Sol burst that coincided with the presence of the burst of activity in the contralateral flexor EMG (l TA) at the slower speed of locomotion, but not during the trial at the higher speed.

The recent finding that certain commissural neurons involved in the production of hopping gaits in genetically modified mice (Talpalar et al., 2013) are configured to be recruited at different speeds of locomotion prompted us to examine the possibility that such neurons might contribute to the appearance of the synchrony microcircuit resulting from treatment with 5-HT agonists. To this end we examined whether the speed of locomotion could alter the appearance of the synchrony microcircuit. **Figure 4** illustrates one example of the results of these experiments, which was a consistent finding in all five cases. Here, the synchrony microcircuit is recruited at the slower speed of locomotion, but not at the higher speed, implicating the $V0_V$ excitatory commissural neurons and their target contralateral inhibitory interneurons that terminate on contralateral motoneurons (Menelaou and McLean, 2013; Talpalar et al., 2013) as the basis for this phenomenon.

DISCUSSION

To summarize the results of the present study, we show here that activation of either 5-HT_2 or 5-HT_7/5-HT_{1A} receptors by application of single doses of either quipazine or 8-OHDPAT facilitates activity of the spinal cord network controlling locomotor hindlimb movements in paraplegic rats in the normal horizontal posture. However, markedly different locomotor effects induced by activation of these two receptors suggest that the observed results are related to the activation of different populations of spinal neurons. Activation of 5-HT_2 receptors led mainly to improvement of extensor muscle activity and less to the improvement of regularity of walking, likely facilitating the output stage of the locomotor system by direct activation of motoneurons. It also may activate the pattern formation interneurons of the locomotor CPG that are the presumed last-order interneurons providing the CPG output to motoneurons. Activation of 5-HT_7 and 5-HT_{1A} receptors that led to improved left-right alternation in hindlimb walking probably facilitated the activity of the locomotor CPG, without direct actions on the output components of the locomotor system, including motoneurons. These results are consistent with the idea that the spinal cord network responsible for locomotor pattern generation consists of different layers that control motoneuron activity, one the rhythm generator (CPG) and another the pattern formation layer

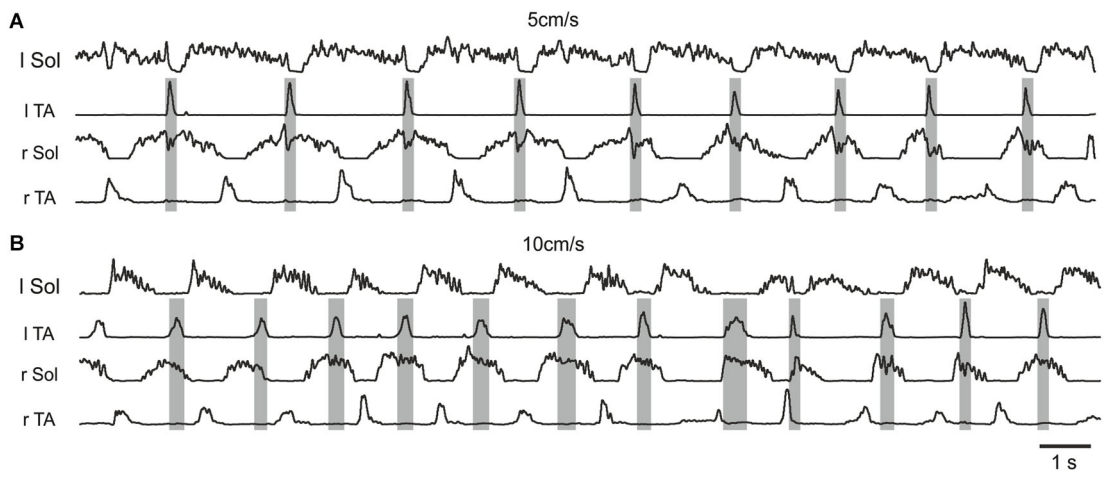

FIGURE 4 | Examples of rectified and filtered EMG records from left and right Sol and TA muscles during hindlimb locomotor movements after a low dose (0. 1 mg/kg, i.p.) of quipazine and a subsequent low dose (0.1 mg/kg, i.p.) of 8-OHDPAT in a rat suspended over a moving treadmill belt at two speeds: 5 cm/s (A) and 10 cm/s (B). Note the presence of a period of inhibition of the r Sol burst that coincided with the presence of the burst of activity in the contralateral flexor EMG (l TA) at the slower speed of locomotion **(A)**, but not during the trial at the higher speed **(B)**.

(McCrea and Rybak, 2007, 2008). This hypothesis is supported also by the results of the combined use of the drugs in our investigations, which show that simultaneous activation of different neuronal populations expressing different 5-HT receptors results in production of improved well-coordinated weight supported locomotion. Moreover, the requirement for a smaller dose of both drugs suggests that both neuronal populations play complementary roles in facilitation of locomotor hindlimb movements in spinalized adult rats. In addition, the data reveal the presence of a previously undescribed system of crossed inhibition of ankle extensor muscles that is time-locked to the occurrence of the contralateral flexor burst that is brought into play when neurons that possess receptors activated by quipazine and 8-OHDPAT are recruited. These results are compatible with the activation of neurons that play a role in the production of synchronous activity across the midline, such as occurs in galloping and hopping, and they are strikingly similar to the results obtained in mutant mice showing a role for V0 interneurons commissural microcircuits involved in speed control of hopping (Talpalar et al., 2013).

Chronic treatment with this combination of drugs has been demonstrated to improve locomotion (Antri et al., 2005), and a number of studies have examined the effects of one or the other of these drugs on the restoration of locomotion after spinal cord injury in adult animals (Landry et al., 2006). Combinations of these drugs along with other interventions, such as locomotor training and epidural stimulation, have been reported to improve functional recovery after spinal cord injury (Courtine et al., 2009; Musienko et al., 2011). Many of these studies have used a paradigm in which the rats are placed in an upright posture, a condition that we have shown has its own ability to facilitate recovery of plantar stepping (Sławińska et al., 2012b,c). Here we focus upon the demonstration that different populations of spinal neurons and coordinating microcircuits are recruited by the drugs acting on different 5-HT receptors to account for restoration of plantar stepping without the need for other interventions.

A central issue to the interpretation of our results is which receptors are likely to be involved in the action of these drugs (Hochman et al., 2001). The action of quipazine could be mediated by a few 5-HT receptors for which it has high affinity. These include 5-HT_{2A}, 5-HT_{2B} or 5-HT_{2C} receptors. 5-HT_{2A} labeling has been demonstrated to be most intense in motoneurons (Maeshima et al., 1998; Cornea-Hébert et al., 1999; Doly et al., 2004), but it also occurs in other neurons of the ventral horn and in dorsal root ganglion cells. The action of quipazine to facilitate locomotion has been attributed to 5-HT_{2A} receptors (Ung et al., 2008), and 5-HT_{2A} receptors have been implicated in the production of locomotion by stimulation of brainstem serotonergic neurons in neonatal rats (Liu and Jordan, 2005). The affinity of quipazine for 5-HT_{2A} receptors is in the order of four fold higher than for 5-HT_{2C} receptors, and over 50-fold higher than for 5-HT_{2B} receptors.[2] Another study (Murray et al., 2010) claimed that the appearance of constitutively active 5-HT_{2C} receptors after staggered hemisection accounts for recovery of locomotor function after spinal cord injury. In contrast, after complete transection of the spinal cord (Navarrett et al., 2012) 5-HT_{2A}R mRNA expression was upregulated below the site of spinal cord injury, but no changes in 5-HT_{2C}R mRNA editing or expression were detected. A similar absence of changes in 5-HT_{2C} receptors below the lesion was reported after contusive injury to the spinal cord (Nakae et al., 2008). It appears possible that the upregulation of constitutively active 5-HT_{2C} receptors as a means of restoring locomotion after spinal cord injury may be limited to the staggered hemisection preparation and/or to the intrathecal application of drugs at the L5-L6 vertebral level (Murray et al., 2010), where locomotor control due to sacrocaudal afferent stimulation (Lev-Tov et al., 2010) might predominate. On the other hand, considerable evidence supports the upregulation of 5-HT_{2A} receptors after complete spinal cord injury, especially in motoneurons (Jordan et al., 2010; Kong et al., 2010, 2011; Navarrett et al., 2012). We have preliminary evidence using intrathecal drug application of antagonists at the lumbar level in intact rats that 5-HT_{2A} but not 5-HT_{2C} receptors are normally involved in the production of locomotion (Sławińska et al., 2012a). Similarly, Fouad et al. (2010) found that blocking constitutive 5-HT_{2C} receptor activity with the potent inverse agonist SB206553 applied intrathecally had no effect on stepping in normal rats.

Motoneuron responses to 5-HT are mediated by a variety of receptors (Schmidt and Jordan, 2000; Heckman et al., 2009; Perrier et al., 2013). The responses are typically an increase in excitability, through several possible mechanisms (E_m depolarization, reduced AHP, hyperpolarization of voltage threshold, production of persistent inward currents, PICs) when 5-HT_2 receptors are activated, and depression of motoneuron output when 5-HT_{1A} receptors are activated (reviewed Perrier et al., 2013). It is clear that increased motoneuron excitability is one of the consequences of 5-HT_2 receptor activation due to quipazine's actions in our experiments. It seems plausible that the sensory feedback from the improved plantar contact may be at least partially responsible for the increased soleus duty cycle rather than or in addition to direct actions of quipazine on extensor motoneurons. It is known that quipazine can increase the excitability of both flexor and extensor motoneurons (Chopek et al., 2013). Such an increase in flexor motoneuron activity could contribute to the improved placement of the paw and subsequent improved afferent feedback to promote plantar stepping (Sławińska et al., 2012b).

The role of 8-OHDPAT in the production of locomotion appears to be mediated by its action at both 5-HT_7 and 5-HT_{1A} receptors (Landry et al., 2006), since it could produce locomotion in 5-HT_7 knockout mice or in the presence of a selective 5-HT_7 antagonist, and since locomotion could be blocked by antagonists of either 5-HT_7 or 5-HT_{1A} receptors. The effect of 8-OHDPAT to facilitate locomotion in our experiments appears to be exerted primarily on the rhythm generating and coordinating elements of the CPG. This is indicated by the data showing that the motoneuron excitability is not significantly altered by this drug (see **Figure 3**; Sławińska et al., 2012b), but its application in low doses together with low doses of quipazine creates a situation where exteroceptive stimulation is no longer necessary to activate the locomotor CPG, and coordination is dramatically improved.

[2]http://pdsp.med.unc.edu/

This is consistent with the fact that locomotor neurons in the spinal cord possess 5-HT_7 and 5-HT_{1A} receptors in abundance (Jordan and Schmidt, 2002; Noga et al., 2009). A prominent role for 5-HT_7 receptors in the control of coordinated muscle activity during locomotion has also been suggested by the investigation of locomotion in 5-HT_7 receptor knockout mice (Liu et al., 2009) and in experiments using the specific 5-HT_7 receptor antagonist SB-269970 (Madriaga et al., 2004; Liu and Jordan, 2005; Pearlstein et al., 2005; Liu et al., 2009). Although there is some overlap of 5-HT_{2A} and 5-HT_7 receptors on locomotor interneurons, there are clearly more 5-HT_7 positive locomotor neurons than 5-HT_{2A} positive ones (Noga et al., 2009). 5-HT_7 receptors have not been clearly shown to be associated with motoneurons, due to antibody specificity problems. Almost all locomotor neurons identified by c-fos immunostaining in the above experiments (Jordan and Schmidt, 2002; Noga et al., 2009) were positive for 5-HT_7 receptors, and a lesser number were positive for 5-HT_{2A} receptors. Faint 5-HT_7 receptor labeling has been reported in motoneurons (Doly et al., 2005), but we have found the same antibody labels motoneurons in 5-HT_7 receptor knockout mice.

We conclude from our results and from the information available in the literature about the effects of activation of the various receptors that are the targets of quipazine and 8-OHDPAT that the effects on locomotion that we observe are due largely to actions on 5-HT_{2A} and 5-HT_7 receptors. We further conclude that the neurons that are activated by 5-HT_{2A} receptors include CPG neurons capable of inducing rhythmic activity, but with incomplete facilitation of coordinating interneurons responsible for inter- and intralimb coordination. Activation of these receptors also results in increased motoneuron excitability and prolonged activity in extensor motoneuron output. The latter effects may be due to the presence of abundant 5-HT_{2A} receptors on motoneurons, especially in order to explain the increased motoneuron excitability. It is more difficult, however, to explain the prolongation of the motoneuron discharge during the stance phase of locomotion on the basis of direct actions of the drug on motoneurons, with the caveat that facilitation of PICs in motoneurons might account for the prolonged activity (Heckman et al., 2009; Perrier et al., 2013). It is also possible that the effects are due in part to the enhancement of activity in CPG neurons of the Pattern Formation layer of the locomotor CPG (McCrea and Rybak, 2007, 2008), which could not only influence the timing of motoneuron activity during the step cycle, but also increase the excitatory drive to motoneurons. If the rhythmic activity produced by quipazine were due to actions directly on motoneurons and on the output stage of the Pattern Formation layer of the CPG, rather than on activation of the Rhythm Generating layer of the CPG, this would explain the incomplete facilitation of intra- and interlimb coordination with this drug. The actions of 5-HT_7 receptors to trigger rhythmic activity and to activate the neurons responsible for intra- and interlimb coordination, without direct actions on motoneuron excitability, suggests these receptors are located on Rhythm Generation layer neurons as well as on the inhibitory interneurons responsible for coordination (see **Figure 2**; Jordan and Sławińska, 2011), that are part of the Pattern Formation layer, but without effect on neurons that control the timing of motoneuron activity. This is consistent with the finding that locomotor interneurons may possess either 5-HT_7 or 5-HT_{2A} receptors, and a smaller proportion possess both (Noga et al., 2009). With this working hypothesis as a basis for further investigation, there are ample opportunities for experiments designed to identify the elements of the CPG responsible for the various functions suggested by our experiments.

Our experiments also provide the first evidence for 5-HT activation of a commissural microcircuit involved in synchronous activation of the hindlimbs (**Figures 3**, **4**). This microcircuit, associated with inhibition of extensor motoneurons at the same time that contralateral flexor motoneurons are active, as well as simultaneous activity in flexor motoneurons bilaterally, may underlie 5-HT modulation of gait. **Figures 3**, **4** provide the first observation of muscle activity that supports the presence of such a microcircuit, activated by certain 5-HT receptors in adult rat that could contribute to the production of gaits involving synchronous movements of the two hindlimbs, such as hopping and gallop. Only the slightest hints of simultaneous flexor activity were observed in our experiments (**Figure 4A**), however, suggesting that the neurons excited by the 5-HT agonists are those involved only in the portion of the microcircuit that inhibits contralateral extensors. The findings in **Figures 3**, **4** suggest that there might be some limitations to the utility of combined treatment with these two agonists for the production of well-coordinated locomotion after spinal cord injury, as we have previously pointed out with respect to the need for a proper balance between drug application and sensory feedback from the sole of the foot (Sławińska et al., 2012b).

The results are consistent with the suggestion that during brainstem evoked locomotion, such as occurs normally, the appropriate coordinating interneurons, including commissural cells, are selected by specific restricted populations of serotonergic neurons to produce the desired gait. In the case of systemic application of serotonergic agonists, this selectivity is lost, so that populations of coordinating commissural cells are activated at inappropriate times.

Serotonergic activation of monosynaptic crossed inhibition might account for our results. Such inhibitory commissural cells terminating on contralateral extensor motoneurons have been demonstrated (Bannatyne et al., 2003). Lamina VIII commissural neurons projecting directly to contralateral ankle extensor motoneurons have been shown to be excited by 5-HT (Hammar et al., 2004). It is clear that many commissural neurons can be excited by 5-HT (Carlin et al., 2006; Zhong et al., 2006a,b; Abbinanti et al., 2012). A disynaptic inhibitory pathway is also a possibility, and some such pathways involve an excitatory commissural cell that activates contralateral Ia inhibitory interneurons (Jankowska et al., 2005).

Another suggestion based on recent work on commissural neuron activity during locomotion involves V0 commissural neurons, which are implicated in the production of a hopping gait. The ipsilateral flexor-related inhibition of contralateral extensor motoneurons could be achieved by selecting for $V0_V$ commissural interneurons, which produce contralateral inhibition via a disynaptic pathway at slow speeds of locomotion. When $V0_V$

cells predominate, the result is hopping at slow speeds (Menelaou and McLean, 2013; Talpalar et al., 2013). In this scenario the balance between $V0_V$ and $V0_D$ neurons might be altered under conditions of systemic serotonin agonist application, so that $V0_V$ neurons predominate, resulting in the observed inhibition of soleus motoneurons during slow locomotion. This suggests that $V0_V$ commissural neurons must receive excitatory input descending serotonergic pathways as well as from excitatory components of the CPG projecting to the contralateral flexors. Our finding that the crossed inhibitory microcircuit activated by 5-HT agonists is apparent during slow but not during faster walking (**Figure 4**) is consistent with the involvement of $V0_V$ neurons in this microcircuit. There is evidence that V0 interneurons possess 5-HT receptors (Dyck et al., 2009; Olsen, 2011), but there have been no direct tests of 5-HT actions on V0 interneurons. Such experiments are warranted by our findings.

The findings described here provide detailed documentation of the differences in EMG activity produced by quipazine and 8-OHDPAT in spinal rats, and make a case for actions of the two drugs on different populations of spinal neurons. An increased understanding of the modes of action of these drugs and the receptors that they activate is of increasing importance because these two drugs have gained prominence in efforts to restore locomotion after spinal cord injury. We also demonstrate that systemic application of these drugs can lead to inappropriate activation of coordinating microcircuits that can interfere with the restoration of the normal locomotor pattern. Further experiments on the involvement of CPG and commissural interneurons in the effects of 5-HT agonists are needed, including recordings from identified interneurons during locomotor activity.

AUTHOR CONTRIBUTIONS

Urszula Sławińska and Larry M. Jordan conceived, designed research and supervised experiments conducted in Warsaw and in Winnipeg respectively. Urszula Sławińska and Krzysztof Miazga performed research in Warsaw, and Larry M. Jordan and Urszula Sławińska performed research in Winnipeg. Urszula Sławińska, Krzysztof Miazga and Larry M. Jordan analyzed the data and wrote the paper.

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REFERENCES

Abbinanti, M. D., Zhong, G., and Harris-Warrick, R. M. (2012). Postnatal emergence of serotonin-induced plateau potentials in commissural interneurons of the mouse spinal cord. *J. Neurophysiol.* 108, 2191–2202. doi: 10.1152/jn.00336.2012

Antri, M., Barthe, J. Y., Mouffle, C., and Orsal, D. (2005). Long-lasting recovery of locomotor function in chronic spinal rat following chronic combined pharmacological stimulation of serotonergic receptors with 8-OHDPAT and quipazine. *Neurosci. Lett.* 384, 162–167. doi: 10.1016/j.neulet.2005.04.062

Antri, M., Mouffle, C., Orsal, D., and Barthe, J. Y. (2003). 5-HT1A receptors are involved in short- and long-term processes responsible for 5-HT-induced locomotor function recovery in chronic spinal rat. *Eur. J. Neurosci.* 18, 1963–1972. doi: 10.1046/j.1460-9568.2003.02916.x

Bannatyne, B. A., Edgley, S. A., Hammar, I., Jankowska, E., and Maxwell, D. J. (2003). Networks of inhibitory and excitatory commissural interneurons mediating crossed reticulospinal actions. *Eur. J. Neurosci.* 18, 2273–2284. doi: 10.1046/j.1460-9568.2003.02973.x

Batschelet, E. (1981). *Circular Statistics in Biology.* New York: Academic Press.

Beato, M., Bracci, E., and Nistri, A. (1997). Contribution of NMDA and non-NMDA glutamate receptors to locomotor pattern generation in the neonatal rat spinal cord. *Proc. Biol. Sci.* 264, 877–884. doi: 10.1098/rspb.1997.0122

Bos, R., Sadlaoud, K., Boulenguez, P., Buttigieg, D., Liabeuf, S., Brocard, C., et al. (2013). Activation of 5-HT2A receptors upregulates the function of the neuronal K-Cl cotransporter KCC2. *Proc. Natl. Acad. Sci. U S A* 110, 348–353. doi: 10.1073/pnas.1213680110

Carlin, K. P., Dai, Y., and Jordan, L. M. (2006). Cholinergic and serotonergic excitation of ascending commissural neurons in the thoraco-lumbar spinal cord of the neonatal mouse. *J. Neurophysiol.* 95, 1278–1284. doi: 10.1152/jn.00963.2005

Cazalets, J. R., Grillner, P., Menard, I., Cremieux, J., and Clarac, F. (1990). Two types of motor rhythm induced by NMDA and amines in an *in vitro* spinal cord preparation of neonatal rat. *Neurosci. Lett.* 111, 116–121. doi: 10.1016/0304-3940(90)90354-c

Cazalets, J. R., Sqalli-Houssaini, Y., and Clarac, F. (1992). Activation of the central pattern generators for locomotion by serotonin and excitatory amino acids in neonatal rat. *J. Physiol.* 455, 187–204.

Chopek, J. W., MacDonell, C. W., Power, K. E., Gardiner, K., and Gardiner, P. F. (2013). Removal of supraspinal input reveals a difference in the flexor and extensor monosynaptic reflex response to quipazine independent of motoneuron excitation. *J. Neurophysiol.* 109, 2056–2063. doi: 10.1152/jn.00405.2012

Cornea-Hébert, V., Riad, M., Wu, C., Singh, S. K., and Descarries, L. (1999). Cellular and subcellular distribution of the serotonin 5-HT2A receptor in the central nervous system of adult rat. *J. Comp. Neurol.* 409, 187–209. doi: 10.1002/(sici)1096-9861(19990628)409:2<187::aid-cne2>3.0.co;2-p

Courtine, G., Gerasimenko, Y., van den Brand, R., Yew, A., Musienko, P., Zhong, H., et al. (2009). Transformation of nonfunctional spinal circuits into functional states after the loss of brain input. *Nat. Neurosci.* 12, 1333–1342. doi: 10.1038/nn.2401

Cowley, K. C., and Schmidt, B. J. (1994). A comparison of motor patterns induced by N-methyl-D-aspartate, acetylcholine and serotonin in the in vitro neonatal rat spinal cord. *Neurosci. Lett.* 171, 147–150. doi: 10.1016/0304-3940(94)90626-2

Cowley, K. C., and Schmidt, B. J. (1997). Regional distribution of the locomotor pattern-generating network in the neonatal rat spinal cord. *J. Neurophysiol.* 77, 247–259.

Cowley, K. C., Zaporozhets, E., Maclean, J. N., and Schmidt, B. J. (2005). Is NMDA receptor activation essential for the production of locomotor-like activity in the neonatal rat spinal cord? *J. Neurophysiol.* 94, 3805–3814. doi: 10.1152/jn.00016.2005

Doly, S., Fischer, J., Brisorgueil, M. J., Verge, D., and Conrath, M. (2005). Pre- and postsynaptic localization of the 5-HT7 receptor in rat dorsal spinal cord: immunocytochemical evidence. *J. Comp. Neurol.* 490, 256–269. doi: 10.1002/cne.20667

Doly, S., Madeira, A., Fischer, J., Brisorgueil, M. J., Daval, G., Bernard, R., et al. (2004). The 5-HT2A receptor is widely distributed in the rat spinal cord and mainly localized at the plasma membrane of postsynaptic neurons. *J. Comp. Neurol.* 472, 496–511. doi: 10.1002/cne.20082

Dunbar, M. J., Tran, M. A., and Whelan, P. J. (2010). Endogenous extracellular serotonin modulates the spinal locomotor network of the neonatal mouse. *J. Physiol.* 588, 139–156. doi: 10.1113/jphysiol.2009.177378

Dyck, J., Olsen, F., and Gosgnach, S. (2009). "The role of the V0 spinal interneurons in generating the locomotor rhythm," in *Society for Neuroscience Meeting 2009* (Chicago, IL: Neuroscience Meeting Planner), Program No. 177.178.

Etlin, A., Blivis, D., Ben-Zwi, M., and Lev-Tov, A. (2010). Long and short multifunicular projections of sacral neurons are activated by sensory input to produce

locomotor activity in the absence of supraspinal control. *J. Neurosci.* 30, 10324–10336. doi: 10.1523/JNEUROSCI.1208-10.2010

Feraboli-Lohnherr, D., Orsal, D., Yakovleff, A., Gimenez y Ribotta, M., and Privat, A. (1997). Recovery of locomotor activity in the adult chronic spinal rat after sublesional transplantation of embryonic nervous cells: specific role of serotonergic neurons. *Exp. Brain Res.* 113, 443–454. doi: 10.1007/pl00005597

Fouad, K., Rank, M. M., Vavrek, R., Murray, K. C., Sanelli, L., and Bennett, D. J. (2010). Locomotion after spinal cord injury depends on constitutive activity in serotonin receptors. *J. Neurophysiol.* 104, 2975–2984. doi: 10.1152/jn.00499.2010

Hammar, I., Bannatyne, B. A., Maxwell, D. J., Edgley, S. A., and Jankowska, E. (2004). The actions of monoamines and distribution of noradrenergic and serotoninergic contacts on different subpopulations of commissural interneurons in the cat spinal cord. *Eur. J. Neurosci.* 19, 1305–1316. doi: 10.1111/j.1460-9568.2004.03239.x

Heckman, C. J., Mottram, C., Quinlan, K., Theiss, R., and Schuster, J. (2009). Motoneuron excitability: the importance of neuromodulatory inputs. *Clin. Neurophysiol.* 120, 2040–2054. doi: 10.1016/j.clinph.2009.08.009

Hochman, S., Garraway, S. M., Machacek, D. W., and Shay, B. L. (2001). "5-HT receptors and the neuromodualatory control of spinal cord function," in *Motor Neurobiology of the Spinal Cord*, ed T. C. Cope (New York: CRC Press), 48–87.

Jankowska, E., Krutki, P., and Matsuyama, K. (2005). Relative contribution of Ia inhibitory interneurones to inhibition of feline contralateral motoneurones evoked via commissural interneurones. *J. Physiol.* 568, 617–628. doi: 10.1113/jphysiol.2005.088351

Jordan, L. M., Fabczak, H., Kisielnicka, E., Leszczyńska, A., Majczynski, H., Nagy, J. I., et al. (2010). "Segmental distribution of 5-HT2A and 5-HT2C receptor up-regulation one month after complete spinal cord injury," in *Society for Neuroscience Meeting* (San Diego, CA: Neuroscience Meeting Planner), Program No. 259.220.

Jordan, L. M., and Schmidt, B. J. (2002). Propriospinal neurons involved in the control of locomotion: potential targets for repair strategies? *Prog. Brain Res.* 137, 125–139. doi: 10.1016/s0079-6123(02)37012-2

Jordan, L. M., and Sławińska, U. (2011). Chapter 12—modulation of rhythmic movement: control of coordination. *Prog. Brain Res.* 188, 181–195. doi: 10.1016/B978-0-444-53825-3.00017-6

Kiehn, O., and Kjaerulff, O. (1996). Spatiotemporal characteristics of 5-HT and dopamine-induced rhythmic hindlimb activity in the in vitro neonatal rat. *J. Neurophysiol.* 75, 1472–1482.

Kjaerulff, O., and Kiehn, O. (1996). Distribution of networks generating and coordinating locomotor activity in the neonatal rat spinal cord in vitro: a lesion study. *J. Neurosci.* 16, 5777–5794.

Kong, X. Y., Wienecke, J., Chen, M., Hultborn, H., and Zhang, M. (2011). The time course of serotonin 2A receptor expression after spinal transection of rats: an immunohistochemical study. *Neuroscience* 177, 114–126. doi: 10.1016/j.neuroscience.2010.12.062

Kong, X. Y., Wienecke, J., Hultborn, H., and Zhang, M. (2010). Robust upregulation of serotonin 2A receptors after chronic spinal transection of rats: an immunohistochemical study. *Brain Res.* 1320, 60–68. doi: 10.1016/j.brainres.2010.01.030

Kudo, N., and Yamada, T. (1987). N-methyl-D,L-aspartate-induced locomotor activity in a spinal cord-hindlimb muscles preparation of the newborn rat studied in vitro. *Neurosci. Lett.* 75, 43–48. doi: 10.1016/0304-3940(87)90072-3

Landry, E. S., Lapointe, N. P., Rouillard, C., Levesque, D., Hedlund, P. B., and Guertin, P. A. (2006). Contribution of spinal 5-HT1A and 5-HT7 receptors to locomotor-like movement induced by 8-OH-DPAT in spinal cord-transected mice. *Eur. J. Neurosci.* 24, 535–546. doi: 10.1111/j.1460-9568.2006.04917.x

Lev-Tov, A., Etlin, A., and Blivis, D. (2010). Sensory-induced activation of pattern generators in the absence of supraspinal control. *Ann. N Y Acad. Sci.* 1198, 54–62. doi: 10.1111/j.1749-6632.2009.05424.x

Liu, J., Akay, T., Hedlund, P. B., Pearson, K. G., and Jordan, L. M. (2009). Spinal 5-HT7 receptors are critical for alternating activity during locomotion: in vitro neonatal and in vivo adult studies using 5-HT7 receptor knockout mice. *J. Neurophysiol.* 102, 337–348. doi: 10.1152/jn.91239.2008

Liu, J., and Jordan, L. M. (2005). Stimulation of the parapyramidal region of the neonatal rat brain stem produces locomotor-like activity involving spinal 5-HT7 and 5-HT2A receptors. *J. Neurophysiol.* 94, 1392–1404. doi: 10.1152/jn.00136.2005

Madriaga, M. A., McPhee, L. C., Chersa, T., Christie, K. J., and Whelan, P. J. (2004). Modulation of locomotor activity by multiple 5-HT and dopaminergic receptor subtypes in the neonatal mouse spinal cord. *J. Neurophysiol.* 92, 1566–1576. doi: 10.1152/jn.01181.2003

Maeshima, T., Ito, R., Hamada, S., Senzaki, K., Hamaguchi-Hamada, K., Shutoh, F., et al. (1998). The cellular localization of 5-HT2A receptors in the spinal cord and spinal ganglia of the adult rat. *Brain Res.* 797, 118–124. doi: 10.1016/s0006-8993(98)00360-6

Majczyński, H., Maleszak, K., Cabaj, A., and Sławińska, U. (2005). Serotonin-related enhancement of recovery of hind limb motor functions in spinal rats after grafting of embryonic raphe nuclei. *J. Neurotrauma* 22, 590–604. doi: 10.1089/neu.2005.22.590

McCrea, D. A., and Rybak, I. A. (2007). Modeling the mammalian locomotor CPG: insights from mistakes and perturbations. *Prog. Brain Res.* 165, 235–253. doi: 10.1016/s0079-6123(06)65015-2

McCrea, D. A., and Rybak, I. A. (2008). Organization of mammalian locomotor rhythm and pattern generation. *Brain Res. Rev.* 57, 134–146. doi: 10.1016/j.brainresrev.2007.08.006

Meisel, R. L., and Rakerd, B. (1982). Induction of hindlimb stepping movements in rats spinally transected as adults or as neonates. *Brain Res.* 240, 353–356. doi: 10.1016/0006-8993(82)90235-9

Menelaou, E., and McLean, D. L. (2013). Speed control: spinal interneurons with crossed purposes. *Curr. Biol.* 23, R716–R718. doi: 10.1016/j.cub.2013.07.064

Murray, K. C., Nakae, A., Stephens, M. J., Rank, M., D'Amico, J., Harvey, P. J., et al. (2010). Recovery of motoneuron and locomotor function after spinal cord injury depends on constitutive activity in 5-HT2C receptors. *Nat. Med.* 16, 694–700. doi: 10.1038/nm.2160

Musienko, P., van den Brand, R., Marzendorfer, O., Roy, R. R., Gerasimenko, Y., Edgerton, V. R., et al. (2011). Controlling specific locomotor behaviors through multidimensional monoaminergic modulation of spinal circuitries. *J. Neurosci.* 31, 9264–9278. doi: 10.1523/JNEUROSCI.5796-10.2011

Nakae, A., Nakai, K., Tanaka, T., Takashina, M., Hagihira, S., Shibata, M., et al. (2008). Serotonin2C receptor mRNA editing in neuropathic pain model. *Neurosci. Res.* 60, 228–231. doi: 10.1016/j.neures.2007.10.004

Navarrett, S., Collier, L., Cardozo, C., and Dracheva, S. (2012). Alterations of serotonin 2C and 2A receptors in response to T10 spinal cord transection in rats. *Neurosci. Lett.* 506, 74–78. doi: 10.1016/j.neulet.2011.10.052

Noga, B. R., Johnson, D. M., Riesgo, M. I., and Pinzon, A. (2009). Locomotor-activated neurons of the cat. I. Serotonergic innervation and co-localization of 5-HT7, 5-HT2A and 5-HT1A receptors in the thoraco-lumbar spinal cord. *J. Neurophysiol.* 102, 1560–1576. doi: 10.1152/jn.91179.2008

Olsen, F. G. (2011). "The V0 Interneurons: First-order Interneurons of the Locomotor CPG?" in *MSc Thesis* (Edmonton: University of Alberta).

Orsal, D., Barthe, J. Y., Antri, M., Feraboli-Lohnherr, D., Yakovleff, A., Gimenez, Y. R. M., et al. (2002). Locomotor recovery in chronic spinal rat: long-term pharmacological treatment or transplantation of embryonic neurons? *Prog. Brain Res.* 137, 213–230. doi: 10.1016/s0079-6123(02)37018-3

Pearlstein, E., Ben Mabrouk, F., Pflieger, J. F., and Vinay, L. (2005). Serotonin refines the locomotor-related alternations in the in vitro neonatal rat spinal cord. *Eur. J. Neurosci.* 21, 1338–1346. doi: 10.1111/j.1460-9568.2005.03971.x

Pearson, K. G., and Rossignol, S. (1991). Fictive motor patterns in chronic spinal cats. *J. Neurophysiol.* 66, 1874–1887.

Perrier, J. F., Rasmussen, H. B., Christensen, R. K., and Petersen, A. V. (2013). Modulation of the intrinsic properties of motoneurons by serotonin. *Curr. Pharm. Des.* 19, 4371–4384. doi: 10.2174/1381612811319990341

Ribotta, M. G., Provencher, J., Feraboli-Lohnherr, D., Rossignol, S., Privat, A., and Orsal, D. (2000). Activation of locomotion in adult chronic spinal rats is achieved by transplantation of embryonic raphe cells reinnervating a precise lumbar level. *J. Neurosci.* 20, 5144–5152.

Rossignol, S., Dubuc, R., and Gossard, J. P. (2006). Dynamic sensorimotor interactions in locomotion. *Physiol. Rev.* 86, 89–154. doi: 10.1152/physrev.00028.2005

Schmidt, B. J., and Jordan, L. M. (2000). The role of serotonin in reflex modulation and locomotor rhythm production in the mammalian spinal cord. *Brain Res. Bull.* 53, 689–710. doi: 10.1016/s0361-9230(00)00402-0

Sławińska, U., Jordan, L. M., Cabaj, A. M., Burger, B., Fabczak, H., and Majczyński, H. (2012a). "Locomotion of intact adult rats is controlled by 5-HT2A and 5-HT7 but not 5-HT2C receptors," in *Society for Neuroscience Meeting* (New Orleans, LA: Neuroscience Meeting Planner), Program No. 85.09.

Sławińska, U., Majczyński, H., Dai, Y., and Jordan, L. M. (2012b). The upright posture improves plantar stepping and alters responses to serotonergic drugs in spinal rats. *J. Physiol.* 590, 1721–1736. doi: 10.1113/jphysiol.2011.224931

Sławińska, U., Majczyński, H., and Djavadian, R. (2000). Recovery of hindlimb motor functions after spinal cord transection is enhanced by grafts of the embryonic raphe nuclei. *Exp. Brain Res.* 132, 27–38. doi: 10.1007/s002219900323

Sławińska, U., Miazga, K., Cabaj, A. M., Leszczyńska, A. N., Majczyński, H., Nagy, J. I., et al. (2013). Grafting of fetal brainstem 5-HT neurons into the sublesional spinal cord of paraplegic rats restores coordinated hindlimb locomotion. *Exp. Neurol.* 247, 572–581. doi: 10.1016/j.expneurol.2013.02.008

Sławińska, U., Miazga, K., and Jordan, L. M. (2014). The role of serotonin in the control of locomotor movements and strategies for restoring locomotion after spinal cord injury. *Acta Neurobiol. Exp. (Wars)* 74, 172–187.

Sławińska, U., Miazga, K., Majczyński, H., Cabaj, A. M., and Jordan, L. M. (2011). Differential effects on locomotor-like movements of paraplegic rats produced by activation of 5-HT2 or 5-HT7 receptors. *Acta Neurobiol. Exp.* 71, 83.

Sławińska, U., Rossignol, S., Bennett, D. J., Schmidt, B. J., Frigon, A., Fouad, K., et al. (2012c). Comment on "Restoring voluntary control of locomotion after paralyzing spinal cord injury". *Science* 338:328. doi: 10.1126/science.1226082

Sqalli-Houssaini, Y., Cazalets, J. R., and Clarac, F. (1993). Oscillatory properties of the central pattern generator for locomotion in neonatal rats. *J. Neurophysiol.* 70, 803–813.

Talpalar, A. E., Bouvier, J., Borgius, L., Fortin, G., Pierani, A., and Kiehn, O. (2013). Dual-mode operation of neuronal networks involved in left-right alternation. *Nature* 500, 85–88. doi: 10.1038/nature12286

Ung, R. V., Landry, E. S., Rouleau, P., Lapointe, N. P., Rouillard, C., and Guertin, P. A. (2008). Role of spinal 5-HT2 receptor subtypes in quipazine-induced hindlimb movements after a low-thoracic spinal cord transection. *Eur. J. Neurosci.* 28, 2231–2242. doi: 10.1111/j.1460-9568.2008.06508.x

van den Brand, R., Heutschi, J., Barraud, Q., Digiovanna, J., Bartholdi, K., Huerlimann, M., et al. (2012). Restoring voluntary control of locomotion after paralyzing spinal cord injury. *Science* 336, 1182–1185. doi: 10.1126/science.1217416

Zar, J. H. (1974). "Circular distribution," in *Biostatistical Analysis*, eds W. D. Mcelroy and C. P. Swanson (Emglewood Cliffs, NJ: Prentice Hall), 310–327.

Zhong, G., Diaz-Rios, M., and Harris-Warrick, R. M. (2006a). Intrinsic and functional differences among commissural interneurons during fictive locomotion and serotonergic modulation in the neonatal mouse. *J. Neurosci.* 26, 6509–6517. doi: 10.1523/jneurosci.1410-06.2006

Zhong, G., Diaz-Rios, M., and Harris-Warrick, R. M. (2006b). Serotonin modulates the properties of ascending commissural interneurons in the neonatal mouse spinal cord. *J. Neurophysiol.* 95, 1545–1555. doi: 10.1152/jn.01103.2005

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Serotonergic modulation of post-synaptic inhibition and locomotor alternating pattern in the spinal cord

Florian Gackière and Laurent Vinay*

Institut de Neurosciences de la Timone, UMR 7289, CNRS, Aix Marseille Université, Marseille, France

Edited by:
Shawn Hochman, Emory University, USA

Reviewed by:
Pete Wenner, Emory University, USA
Jorge Noel Quevedo, Centro de Investigación y de Estudios Avanzados (CINVESTAV), Mexico
Michael O'Donovan, National Institutes of Health, USA

***Correspondence:**
Laurent Vinay, Institut de Neurosciences de la Timone, UMR 7289, CNRS, Aix Marseille Université, Campus Santé Timone, 27, Boulevard Jean Moulin, 13385 Marseille cx 5, France
e-mail: laurent.vinay@univ-amu.fr

The central pattern generators (CPGs) for locomotion, located in the lumbar spinal cord, are functional at birth in the rat. Their maturation occurs during the last few days preceding birth, a period during which the first projections from the brainstem start to reach the lumbar enlargement of the spinal cord. Locomotor burst activity in the mature intact spinal cord alternates between flexor and extensor motoneurons through reciprocal inhibition and between left and right sides through commisural inhibitory interneurons. By contrast, all motor bursts are in phase in the fetus. The alternating pattern disappears after neonatal spinal cord transection which suppresses supraspinal influences upon the locomotor networks. This article will review the role of serotonin (5-HT), in particular 5-HT_2 receptors, in shaping the alternating pattern. For instance, pharmacological activation of these receptors restores the left-right alternation after injury. Experiments aimed at either reducing the endogenous level of serotonin in the spinal cord or blocking the activation of 5-HT_2 receptors. We then describe recent evidence that the action of 5-HT_2 receptors is mediated, at least in part, through a modulation of chloride homeostasis. The postsynaptic action of GABA and glycine depends on the intracellular concentration of chloride ions which is regulated by a protein in the plasma membrane, the K^+-Cl^- cotransporter (KCC2) extruding both K^+ and Cl^- ions. Absence or reduction of KCC2 expression leads to a depolarizing action of GABA and glycine and a marked reduction in the strength of postsynaptic inhibition. This latter situation is observed early during development and in several pathological conditions, such as after spinal cord injury, thereby causing spasticity and chronic pain. It was recently shown that specific activation of 5-HT_{2A} receptors is able to up-regulate KCC2, restore endogenous inhibition and reduce spasticity.

Keywords: 5-HT2A receptor, 5-HT7 receptor, chloride homeostasis, KCC2 transporter, reciprocal inhibition

INTRODUCTION

It is well established that the basic rhythmic activity underlying locomotion is generated by interneuronal networks within the spinal cord called central pattern generators (CPGs; Grillner and Wallén, 1985). These are functional at birth in the rat as shown by experiments using *in vitro* spinal cord preparations isolated from neonates (Cazalets et al., 1992). In these preparations, the most effective pharmacological cocktails to induce fictive locomotion include serotonin (5-HT; Cazalets et al., 1992; Madriaga et al., 2004). There is considerable evidence that 5-HT plays a key role in locomotion. Chronic recordings from 5-HT neurons in awake cats demonstrated a correlation between single unit activity and locomotor activity (Veasey et al., 1995) suggesting that the 5-HT system facilitates motor output and concurrently inhibits sensory information processing (Jacobs and Fornal, 1993). Stimulation of a discrete population of 5-HT neurons in the parapyramidal region (PPR) of the medulla elicits locomotor-like activity in the neonatal rat isolated brain stem-spinal cord preparation (Liu and Jordan, 2005). Most locomotor-activated cells, as revealed by expression of the activity-dependent marker *c-fos*, co-localize with 5-HT_7, 5-HT_{2A}, and 5-HT_{1A} receptors (Noga et al., 2009). The intrinsic 5-HT system contributes significantly to locomotor pattern generation (Zhang and Grillner, 2000; Pearlstein et al., 2005, 2011). In addition, there is increasing evidence that recovery of locomotion after spinal cord injury (SCI) can be facilitated by systemic or intrathecal application of 5-HT or various 5-HT receptor agonists (Feraboli-Lohnherr et al., 1999; Kim et al., 2001; Antri et al., 2002, 2005; Landry et al., 2006), or transplantation of embryonic 5-HT neurons into the spinal cord caudal to the lesion (Feraboli-Lohnherr et al., 1997; Ribotta et al., 1998a,b, 2000; Kim et al., 1999; Sławińska et al., 2000, 2013; Majczyński et al., 2005).

Serotonin has a number of effects on the spinal cord, including the control of motoneuron and interneuron excitability and afferent transmission (Schmidt and Jordan, 2000; Abbinanti and Harris-Warrick, 2012; Abbinanti et al., 2012). The present review will focus on the contribution of 5-HT, with emphasis on 5-HT_2 and 5-HT_7 receptors, in shaping the alternating pattern, and on one of the mechanisms underlying this effect, strengthening of post-synaptic inhibition, by modulation of chloride homeostasis.

ENDOGENOUS SEROTONIN IS IMPORTANT FOR THE EXPRESSION OF A LEFT-RIGHT ALTERNATING PATTERN

Pharmacological activation of the CPGs *in vitro* in newborn animals evokes a fictive locomotor pattern consisting of alternation of motor bursts between both the left and right sides of the lumbar spinal cord, and flexors and extensors on one side (Cazalets et al., 1992; Kiehn and Kjaerulff, 1996). On the embryonic day (E)16 (i.e., 5 days prior to birth), the same kind of experiments reveal a motor pattern with all bursts in phase (Iizuka et al., 1998; Nakayama et al., 2002). In rats, the transition from left-right synchrony to alternation occurs around E18 and is due to the maturation of inhibitory connections between the two sides, and a shift in GABA/glycine synaptic potentials from excitation to inhibition (Wu et al., 1992; see below).

These major changes in locomotor network operation occur shortly after the arrival in the lumbar enlargement of the first axons descending from the brainstem, suggesting that descending pathways may contribute to the maturation of spinal networks (Vinay et al., 2000, 2002). Serotonergic fibers start to arrive in the lumbar gray matter by E17 (Bregman, 1987; Rajaofetra et al., 1989). Projections arising from the raphe nuclei are among the earliest axons to reach the upper lumbar segments in the rat (Lakke, 1997). They are the source of almost all the 5-HT in the lumbar spinal cord in mammals (reviewed by Schmidt and Jordan, 2000).

A number of experiments support the conclusion that descending pathways, in particular 5-HT projections, play a role in the maturation and/or the operation of the lumbar CPG. Daily *in vivo* injections of p-chloro-phenylalanine (PCPA), a 5-HT synthesis inhibitor, starting the day of birth markedly reduce 5-HT immunoreactivity in the lumbar enlargement within 3–4 days (Pflieger et al., 2002). Depletion of endogenous 5-HT during early postnatal development induces an asymmetry of posture (Pflieger et al., 2002) and deficits in locomotion (Myoga et al., 1995), both of which indicate that the interlimb coordination is impaired. In addition, kittens or rats that have undergone a complete spinal cord transection at birth exhibit synchronous air stepping during the first postnatal week (Bradley and Smith, 1988a,b; Norreel et al., 2003). Quipazine, a 5-HT_2 receptor agonist promotes alternating air stepping in intact neonatal rats (Brumley et al., 2012). The 5-HT_7 receptors also appear to play an important role as the antagonist, SB-269970, applied directly to the spinal cord consistently disrupts locomotion in adult mice (Liu et al., 2009).

In vitro experiments showed that 5-HT, when added with *N*-methyl-d,l-aspartate (NMA) to neonatal rat isolated spinal cord preparations, strongly strengthens left/right and flexor/extensor alternation, an effect that is at least partly dependent on activation of 5-HT_2 receptors (**Figures 1A,B**; Pearlstein et al., 2005). The NMA-induced motor pattern is strongly affected in PCPA-treated animals (Pearlstein et al., 2005). Both left/right and L3-L5 alternations are weak but recover after adding 5-HT (**Figure 1B**). A contribution of endogenous 5-HT is further supported by the observations that ketanserin (a 5-HT_2 receptor antagonist, **Figure 1B**) or SB-269970 (a 5-HT_7 receptor antagonist, **Figure 1B**) disorganizes the locomotor pattern (makes the cross-correlation coefficient less negative) induced by either NMA (Pearlstein et al., 2005; Liu et al., 2009; Jordan and Slawinska, 2011) or electrical stimulation of the brainstem (Liu and Jordan, 2005). Finally, in spinal cords isolated from 5-HT_7 receptor knock-out mice, 5-HT produces either uncoordinated rhythmic activity or results in synchronous discharges of the ventral roots (Liu et al., 2009).

Together, these observations suggest that 5-HT_2 and 5-HT_7 receptors appear to mediate the effect of serotonin to enhance and stabilize both left-right and flexor-extensor alternation. Other 5-HT receptors, such as 5-HT_{1A}, may have an opposite effect to reduce reciprocal inhibition as shown in *Xenopus laevis* (Wedderburn and Sillar, 1994; McDearmid et al., 1997).

THE STRENGTH OF POSTSYNAPTIC INHIBITION IS REDUCED AFTER SCI DUE TO A DYSREGULATION OF CHLORIDE HOMEOSTASIS

Ipsilateral co-contraction of flexors and extensors is commonly observed in SCI patients (Harkema, 2008). The strength of several well-characterized inhibitory mechanisms such as presynaptic (Katz, 1999), recurrent (Mazzocchio and Rossi, 1997) and reciprocal (Boorman et al., 1996) inhibition is reduced after SCI. A reciprocal facilitation, instead of reciprocal inhibition, may even appear (Crone et al., 2003). Similarly, crossed inhibition of contralateral motoneurons by group II muscle afferents in intact cats is inverted to crossed excitation in spinal cats (Aggelopoulos et al., 1996). The mechanisms responsible for the decrease in strength of postsynaptic inhibition were recently identified. Briefly, in healthy mature cells, activation of $GABA_A$ and glycine receptors leads to chloride entry which causes membrane hyperpolarization. This occurs because the intracellular concentration of chloride ions ($[Cl^-]_i$) is maintained at low levels by the potassium-chloride co-transporters KCC2 that extrude chloride from the cell (Payne et al., 2003; Vinay and Jean-Xavier, 2008; Blaesse et al., 2009; Chamma et al., 2012). There is now abundant evidence that an increase in $[Cl^-]_i$, most often recorded/visualized as a depolarizing shift of the chloride equilibrium potential, reduces the strength of postsynaptic inhibition or may even switch it towards excitation or promote facilitation of concomitant excitatory inputs (van den Pol et al., 1996; Gao et al., 1998b; Gulledge and Stuart, 2003; Prescott et al., 2006; Jean-Xavier et al., 2007; Doyon et al., 2011).

The expression of KCC2 in the plasma membrane of lumbar motoneurons below the lesion is reduced after spinal cord injury, thereby causing a depolarizating shift in the chloride equilibrium potential (Boulenguez et al., 2010). Similar results were described in the superficial layers of the dorsal horn either after SCI (Cramer et al., 2008; Lu et al., 2008) or following peripheral nerve injury (Coull et al., 2003, 2005). These observations were shown to contribute to spasticity and chronic pain, respectively. To conclude, dysregulation of chloride homeostasis can account for the reduction in strength of postsynaptic inhibition or even a switch to facilitation after SCI.

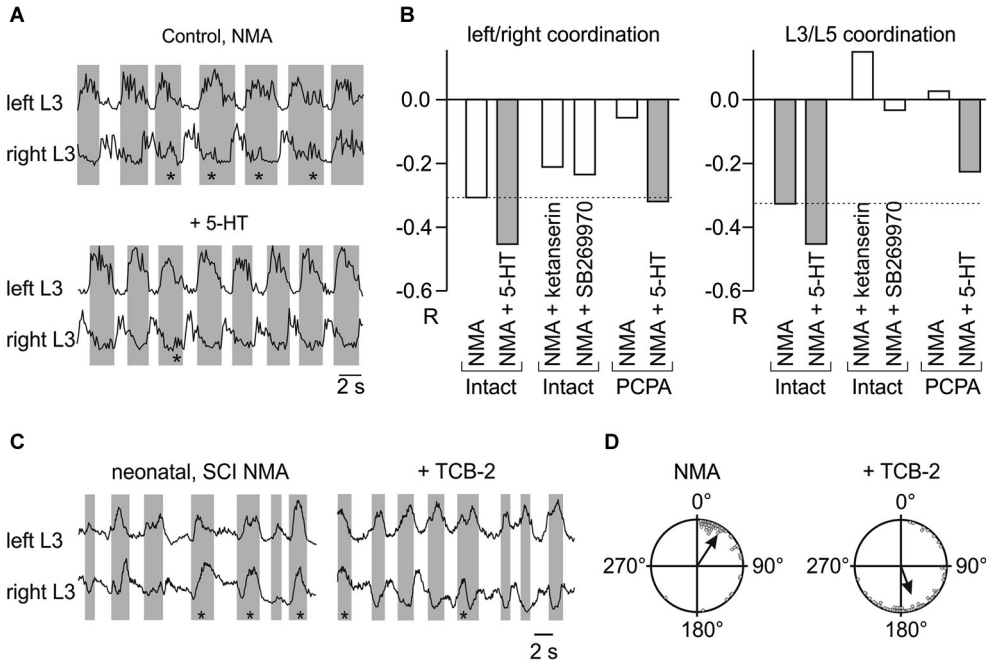

FIGURE 1 | 5-HT enhances the NMA-induced fictive locomotor pattern in the isolated neonatal rat spinal cord. (A) Activity recorded from the 3rd left and right lumbar ventral roots after rectification and integration. Bath application of NMA (18 μM) induced fictive locomotion characterized by left-right alternation. Addition of 5-HT (5 μM) stabilized these alternations. Note the lower occurrence of left-right concomitant bursting (asterisks). **(B)** Mean correlation coefficients (R) for left-right and L3/L5 relationships in various experimental conditions, after adding 5-HT, blocking 5-HT_{2A} receptors with ketanserin or 5-HT_7 receptors with SB269970, or blocking 5-HT synthesis by PCPA. **(A)** and **(B)** are adapted from Pearlstein et al. (2005). **(C,D)** Activation of 5-HT_{2A} receptors *in vitro* restores the left-right alternating locomotor pattern 5 days after neonatal spinal cord transection. **(C)** Integrated recordings from the left and right L3 ventral roots at P5 in the presence of NMA (16 μM) alone (note the high occurrence of synchronous bursts, asterisks) or together with 5-HT_{2A} receptor agonist (4-bromo-3,6-dimethoxybenzocyclobuten-1-yl) methylamine hydrobromide (TCB-2): (0.1 μM). **(D)** Distribution of phase relationships between left and right ventral root bursts in NMA alone (Left) and NMA plus TCB-2 (Right) in all of the spinal animals (P5–P6; $n = 6$). **(C)** and **(D)** are adapted from Bos et al. (2013).

SEROTONIN ENABLES RESTORATION OF COORDINATED LOCOMOTION AFTER SPINAL CORD INJURY

Following a neonatal spinal cord transection that disorganizes the left-right alternating pattern, left-right hindlimb alternation is restored after injecting (±)-2,5-dimethoxy-4-iodoamphetamine hydrochloride (DOI), a 5-HT_2 receptor agonist (Norreel et al., 2003). In addition, however, sensory inputs from the moving limbs *in vivo* can also promote left-right alternations under certain circumstances. In kittens with spinal cord transection at birth, alternation is more pronounced during treadmill stepping (∼40% of alternating steps) than during air stepping (∼3%), suggesting that rhythmic ground contact may promote an alternating gait (Bradley and Smith, 1988b). However, in recent experiments in intact neonatal rats, in which a substrate (elastic, stiff, or none) was placed beneath their limbs so that the feet could make plantar surface contact with the substrate, pups treated with quipazine showed significantly more alternating fore- and hindlimb steps than pups treated with saline (Brumley et al., 2012). In rats with a neonatal spinal cord transection, the fictive locomotor pattern induced by excitatory amino acids does not exhibit any left/right alternation. However, strong alternation is restored when 5-HT is added to the bath (Norreel et al., 2003).

Serotonergic 5-HT_2 and 5-HT_7 receptor agonists have repeatedly been shown to promote locomotor recovery after SCI in adult rodents (Barbeau and Rossignol, 1990; Antri et al., 2002; Kao et al., 2006; Landry et al., 2006; Ung et al., 2008; Courtine et al., 2009; Murray et al., 2010; Jordan and Slawinska, 2011; Musienko et al., 2011; van den Brand et al., 2012). Combined activation of both receptor subtypes is more effective than activation of either receptor alone (Antri et al., 2005; Landry et al., 2006; Courtine et al., 2009; Sławińska et al., 2012). In rats with a complete thoracic spinal cord transection, grafts of embryonic serotonergic neurons improve locomotor recovery (Feraboli-Lohnherr et al., 1997; Ribotta et al., 2000; Majczyński et al., 2005; Sławińska et al., 2013). Importantly, both inter- and intralimb coordinations are improved by grafting embryonic 5-HT neurons after SCI in adult rats and the effectiveness of the transplants arises from intrinsic activation of 5-HT_2 and 5-HT_7 receptors (Sławińska et al., 2000, 2013).

MODULATION OF INHIBITORY SYNAPTIC TRANSMISSION BY 5-HT_2 AND 5-HT_7 RECEPTORS

There are, in principle, various ways through which 5-HT may strengthen inhibitory synaptic transmission. The most obvious explanation for the improvement of inter- and intralimb

alternating motor activity following activation of 5-HT_2 and 5-HT_7 receptors is that this activation excites inhibitory interneurons responsible for coordinating flexor/extensor and left/right activity (Aggelopoulos et al., 1996; Pearlstein et al., 2005; Sławińska et al., 2013). Exogenously applied 5-HT (Lewis et al., 1993; Shen and Andrade, 1998; Abi-Saab et al., 1999; Xie et al., 2012) and endogenous 5-HT (Iwasaki et al., 2013) have been shown to activate GABAergic and/or glycinergic interneurons *via* 5-HT_2 receptors in the CNS including the spinal cord. Activation of 5-$HT_{2A/2C}$ receptors enhances glycine and/or GABA responses in spinal neurons in the rat (Xu et al., 1996, 1998; Li et al., 2000) and spontaneous inhibitory postsynaptic currents in the substantia gelatinosa (Xie et al., 2012). These effects involve, at least in part, a presynaptic facilitation of GABA/glycine release (Wang and Zucker, 1998; Xie et al., 2012). It has been shown that 5-HT_7 receptor activation in the hippocampal CA1 area results in an enhancement of GABAergic transmission *via* two mechanisms (Tokarski et al., 2011). The first one involves an enhancement of excitatory glutamatergic input to GABAergic interneurons and is likely to be mediated by presynaptic 5-HT_7 receptors. The second effect, most likely related to the activation of 5-HT_7 receptors located on interneurons, results in an enhancement of GABA release.

Developmental studies provide interesting information about 5-HT modulation of inhibitory synaptic transmission. Maturation of inhibition in the lumbar spinal cord occurs during perinatal development in rodents. The key events are as follows: (1) the density of glycine currents (Gao and Ziskind-Conhaim, 1995) and receptors (Sadlaoud et al., 2010) increases whereas that of $GABA_A$ currents and receptors drop concomitantly. (2) Inhibitory postsynaptic potentials switch from depolarizing to hyperpolarizing (Takahashi, 1984; Wu et al., 1992; Gao et al., 1998a; Jean-Xavier et al., 2006; Delpy et al., 2008; Stil et al., 2011), mostly due to the up-regulation of KCC2 expression (Jean-Xavier et al., 2006; Stil et al., 2009). A neonatal spinal cord transection at birth, which removes all descending modulatory influences from the brainstem, prevents both the depolarization-to-hyperpolarization switch (Jean-Xavier et al., 2006; Bos et al., 2013) and the developmental down-regulation of $GABA_A$ currents and receptors (Sadlaoud et al., 2010). Interestingly, up-regulation of glycine receptors is not affected by spinal transection. Chronic treatment with the 5-HT_2 receptor agonist, DOI, throughout the first postnatal week restores the hyperpolarizing shift of the chloride equilibrium potential (Bos et al., 2013) and the down-regulation of $GABA_A$ receptors, without any significant effect on glycine receptors (Sadlaoud et al., 2010). These data suggested that 5-HT plays a role in the maturation of GABAergic synaptic transmission but that the up-regulation of glycinergic receptors does not depend on descending modulation from the brainstem.

Because the strength of inhibition depends on $[Cl^-]_i$, 5-HT, in principle, may strengthen inhibitory synaptic transmission by increasing KCC2 function. This hypothesis is supported by recent results showing that activation of 5-HT_{2A} receptors shifts the chloride equilibrium potential in the hyperpolarizing direction (Bos et al., 2013). This effect is mediated by an up-regulation of KCC2 function and involves a protein kinase C (PKC)-dependent mechanism. After SCI, acute addition of a specific 5-HT_{2A} receptor agonist, TCB-2, restores endogenous inhibition and thereby reduces spasticity and restores left-right alternation during fictive locomotion (**Figures 1C,D**; Bos et al., 2013). Interestingly, 5-HT_{2A} and 5-$HT_{2B/2C}$ receptors were shown in the latter study to have opposite effects on KCC2 function. Consistent with these observations, 5-HT_{2A} and 5-HT_{2C} receptors exert opposing effects on both locomotor activity in mice and spinal reflexes in rats (Machacek et al., 2001; Halberstadt et al., 2009). As 5-$HT_{2B/2C}$ receptors become constitutively active (spontaneously active without 5-HT) after SCI (Murray et al., 2010, 2011), this constitutive activity may be partly responsible for the depolarizing shift of the chloride equilibrium potential after SCI (Boulenguez et al., 2010).

CONCLUSIONS AND FUTURE DIRECTIONS

This review has shown that 5-HT plays a critical role in shaping the locomotor pattern by promoting left-right and flexor-extensor alternation, thereby raising the question of whether serotonin descending systems should be formally included as components of the CPGs for locomotion (Jordan and Slawinska, 2011). According to the initial definition of CPGs (Grillner and Wallén, 1985), "the term CPGs refers to function, not a circumscribed anatomical entity. The individual neurons that constitute the CPG may in principle be located in widely separate parts of the central nervous system".

As combined exogenous application of both 5-HT_7 and 5-HT_2 receptor agonists is more effective than activation of either of these receptors alone (Antri et al., 2005; Landry et al., 2006; Musienko et al., 2011; Sławińska et al., 2012), it will be important to identify whether the mechanisms by which 5-HT_7 receptors affect alternating motor activities also involve chloride homeostasis as is the case for 5-HT_{2A} receptors. Although the present review focused on serotonin, SCI removes not only serotonergic inputs but also dopaminergic and noradrenergic inputs to neurons below the lesion. The contribution of these pathways to the alternating locomotor pattern and regulation of chloride homeostasis should be investigated further.

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REFERENCES

Abbinanti, M. D., and Harris-Warrick, R. M. (2012). Serotonin modulates multiple calcium current subtypes in commissural interneurons of the neonatal mouse. *J. Neurophysiol.* 107, 2212–2219. doi: 10.1152/jn.00768.2011

Abbinanti, M. D., Zhong, G., and Harris-Warrick, R. M. (2012). Postnatal emergence of serotonin-induced plateau potentials in commissural interneurons of the mouse spinal cord. *J. Neurophysiol.* 108, 2191–2202. doi: 10.1152/jn.00336.2012

Abi-Saab, W. M., Bubser, M., Roth, R. H., and Deutch, A. Y. (1999). 5-HT2 receptor regulation of extracellular GABA levels in the prefrontal cortex. *Neuropsychopharmacology* 20, 92–96. doi: 10.1016/s0893-133x(98)00046-3

Aggelopoulos, N. C., Burton, M. J., Clarke, R. W., and Edgley, S. A. (1996). Characterization of a descending system that enables crossed group II inhibitory reflex pathways in the cat spinal cord. *J. Neurosci.* 16, 723–729.

Antri, M., Barthe, J. Y., Mouffle, C., and Orsal, D. (2005). Long-lasting recovery of locomotor function in chronic spinal rat following chronic combined pharmacological stimulation of serotonergic receptors with 8-OHDPAT and quipazine. *Neurosci. Lett.* 384, 162–167. doi: 10.1016/j.neulet.2005.04.062

Antri, M., Orsal, D., and Barthe, J. Y. (2002). Locomotor recovery in the chronic spinal rat: effects of long-term treatment with a 5-HT2 agonist. *Eur. J. Neurosci.* 16, 467–476. doi: 10.1046/j.1460-9568.2002.02088.x

Barbeau, H., and Rossignol, S. (1990). The effects of serotonergic drugs on the locomotor pattern and on cutaneous reflexes of the adult chronic spinal cat. *Brain Res.* 514, 55–67. doi: 10.1016/0006-8993(90)90435-e

Blaesse, P., Airaksinen, M. S., Rivera, C., and Kaila, K. (2009). Cation-chloride cotransporters and neuronal function. *Neuron* 61, 820–838. doi: 10.1016/j.neuron.2009.03.003

Boorman, G. I., Lee, R. G., Becker, W. J., and Windhorst, U. R. (1996). Impaired "natural reciprocal inhibition" in patients with spasticity due to incomplete spinal cord injury. *Electroencephalogr. Clin. Neurophysiol.* 101, 84–92. doi: 10.1016/0924-980x(95)00262-j

Bos, R., Sadlaoud, K., Boulenguez, P., Buttigieg, D., Liabeuf, S., Brocard, C., et al. (2013). Activation of 5-HT2A receptors upregulates the function of the neuronal K-Cl cotransporter KCC2. *Proc. Natl. Acad. Sci. U S A* 110, 348–353. doi: 10.1073/pnas.1213680110

Boulenguez, P., Liabeuf, S., Bos, R., Bras, H., Jean-Xavier, C., Brocard, C., et al. (2010). Down-regulation of the potassium-chloride cotransporter KCC2 contributes to spasticity after spinal cord injury. *Nat. Med.* 16, 302–307. doi: 10.1038/nm.2107

Bradley, N. S., and Smith, J. L. (1988a). Neuromuscular patterns of stereotypic hindlimb behaviors in the first two postnatal months. I. Stepping in normal kittens. *Brain Res.* 466, 37–52. doi: 10.1016/0165-3806(88)90084-3

Bradley, N. S., and Smith, J. L. (1988b). Neuromuscular patterns of stereotypic hindlimb behaviors in the first two postnatal months. II. Stepping in spinal kittens. *Brain Res.* 466, 53–67. doi: 10.1016/0165-3806(88)90085-5

Bregman, B. S. (1987). Development of serotonin immunoreactivity in the rat spinal cord and its plasticity after neonatal spinal cord lesions. *Brain Res.* 431, 245–263.

Brumley, M. R., Roberto, M. E., and Strain, M. M. (2012). Sensory feedback modulates quipazine-induced stepping behavior in the newborn rat. *Behav. Brain Res.* 229, 257–264. doi: 10.1016/j.bbr.2012.01.006

Cazalets, J. R., Sqalli-Houssaini, Y., and Clarac, F. (1992). Activation of the central pattern generators for locomotion by serotonin and excitatory amino acids in neonatal rat. *J. Physiol.* 455, 187–204.

Chamma, I., Chevy, Q., Poncer, J. C., and Levi, S. (2012). Role of the neuronal K-Cl co-transporter KCC2 in inhibitory and excitatory neurotransmission. *Front. Cell. Neurosci.* 6:5. doi: 10.3389/fncel.2012.00005

Coull, J. A., Beggs, S., Boudreau, D., Boivin, D., Tsuda, M., Inoue, K., et al. (2005). BDNF from microglia causes the shift in neuronal anion gradient underlying neuropathic pain. *Nature* 438, 1017–1021. doi: 10.1038/nature04223

Coull, J. A., Boudreau, D., Bachand, K., Prescott, S. A., Nault, F., Sik, A., et al. (2003). Trans-synaptic shift in anion gradient in spinal lamina I neurons as a mechanism of neuropathic pain. *Nature* 424, 938–942. doi: 10.1038/nature01868

Courtine, G., Gerasimenko, Y., van den Brand, R., Yew, A., Musienko, P., Zhong, H., et al. (2009). Transformation of nonfunctional spinal circuits into functional states after the loss of brain input. *Nat. Neurosci.* 12, 1333–1342. doi: 10.1038/nn.2401

Cramer, S. W., Baggott, C., Cain, J., Tilghman, J., Allcock, B., Miranpuri, G., et al. (2008). The role of cation-dependent chloride transporters in neuropathic pain following spinal cord injury. *Mol. Pain* 4:36. doi: 10.1186/1744-8069-4-36

Crone, C., Johnsen, L. L., Biering-Sorensen, F., and Nielsen, J. B. (2003). Appearance of reciprocal facilitation of ankle extensors from ankle flexors in patients with stroke or spinal cord injury. *Brain* 126, 495–507. doi: 10.1093/brain/awg036

Delpy, A., Allain, A. E., Meyrand, P., and Branchereau, P. (2008). NKCC1 cotransporter inactivation underlies embryonic development of chloride-mediated inhibition in mouse spinal motoneuron. *J. Physiol.* 586, 1059–1075. doi: 10.1113/jphysiol.2007.146993

Doyon, N., Prescott, S. A., Castonguay, A., Godin, A. G., Kroger, H., and De Koninck, Y. (2011). Efficacy of synaptic inhibition depends on, multiple., dynamically interacting mechanisms implicated in chloride homeostasis. *PLoS Comput. Biol.* 7:e1002149. doi: 10.1371/journal.pcbi.1002149

Feraboli-Lohnherr, D., Barthe, J. Y., and Orsal, D. (1999). Serotonin-induced activation of the network for locomotion in adult spinal rats. *J. Neurosci. Res.* 55, 87–98. doi: 10.1002/(sici)1097-4547(19990101)55:1<87::aid-jnr10>3.3.co;2-r

Feraboli-Lohnherr, D., Orsal, D., Yakovleff, A., Giménez y Ribotta, M., and Privat, A. (1997). Recovery of locomotor activity in the adult chronic spinal rat after sublesional transplantation of embryonic nervous cells: specific role of serotonergic neurons. *Exp. Brain Res.* 113, 443–454. doi: 10.1007/pl00005597

Gao, X. B., Chen, G., and van den Pol, A. N. (1998b). GABA-dependent firing of glutamate-evoked action potentials at AMPA/kainate receptors in developing hypothalamic neurons. *J. Neurophysiol.* 79, 716–726.

Gao, B. X., Cheng, G., and Ziskind-Conhaim, L. (1998a). Development of spontaneous synaptic transmission in the rat spinal cord. *J. Neurophysiol.* 79, 2277–2287.

Gao, B.-X., and Ziskind-Conhaim, L. (1995). Development of glycine- and GABA-gated currents in rat spinal motoneurons. *J. Neurophysiol.* 74, 113–121.

Grillner, S., and Wallén, P. (1985). Central pattern generators for locomotion, with special reference to vertebrates. *Annu. Rev. Neurosci.* 8, 233–261. doi: 10.1146/annurev.neuro.8.1.233

Gulledge, A. T., and Stuart, G. J. (2003). Excitatory actions of GABA in the cortex. *Neuron* 37, 299–309. doi: 10.1016/s0896-6273(02)01146-7

Halberstadt, A. L., van der Heijden, I., Ruderman, M. A., Risbrough, V. B., Gingrich, J. A., Geyer, M. A., et al. (2009). 5-HT(2A) and 5-HT(2C) receptors exert opposing effects on locomotor activity in mice. *Neuropsychopharmacology* 34, 1958–1967. doi: 10.1038/npp.2009.29

Harkema, S. J. (2008). Plasticity of interneuronal networks of the functionally isolated human spinal cord. *Brain Res. Rev.* 57, 255–264. doi: 10.1016/j.brainresrev.2007.07.012

Iizuka, M., Nishimaru, H., and Kudo, N. (1998). Development of the spatial pattern of 5-HT-induced locomotor rhythm in the lumbar spinal cord of rat fetuses in vitro. *Neurosci. Res.* 31, 107–111. doi: 10.1016/s0168-0102(98)00029-7

Iwasaki, T., Otsuguro, K., Kobayashi, T., Ohta, T., and Ito, S. (2013). Endogenously released 5-HT inhibits A and C fiber-evoked synaptic transmission in the rat spinal cord by the facilitation of GABA/glycine and 5-HT release via 5-HT(2A) and 5-HT(3) receptors. *Eur. J. Pharmacol.* 702, 149–157. doi: 10.1016/j.ejphar.2013.01.058

Jacobs, B. L., and Fornal, C. A. (1993). 5-HT and motor control: a hypothesis. *Trends Neurosci.* 16, 346–352. doi: 10.1016/0166-2236(93)90090-9

Jean-Xavier, C., Mentis, G. Z., O'Donovan, M., Cattaert, D., and Vinay, L. (2007). Dual personality of GABA/glycine-mediated depolarizations in the immature spinal cord. *Proc. Natl. Acad. Sci. U S A* 104, 11477–11482. doi: 10.1073/pnas.0704832104

Jean-Xavier, C., Pflieger, J.-F., Liabeuf, S., and Vinay, L. (2006). Inhibitory postsynaptic potentials in lumbar motoneurons remain depolarizing after neonatal spinal cord transection in the rat. *J. Neurophysiol.* 96, 2274–2281. doi: 10.1152/jn.00328.2006

Jordan, L. M., and Slawinska, U. (2011). Chapter 12—modulation of rhythmic movement: control of coordination. *Prog. Brain Res.* 188, 181–195. doi: 10.1016/B978-0-444-53825-3.00017-6

Kao, T., Shumsky, J. S., Jacob-Vadakot, S., Himes, B. T., Murray, M., and Moxon, K. A. (2006). Role of the 5-HT2C receptor in improving weight-supported stepping in adult rats spinalized as neonates. *Brain Res.* 1112, 159–168. doi: 10.1016/j.brainres.2006.07.020

Katz, R. (1999). Presynaptic inhibition in humans: a comparison between normal and spastic patients. *J. Physiol. Paris* 93, 379–385. doi: 10.1016/s0928-4257(00)80065-0

Kiehn, O., and Kjaerulff, O. (1996). Spatiotemporal characteristics of 5-HT and dopamine-induced rhythmic hindlimb activity in the in vitro neonatal rat. *J. Neurophysiol.* 75, 1472–1482.

Kim, D., Adipudi, V., Shibayama, M., Giszter, S., Tessler, A., Murray, M., et al. (1999). Direct agonists for serotonin receptors enhance locomotor function in rats that received neural transplants after neonatal spinal transection. *J. Neurosci.* 19, 6213–6224.

Kim, D., Murray, M., and Simansky, K. J. (2001). The serotonergic 5-HT(2C) agonist m-chlorophenylpiperazine increases weight-supported locomotion without development of tolerance in rats with spinal transections. *Exp. Neurol.* 169, 496–500. doi: 10.1006/exnr.2001.7660

Lakke, E. A. J. F. (1997). The projections to the spinal cord of the rat during development: a time-table of descent. *Adv. Anat. Embryol. Cell Biol.* 135, 1–143. doi: 10.1007/978-3-642-60601-4

Landry, E. S., Lapointe, N. P., Rouillard, C., Levesque, D., Hedlund, P. B., and Guertin, P. A. (2006). Contribution of spinal 5-HT1A and 5-HT7 receptors to locomotor-like movement induced by 8-OH-DPAT in spinal cord-transected mice. *Eur. J. Neurosci.* 24, 535–546. doi: 10.1111/j.1460-9568.2006.04917.x

Lewis, D. I., Sermasi, E., and Coote, J. H. (1993). Excitatory and indirect inhibitory actions of 5-hydroxytryptamine on sympathetic preganglionic neurones in the neonate rat spinal cord in vitro. *Brain Res.* 610, 267–275. doi: 10.1016/0006-8993(93)91410-t

Li, H., Lang, B., Kang, J. F., and Li, Y. Q. (2000). Serotonin potentiates the response of neurons of the superficial laminae of the rat spinal dorsal horn to gamma-aminobutyric acid. *Brain Res. Bull.* 52, 559–565. doi: 10.1016/s0361-9230(00)00297-5

Liu, J., Akay, T., Hedlund, P. B., Pearson, K. G., and Jordan, L. M. (2009). Spinal 5-HT7 receptors are critical for alternating activity during locomotion: in vitro neonatal and in vivo adult studies using 5-HT7 receptor knockout mice. *J. Neurophysiol.* 102, 337–348. doi: 10.1152/jn.91239.2008

Liu, J., and Jordan, L. M. (2005). Stimulation of the parapyramidal region of the neonatal rat brain stem produces locomotor-like activity involving spinal 5-HT7 and 5-HT2A receptors. *J. Neurophysiol.* 94, 1392–1404. doi: 10.1152/jn.00136.2005

Lu, Y., Zheng, J., Xiong, L., Zimmermann, M., and Yang, J. (2008). Spinal cord injury-induced attenuation of GABAergic inhibition in spinal dorsal horn circuits is associated with down-regulation of the chloride transporter KCC2 in rat. *J. Physiol.* 586, 5701–5715. doi: 10.1113/jphysiol.2008.152348

Machacek, D. W., Garraway, S. M., Shay, B. L., and Hochman, S. (2001). Serotonin 5-HT(2) receptor activation induces a long-lasting amplification of spinal reflex actions in the rat. *J. Physiol.* 537, 201–207. doi: 10.1111/j.1469-7793.2001.0201k.x

Madriaga, M. A., McPhee, L. C., Chersa, T., Christie, K. J., and Whelan, P. J. (2004). Modulation of locomotor activity by multiple 5-HT and dopaminergic receptor subtypes in the neonatal mouse spinal cord. *J. Neurophysiol.* 92, 1566–1576. doi: 10.1152/jn.01181.2003

Majczyński, H., Maleszak, K., Cabaj, A., and Slawinska, U. (2005). Serotonin-related enhancement of recovery of hind limb motor functions in spinal rats after grafting of embryonic raphe nuclei. *J. Neurotrauma* 22, 590–604. doi: 10.1089/neu.2005.22.590

Mazzocchio, R., and Rossi, A. (1997). Involvement of spinal recurrent inhibition in spasticity. Further insight into the regulation of Renshaw cell activity. *Brain* 120(Pt. 6), 991–1003. doi: 10.1093/brain/120.6.991

McDearmid, J. R., Scrymgeour-Wedderburn, J. F., and Sillar, K. T. (1997). Aminergic modulation of glycine release in a spinal network controlling swimming in Xenopus laevis. *J. Physiol.* 503(Pt. 1), 111–117. doi: 10.1111/j.1469-7793.1997.111bi.x

Murray, K. C., Nakae, A., Stephens, M. J., Rank, M., D'Amico, J., Harvey, P. J., et al. (2010). Recovery of motoneuron and locomotor function after spinal cord injury depends on constitutive activity in 5-HT2C receptors. *Nat. Med.* 16, 694–700. doi: 10.1038/nm.2160

Murray, K. C., Stephens, M. J., Ballou, E. W., Heckman, C. J., and Bennett, D. J. (2011). Motoneuron excitability and muscle spasms are regulated by 5-HT2B and 5-HT2C receptor activity. *J. Neurophysiol.* 105, 731–748. doi: 10.1152/jn.00774.2010

Musienko, P., van den Brand, R., Marzendorfer, O., Roy, R. R., Gerasimenko, Y., Edgerton, V. R., et al. (2011). Controlling specific locomotor behaviors through multidimensional monoaminergic modulation of spinal circuitries. *J. Neurosci.* 31, 9264–9278. doi: 10.1523/JNEUROSCI.5796-10.2011

Myoga, H., Nonaka, S., Matsuyama, K., and Mori, S. (1995). Postnatal development of locomotor movements in normal and para-chlorophenylalanine-treated newborn rats. *Neurosci. Res.* 21, 211–221. doi: 10.1016/0168-0102(94)00857-c

Nakayama, K., Nishimaru, H., and Kudo, N. (2002). Basis of changes in left-right coordination of rhythmic motor activity during development in the rat spinal cord. *J. Neurosci.* 22, 10388–10398.

Noga, B. R., Johnson, D. M., Riesgo, M. I., and Pinzon, A. (2009). Locomotor-activated neurons of the cat. I. Serotonergic innervation and co-localization of 5-HT7, 5-HT2A and 5-HT1A receptors in the thoraco-lumbar spinal cord. *J. Neurophysiol.* 102, 1560–1576. doi: 10.1152/jn.91179.2008

Norreel, J.-C., Pflieger, J.-F., Pearlstein, E., Simeoni-Alias, J., Clarac, F., and Vinay, L. (2003). Reversible disorganization of the locomotor pattern after neonatal spinal cord transection in the rat. *J. Neurosci.* 23, 1924–1932.

Payne, J. A., Rivera, C., Voipio, J., and Kaila, K. (2003). Cation-chloride co-transporters in neuronal communication, development and trauma. *Trends Neurosci.* 26, 199–206. doi: 10.1016/s0166-2236(03)00068-7

Pearlstein, E., Ben-Mabrouk, F., Pflieger, J.-F., and Vinay, L. (2005). Serotonin refines the locomotor-related alternations in the in vitro neonatal rat spinal cord. *Eur. J. Neurosci.* 21, 1338–1346. doi: 10.1111/j.1460-9568.2005.03971.x

Pearlstein, E., Bras, H., Deneris, E. S., and Vinay, L. (2011). Contribution of 5-HT to locomotion - the paradox of Pet-1(-/-) mice. *Eur. J. Neurosci.* 33, 1812–1822. doi: 10.1111/j.1460-9568.2011.07679.x

Pflieger, J. F., Clarac, F., and Vinay, L. (2002). Postural modifications and neuronal excitability changes induced by a short-term serotonin depletion during neonatal development in the rat. *J. Neurosci.* 22, 5108–5117.

Prescott, S. A., Sejnowski, T. J., and De Koninck, Y. (2006). Reduction of anion reversal potential subverts the inhibitory control of firing rate in spinal lamina I neurons: towards a biophysical basis for neuropathic pain. *Mol. Pain* 2:32. doi: 10.1186/1744-8069-2-32

Rajaofetra, N., Sandillon, F., Geffard, M., and Privat, A. (1989). Pre- and post-natal ontogeny of serotonergic projections to the rat spinal cord. *J. Neurosci. Res.* 22, 305–321. doi: 10.1002/jnr.490220311

Ribotta, M. G. Y., Orsal, D., Feraboli-Lohnherr, D., and Privat, A. (1998a). Recovery of locomotion following transplantation of monoaminergic neurons in the spinal cord of paraplegic rats. *Ann. N Y Acad. Sci.* 860, 393–411. doi: 10.1111/j.1749-6632.1998.tb09064.x

Ribotta, M. G. Y., Orsal, D., Feraboli-Lohnherr, D., Privat, A., Provencher, J., and Rossignol, S. (1998b). Kinematic analysis of recovered locomotor movements of the hindlimbs in paraplegic rats transplanted with monoaminergic embryonic neurons. *Ann. N Y Acad. Sci.* 860, 521–523. doi: 10.1111/j.1749-6632.1998.tb09093.x

Ribotta, M. G., Provencher, J., Feraboli-Lohnherr, D., Rossignol, S., Privat, A., and Orsal, D. (2000). Activation of locomotion in adult chronic spinal rats is achieved by transplantation of embryonic raphe cells reinnervating a precise lumbar level. *J. Neurosci.* 20, 5144–5152.

Sadlaoud, K., Tazerart, S., Brocard, C., Jean-Xavier, C., Portalier, P., Brocard, F., et al. (2010). Differential plasticity of the GABAergic and glycinergic synaptic transmission to rat lumbar motoneurons after spinal cord injury. *J. Neurosci.* 30, 3358–3369. doi: 10.1523/JNEUROSCI.6310-09.2010

Schmidt, B. J., and Jordan, L. M. (2000). The role of serotonin in reflex modulation and locomotor rhythm production in the mammalian spinal cord. *Brain Res. Bull.* 53, 689–710. doi: 10.1016/s0361-9230(00)00402-0

Shen, R. Y., and Andrade, R. (1998). 5-Hydroxytryptamine2 receptor facilitates GABAergic neurotransmission in rat hippocampus. *J. Pharmacol. Exp. Ther.* 285, 805–812.

Sławińska, U., Majczyński, H., Dai, Y., and Jordan, L. M. (2012). The upright posture improves plantar stepping and alters responses to serotonergic drugs in spinal rats. *J. Physiol.* 590, 1721–1736. doi: 10.1113/jphysiol.2011.224931

Sławińska, U., Majczyński, H., and Djavadian, R. (2000). Recovery of hindlimb motor functions after spinal cord transection is enhanced by grafts of the embryonic raphe nuclei. *Exp. Brain Res.* 132, 27–38. doi: 10.1007/s002219900323

Sławińska, U., Miazga, K., Cabaj, A. M., Leszczynska, A. N., Majczyński, H., Nagy, J. I., et al. (2013). Grafting of fetal brainstem 5-HT neurons into the sublesional spinal cord of paraplegic rats restores coordinated hindlimb locomotion. *Exp. Neurol.* 247, 572–581. doi: 10.1016/j.expneurol.2013.02.008

Stil, A., Jean-Xavier, C., Liabeuf, S., Brocard, C., Delpire, E., Vinay, L., et al. (2011). Contribution of the potassium-chloride co-transporter KCC2 to the modulation of lumbar spinal networks in mice. *Eur. J. Neurosci.* 33, 1212–1222. doi: 10.1111/j.1460-9568.2010.07592.x

Stil, A., Liabeuf, S., Jean-Xavier, C., Brocard, C., Viemari, J. C., and Vinay, L. (2009). Developmental up-regulation of the potassium-chloride cotransporter type 2 in the rat lumbar spinal cord. *Neuroscience* 164, 809–821. doi: 10.1016/j.neuroscience.2009.08.035

Takahashi, T. (1984). Inhibitory miniature synaptic potentials in rat motoneurons. *Proc. R. Soc. Lond. B Biol. Sci.* 221, 103–109. doi: 10.1098/rspb.1984.0025

Tokarski, K., Kusek, M., and Hess, G. (2011). 5-HT7 receptors modulate GABAergic transmission in rat hippocampal CA1 area. *J. Physiol. Pharmacol.* 62, 535–540.

Ung, R. V., Landry, E. S., Rouleau, P., Lapointe, N. P., Rouillard, C., and Guertin, P. A. (2008). Role of spinal 5-HT2 receptor subtypes in quipazine-induced hindlimb movements after a low-thoracic spinal cord transection. *Eur. J. Neurosci.* 28, 2231–2242. doi: 10.1111/j.1460-9568.2008.06508.x

van den Brand, R., Heutschi, J., Barraud, Q., Digiovanna, J., Bartholdi, K., Huerlimann, M., et al. (2012). Restoring voluntary control of locomotion after paralyzing spinal cord injury. *Science* 336, 1182–1185. doi: 10.1126/science.1217416

van den Pol, A. N., Obrietan, K., and Chen, G. (1996). Excitatory actions of GABA after neuronal trauma. *J. Neurosci.* 16, 4283–4292.

Veasey, S. C., Fornal, C. A., Metzler, C. W., and Jacobs, B. L. (1995). Response of serotonergic caudal raphe neurons in relation to specific motor activities in freely moving cats. *J. Neurosci.* 15, 5346–5359.

Vinay, L., Brocard, F., Clarac, F., Norreel, J. C., Pearlstein, E., and Pflieger, J. F. (2002). Development of posture and locomotion: an interplay of endogenously generated activities and neurotrophic actions by descending pathways. *Brain Res. Brain Res. Rev.* 40, 118–129. doi: 10.1016/s0165-0173(02)00195-9

Vinay, L., Brocard, F., Pflieger, J., Simeoni-Alias, J., and Clarac, F. (2000). Perinatal development of lumbar motoneurons and their inputs in the rat. *Brain Res. Bull.* 53, 635–647. doi: 10.1016/s0361-9230(00)00397-x

Vinay, L., and Jean-Xavier, C. (2008). Plasticity of spinal cord locomotor networks and contribution of cation-chloride cotransporters. *Brain Res. Rev.* 57, 103–110. doi: 10.1016/j.brainresrev.2007.09.003

Wang, C., and Zucker, R. S. (1998). Regulation of synaptic vesicle recycling by calcium and serotonin. *Neuron* 21, 155–167. doi: 10.1016/s0896-6273(00)80523-1

Wedderburn, J. F., and Sillar, K. T. (1994). Modulation of rhythmic swimming activity in post-embryonic Xenopus laevis tadpoles by 5-hydroxytryptamine acting at 5HT1a receptors. *Proc. R. Soc. Lond. B Biol. Sci.* 257, 59–66. doi: 10.1098/rspb.1994.0094

Wu, W.-L., Ziskind-Conhaim, L., and Sweet, M. A. (1992). Early development of glycine- and GABA-mediated synapses in rat spinal cord. *J. Neurosci.* 12, 3935–3945.

Xie, D. J., Uta, D., Feng, P. Y., Wakita, M., Shin, M. C., Furue, H., et al. (2012). Identification of 5-HT receptor subtypes enhancing inhibitory transmission in the rat spinal dorsal horn in vitro. *Mol. Pain* 8:58. doi: 10.1186/1744-8069-8-58

Xu, T. L., Nabekura, J., and Akaike, N. (1996). Protein kinase C-mediated enhancement of glycine response in rat sacral dorsal commissural neurones by serotonin. *J. Physiol.* 496, 491–501.

Xu, T. L., Pang, Z. P., Li, J. S., and Akaike, N. (1998). 5-HT potentiation of the GABA(A) response in the rat sacral dorsal commissural neurones. *Br. J. Pharmacol.* 124, 779–787. doi: 10.1038/sj.bjp.0701896

Zhang, W., and Grillner, S. (2000). The spinal 5-HT system contributes to the generation of fictive locomotion in lamprey. *Brain Res.* 879, 188–192. doi: 10.1016/s0006-8993(00)02747-5

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Metaplasticity and behavior: how training and inflammation affect plastic potential within the spinal cord and recovery after injury

James W. Grau[1]*, J. Russell Huie[2], Kuan H. Lee[1], Kevin C. Hoy[3], Yung-Jen Huang[1], Joel D. Turtle[1], Misty M. Strain[1], Kyle M. Baumbauer[4], Rajesh M. Miranda[5], Michelle A. Hook[5], Adam R. Ferguson[2] and Sandra M. Garraway[6]

[1] *Cellular and Behavioral Neuroscience, Department of Psychology, Texas A&M University, College Station, TX, USA*
[2] *Department of Neurological Surgery, Brain and Spinal Injury Center, University of California San Francisco, San Francisco, CA, USA*
[3] *Department of Neurosciences, MetroHealth Medical Center and Case Western Reserve University, Cleveland, OH, USA*
[4] *School of Nursing, University of Connecticut, Storrs, CT, USA*
[5] *Department of Neuroscience and Experimental Therapeutics, Texas A&M Health Science Center, Bryan, TX, USA*
[6] *Department of Physiology, Emory University School of Medicine, Atlanta, GA, USA*

Edited by:
Shawn Hochman, Emory University, USA

Reviewed by:
David Parker, Cambridge University, UK
Ray D. Leon, California State University, Los Angeles, USA

****Correspondence:***
James W. Grau, Cellular and Behavioral Neuroscience, Department of Psychology, Texas A&M University, College Station, TX 77843-4235, USA
e-mail: j-grau@tamu.edu

Research has shown that spinal circuits have the capacity to adapt in response to training, nociceptive stimulation and peripheral inflammation. These changes in neural function are mediated by physiological and neurochemical systems analogous to those that support plasticity within the hippocampus (e.g., long-term potentiation and the NMDA receptor). As observed in the hippocampus, engaging spinal circuits can have a lasting impact on plastic potential, enabling or inhibiting the capacity to learn. These effects are related to the concept of metaplasticity. Behavioral paradigms are described that induce metaplastic effects within the spinal cord. Uncontrollable/unpredictable stimulation, and peripheral inflammation, induce a form of maladaptive plasticity that inhibits spinal learning. Conversely, exposure to controllable or predictable stimulation engages a form of adaptive plasticity that counters these maladaptive effects and enables learning. Adaptive plasticity is tied to an up-regulation of brain derived neurotrophic factor (BDNF). Maladaptive plasticity is linked to processes that involve kappa opioids, the metabotropic glutamate (mGlu) receptor, glia, and the cytokine tumor necrosis factor (TNF). Uncontrollable nociceptive stimulation also impairs recovery after a spinal contusion injury and fosters the development of pain (allodynia). These adverse effects are related to an up-regulation of TNF and a down-regulation of BDNF and its receptor (TrkB). In the absence of injury, brain systems quell the sensitization of spinal circuits through descending serotonergic fibers and the serotonin 1A (5HT 1A) receptor. This protective effect is blocked by surgical anesthesia. Disconnected from the brain, intracellular Cl^- concentrations increase (due to a down-regulation of the cotransporter KCC2), which causes GABA to have an excitatory effect. It is suggested that BDNF has a restorative effect because it up-regulates KCC2 and re-establishes GABA-mediated inhibition.

Keywords: plasticity, learning, inflammation, spinal cord injury, nociception, BDNF, TNF, opioid

INTRODUCTION

Research has shown that brain systems modulate the operation of spinal circuits. For example, afferent pain (nociceptive) signals can be inhibited, yielding an *anti*-nociception that attenuates both spinally mediated withdrawal and brain-mediated indices of pain (Fields, 2000). This provides a form of top-down processing that allows the organism to dynamically modulate incoming pain signals on the basis of expectation (Grau, 1987; McNally et al., 2011). This type of regulatory effect is characterized as a form of *neuromodulation* because it does not initiate a sensory/motor response, but instead regulates signal amplitude within a spinal circuit to facilitate or inhibit neural transmission. Evidence suggests that how and when these descending systems are engaged is tuned by experience, providing a mechanism whereby brain-mediated learning can influence spinal function (also see: Wolpaw, 2010; Thompson and Wolpaw, 2014).

Here we focus on a different question: can spinal systems learn without input from the brain and is this learning affected by past experience? We will show that how spinal circuits operate depends upon both environmental relations (e.g., the temporal regularity of sensory stimuli) and behavioral control (e.g., a consistent relation between limb position and an environmental stimulus). More importantly, we provide evidence that spinal cord learning affects the propensity to learn in future situations and suggest that this reflects a form of *metaplasticity* (Abraham and Bear, 1996). We will link these metaplastic effects to particular neurochemical systems [e.g., the metabotropic glutamate receptor (mGluR),

tumor necrosis factor (TNF), and brain-derived neurotrophic factor (BDNF)]. We will also explore how these processes influence recovery after a spinal contusion injury and how a spinal injury affects their function.

DRAWING ON PARALLELS TO BRAIN-MEDIATED PROCESSES

NEURAL PLASTICITY IN THE HIPPOCAMPUS AND SPINAL CORD INVOLVE COMMON MECHANISMS

Our analysis is informed by studies of learning and memory within the brain. Of particular interest are studies of neural plasticity within the hippocampus. Behavioral evidence that this structure is involved in learning and memory (Squire and Wixted, 2011), combined with the physiological findings that this system supports lasting changes in synaptic function [e.g., long-term potentiation (LTP) and long-term depression (LTD); Bear, 2003], have fueled interest in this structure. This work has linked alterations in synaptic function to the NMDA receptor (NMDAR), which acts as a coincidence detector (Collingridge and Bliss, 1987; Dudai, 1989). From this perspective, modifiable (plastic) changes in neural function are identified with synaptic events. While most would acknowledge that neural connections can be altered in a variety of ways, the preponderance of glutamatergic transmission has focused attention on the role of NMDAR-mediated LTP and LTD (Morris, 2013).

Other regions of the central nervous system, including the spinal cord, support NMDAR-mediated plasticity. For example, peripheral injury and inflammation can produce a lasting increase in neural excitability within the spinal cord, a phenomena called *central sensitization* (Woolf, 1983; Willis, 2001; Ji et al., 2003; Latremoliere and Woolf, 2009). Central sensitization lowers the threshold at which stimulation engages a defensive withdrawal response. Indeed, after the system is sensitized, even non-noxious tactile stimulation may elicit a response. Evidence suggests that central sensitization fosters pain transmission to the brain, and for this reason it is thought to contribute to the development of chronic pain. Interestingly, the induction of central sensitization depends upon a form of NMDAR-mediated plasticity that lays down a memory-like alteration that maintains the sensitized state through neurobiological processes analogous to those involved in hippocampal-dependent learning and memory (Dickenson and Sullivan, 1987; Sandkühler, 2000; Ji et al., 2003).

NEUROMODULATION AND METAPLASTICITY

There is now ample evidence that spinal systems can support some simple forms of learning and memory (reviewed in Grau, 2014). For example, if a rat is spinally transected in the thoracic region and then given a noxious shock to one hindlimb whenever the leg is extended, it learns to maintain the leg in a flexed position (thereby reducing net shock exposure; Grau et al., 1998). Here, learning brings about a modification within a particular stimulus-response (S-R) pathway. What is of greater interest for the present review is that this process of spinal learning can have an effect that impacts the capacity to learn when stimulation is later applied at other sites on the body. For example, experience with controllable stimulation on one leg can foster learning on the contralateral leg whereas exposure to uncontrollable stimulation to either one leg or tail has a lasting inhibitory effect on learning for both legs (Crown et al., 2002a; Joynes et al., 2003).

Correlates to these behavioral observations can be found at the cellular level. For example, electrophysiological stimulation of a neuron can produce a downstream modification (e.g., LTP) that only affects how that particular neural pathway operates. Neural activity can also engage cellular systems that have a remote effect on other neural circuits, providing a form of extrinsic modulation that alters how another neural pathway functions. Our assumption is that environmental stimulation and behavioral training can engage a form of extrinsic modulation that can affect learning (neural plasticity) when stimuli are applied to other regions of the body (and which engage a distinct neural circuit).

How extrinsic processes affect neural function can vary over time. In some cases, a modulatory process may be reflexively elicited in an unconditioned (unlearned) manner and have an acute effect that passively decays over the course of minutes to hours. In other cases, the impact of the modulatory process may continue beyond the events that induced it, to have a long-term effect on how a neural circuit operates. In this case, the initiating event must engage a process that maintains the modulatory process over time, and in this way it involves a kind of memory.

A long-term modulatory effect can impact how a neural circuit operates (performs) or its capacity to change (plasticity). Our focus is on the latter, a phenomenon known as *metaplasticity* (Abraham and Bear, 1996). Metaplasticity is a concept that emerged from work with the hippocampal slice preparation and describes a class of phenomena that have a lasting effect on neural plasticity (**Figure 1A**). Here, neural plasticity is typically assessed using electrophysiological processes (e.g., the development of LTP or LTD). What researchers discovered is that a variety of treatments (environmental enrichment, dark rearing, conditioning) can have a lasting effect on the rate at which LTP or LTD develops and may do so without impacting baseline measures of neural excitability (Abraham, 2008). The criteria for metaplasticity include: (1) it extends beyond the treatments used to induce [i.e., it has a lasting effect that spans minutes to days (Abraham, 2008)]; and (2) it impacts the capacity to change (plasticity), not just the responsiveness of the system (performance). To this, we could add another criterion: (3) the phenomenon is reversible (and not

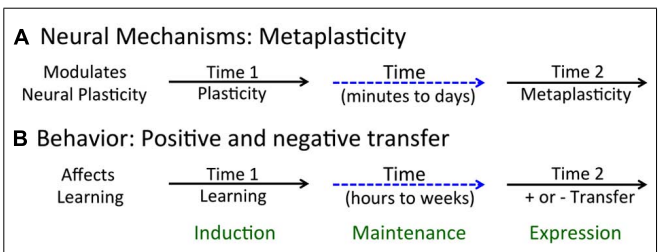


FIGURE 1 | Acute and long-term effects of neurobiological and behavioral processes. (A) Metaplasticity arises when an initial event (at Time 1) brings about a lasting change in neural function that affects plastic potential (at Time 2). **(B)** Learning can affect the future capacity to learn, enhancing learning about new events and relations (positive transfer) or inhibiting this process (negative transfer).

due to dysfunction or injury). From this view, an experimental manipulation that permanently alters plastic potential because it kills cells would not be considered an example of metaplasticity.

EXPERIENCE-DEPENDENT CHANGES IN SPINAL FUNCTION

Our approach begins with a detailed description of the behavioral phenomena and seeks to understand the underlying neurobiological mechanisms. We see this as a complement to physiological approaches that use cellular techniques (e.g., electrophysiology) to detail how components of the system operate. An advantage of the spinal cord preparation is that the link between sensory/motor processes and the underlying neurobiology is (relative to the brain) simpler. For this reason, it may be easier to draw parallels between behavioral effects and neurobiological modifications. In the sections that follow, we show how behavioral manipulations can influence learning potential within the spinal cord and relate these effects to the concept of metaplasticity.

DEFINING LEARNING

To demonstrate spinal learning requires an operational definition of the process (Grau et al., 1998; Grau, 2010). Learning is implicated when an experience at time 1 has a lasting effect at time 2 (Rescorla, 1988). We formalized this idea by proposing that learning: (1) involves a form of neural plasticity; (2) depends upon the organism's experiential history; and (3) outlasts (extends beyond) the environmental contingencies used to induce.

While we recognize that non-neural processes (e.g., glia) play an important role, our focus is on how these processes influence neural function (criterion 1). Likewise, while it is recognized that a wide range of events (including development and injury) can engage forms of neural plasticity (Onifer et al., 2011), learning is limited to those engaged by experience (criterion 2). The final requirement (3) is that the process has a lasting effect (which implies a form of memory). From this view, learning reflects the process used to establish a lasting change in neural/behavioral function (memory) and, like most, we assume that the latter generally involves a protein synthesis dependent structural modification (Dudai, 2004).

Whether spinal systems can learn has both theoretical and clinical implications (Grau et al., 2006, 2012; Hook and Grau, 2007; Grau, 2014). Theoretically, it would imply that learning is not the province of particular neural structures within the brain, but instead, is more widely distributed throughout the CNS, including the spinal cord. From this view, the question is not whether a particular system can learn, the question is: how does learning within this system compare to that shown by other structures? Not surprisingly, spinal learning is (relative to the brain) less flexible and more biologically constrained (Grau et al., 2012). Spinal learning is also important because it has implications for physical therapy. Indeed, physical therapy can be seen as a form of directed learning, the aim of which is to establish a lasting change in neural/behavioral function.

Learning phenomena are typically classified based upon the environmental manipulations used to establish the behavioral change (Grau, 2014; Domjan, 2015). For example, Pavlovian conditioning depends upon the relation between two stimulus events whereas instrumental learning is tied to the relation between a behavioral response (R) and an environmental event [the outcome (O); aka reinforcer]. Recognizing that physical therapy typically involves a kind of instrumental training, we asked whether neurons within the lumbosacral spinal cord are sensitive to response–outcome (R–O) relations (Grau et al., 1998, 2006).

SPINALLY MEDIATED INSTRUMENTAL LEARNING

The first clear evidence that instrumental learning can produce a lasting modification in spinal function was provided by Wolpaw and Carp (1990) and Wolpaw (2010). The response involved a modification of the spinal stretch reflex [the Hoffman (H) reflex] and change in reflex magnitude was reinforced with food. For example, in some subjects the H-reflex was repeatedly elicited and they were reinforced for exhibiting an increase in response strength. This training brought about an increase in the H-reflex. Remarkably, after extended training, this response modification survived a spinal transection. This work demonstrates that instrumental learning can modify spinal function. Here, brain mechanisms mediate the abstraction of the instrumental relation [between H reflex amplitude (the R) and the food reinforcer (the O)]. With extended training, this R–O relation induces (through descending fibers) a lasting change in how a spinal circuit operates. In this case, learning is mediated by the brain and the consequence of this process (the memory) is stored within the spinal cord.

Our studies pushed spinal systems further, to explore whether neurons within the lumbosacral cord can learn (i.e., abstract R–O relations) when isolated from the brain. Rats underwent a thoracic (T2) transection and were trained the following day while loosely restrained (**Figure 2A**). Leg position is monitored by means of a contact electrode that is taped to the base of the hindpaw. When the leg is extended, the tip of the contact electrode touches the underlying salt solution and completes a computer-monitored circuit. A R–O relation is then established by applying shock to the tibialis anterior muscle whenever the leg is extended. Over the course of 30 min of training, subjects exhibit a progressive increase in flexion duration that minimizes net shock exposure (**Figure 2B**; Grau et al., 1998). This learning depends upon glutamatergic systems within the spinal cord, and is blocked when an AMPAR (CNQX) or NMDAR (APV; MK-801) antagonist is injected into the spinal cord [an intrathecal (i.t.) injection] prior to training (Joynes et al., 2004; Ferguson et al., 2006; Hoy et al., 2013).

To show that the R–O relation matters, other subjects received shock independent of leg position. This was accomplished by coupling (yoking) the experimental treatments across subjects, so that a yoked rat received shock every time its master partner was shocked. Notice that, for the yoked rat, there is no relation between shock exposure and leg position – the shock is uncontrollable. Subjects in the yoked group do not exhibit an increase in flexion duration (**Figure 2B**), which provides one indication that the R–O relation matters.

To demonstrate learning, we must show that the experience has a lasting effect, that impacts performance when subjects are tested under common conditions. We accomplished this by testing rats that had previously received controllable shock (Master), uncontrollable shock (Yoked), or nothing (Unshocked) with response

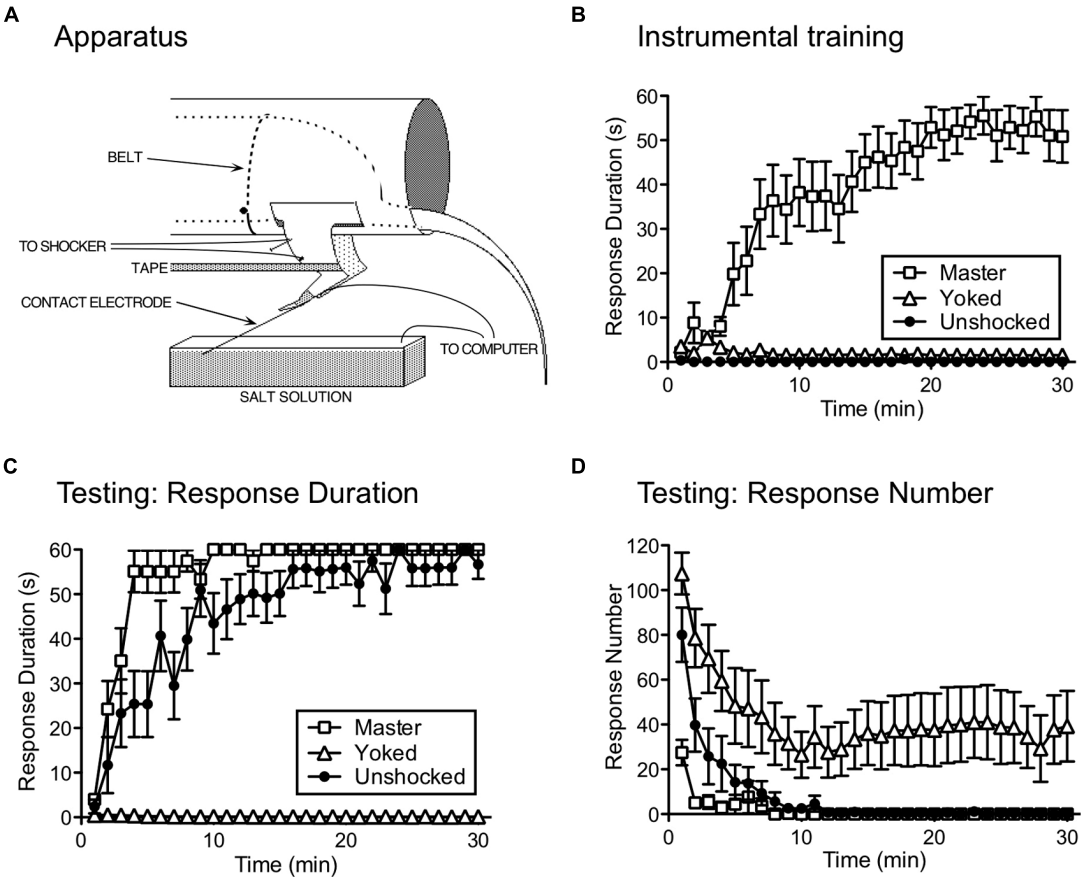


FIGURE 2 | Instrumental learning in spinally transected rats. (A) The apparatus used to study instrumental learning. A spinally transected rat lies in an opaque tube with its hindquarters gently secured with a belt. An insulated contact electrode is taped to the rat's paw and the exposed tip is submerged in a salt solution. Electrical stimulation is applied to the tibialis anterior muscle through a pair of electrodes and tape is used to stabilize the leg. When shock is applied, a flexion response is elicited that raises the contact electrode, breaking a circuit that is monitored by a computer. A response–outcome (R–O) relation is instituted by applying shock whenever the contact electrode touches the underlying salt solution. The task can be made more difficult by increasing the initial depth of the contact electrode (from 4 to 8 mm). **(B)** A system capable of learning the R–O relation should exhibit an increase in flexion (response) duration that minimizes solution contact (and net shock exposure). Response duration (y-axis) is calculated in 1-min time bins using the following formula: Response duration = [60-time (s) in solution]/(flexion number + 1). Over the course of 30 min of testing (x-axis), spinally transected rats that received shock whenever the leg was extended (Master) exhibited a progressive increase in response duration. Other rats are experimentally coupled (Yoked) to the master subjects and receive shock at the same time, but independent of leg position (uncontrollable stimulation). Yoked rats do not exhibit an increase in response duration. The error bars indicate the standard error of the mean. **(C)** Master, Yoked, and previously unshocked rats are then tested under common conditions with controllable shock. Master rats learn more rapidly (positive transfer) than the previously untreated (Unshocked) controls. Rats that had previously received shock independent of leg position (Yoked) fail to learn. Similar results are observed independent of whether subjects are tested on the previously trained (ipsilateral) leg or the contralateral leg. **(D)** As Master rats learn to maintain their leg in a flexed position, response number declines. In Yoked rats, shock elicits a high response rate, but does not produce an increase in flexion duration. Adapted from Grau et al. (1998).

contingent legshock. We were concerned that yoked rats might do poorly during testing simply because they were less responsive to shock or the contact electrode was submerged at a greater depth. To discount these factors, we adjusted shock intensity across subjects so that it elicited an equally strong flexion response and equated contact electrode depth (to 4 mm). We verified the success of these procedures by measuring by measuring the duration of the first shock-elicited flexion response. As expected, there were no differences in performance at the start of testing. Nonetheless, subjects that had previously experienced controllable stimulation learned faster than previously unshocked controls (**Figure 2C**; Grau et al., 1998). This savings effect (positive transfer; **Figure 1B**) provides one indication that training with response-contingent stimulation has a lasting effect. What was more surprising is that rats that had previously received uncontrollable stimulation (Yoked) failed to learn when later tested with controllable shock (negative transfer). Moreover, they failed to learn even though they exhibited a high rate of responding and repeatedly experienced the R–O relation (**Figure 2D**).

UNCONTROLLABLE STIMULATION AND INFLAMMATION INDUCE A LASTING LEARNING IMPAIRMENT

Does the learning impairment observed after uncontrollable stimulation to one hind limb reflect a local (limb-specific) effect or a

general inhibition of learning? We addressed this issue by testing yoked subjects on the same (ipsilateral) or opposite (contralateral) leg. The learning impairment was just as robust when subjects were tested on the contralateral leg (Joynes et al., 2003). Next, we developed a computer program that emulated the variable shock schedule produced by a typical master rat. This program applies 80 ms shocks with a variable inter-stimulus interval (ISI) between 0.2 and 3.8 s (mean ISI = 2 s). We found that just 6 min of variable intermittent shock (VIS) to the leg or tail induced a learning impairment and that this effect lasts up to 48 h (Crown et al., 2002b). Thus, exposure to uncontrollable stimulation induces a lasting effect that generally inhibits instrumental learning. We have suggested that this learning deficit reflects a form of metaplasticity (Ferguson et al., 2008, 2012a).

We reasoned that uncontrollable stimulation could inhibit learning because it induces a form of antinociception that attenuates the effectiveness of the shock reinforcer. However, we found no evidence that VIS inhibits reactivity to noxious stimulation (Crown et al., 2002b). If fact, a test of mechanical reactivity (von Frey stimuli applied to the plantar surface of the hind paws) showed that VIS treated subjects were more responsive (Ferguson et al., 2006). Enhanced mechanical reactivity (EMR) is of interest because it is observed after a variety of treatments known to sensitize nociceptive systems within the spinal cord (central sensitization).

As noted above, central sensitization involves neurochemical mechanisms implicated in hippocampal-dependent learning and memory and its induction depends upon glutamate transmission and the NMDAR (Ji et al., 2003). We hypothesized that this state could interfere with instrumental learning by saturating NMDAR-dependent plasticity (Ferguson et al., 2006). Alternatively, the induction of central sensitization could engage a secondary process that inhibits NMDAR-mediated learning, effectively "locking" the system in its current state. In either case, blocking the NMDAR should interfere with the induction of the learning impairment. Supporting this, we found that rats given MK-801 prior to VIS showed no learning impairment when tested with controllable stimulation 24 h later (Ferguson et al., 2006). Pretreatment with the AMPAR antagonist CNQX had a similar effect (Hoy et al., 2013).

The proposed link to central sensitization suggests that treatments that induce this state should impair instrumental learning. To test this, we applied the irritant capsaicin to one hind paw, which induces peripheral inflammation and central sensitization (Willis, 2001). Capsaicin also induced a learning impairment and this effect, like the VIS-induced deficit, was evident 24 h later when subjects were tested on the contralateral leg (Hook et al., 2008; for evidence other inflammatory agents inhibit learning see Ferguson et al., 2006, 2012b; Huie et al., 2012a).

CONTROLLABLE STIMULATION FOSTERS LEARNING AND HAS A LASTING PROTECTIVE EFFECT

Whereas uncontrollable stimulation and peripheral inflammation disable learning, controllable stimulation enables instrumental learning (Crown et al., 2002a). Evidence for this comes from studies using a higher response criterion, achieved by increasing contact electrode depth (from 4 to 8 mm). Under these conditions, previously untrained rats fail to learn whereas those that had received controllable stimulation can learn and this is true independent of whether they are tested on the same or opposite leg.

Controllable stimulation also exerts a protective effect that counters the consequences of uncontrollable shock. If controllable stimulation is given prior to VIS (to the same leg or the tail), it blocks the induction of the learning impairment (Crown and Grau, 2001). Conversely, after the learning impairment is induced, training with controllable shock [in conjunction with a drug treatment (naltrexone) that temporarily reverses the impairment (see below)] restores the capacity to learn (when subjects are subsequently tested in a drug-free state). Exposure to controllable shock also prevents, and reverses, the learning impairment and EMR induced by peripheral capsaicin (Hook et al., 2008).

The fact controllable stimulation enables learning when subjects are tested on the opposite leg, and prevents the learning impairment when VIS is applied to the tail, implies that controllable stimulation generally modulates the capacity to learn. Further, we have shown that instrumental training has a lasting effect that can block the induction of the learning deficit when VIS is given 24 h later. Taken together, these findings suggest that exposure to controllable stimulation also induces a metaplastic effect, one that promotes instrumental learning.

We, of course, are not the first to show that behavioral control can profoundly affect how an aversive stimulus is processed. Indeed, the overall pattern of results is remarkably similar to what is observed in intact subjects in studies of learned helplessness (Maier and Seligman, 1976). These observations suggest that that the underlying principles have considerable generality and may apply to any neural system capable of encoding R–O relations. At the same time, it is also recognized that higher neural systems allow for a much wider range of behavioral effects (Maier and Watkins, 2005) and that spinal learning is more biologically constrained (Grau et al., 2012).

TEMPORAL REGULARITY (PREDICTABILITY) HAS AN EFFECT ANALOGOUS TO BEHAVIORAL CONTROL

We recently discovered that uncontrollable intermittent shock does not always induce a learning impairment. If stimulation is given at a regular (predictable) interval, an extended exposure to intermittent shock has no adverse effect (Baumbauer et al., 2008). Interestingly, the emergence of this effect requires extended training (720–900 shocks); if subjects receive less training (180 shocks), intermittent shock induces a learning impairment independent of whether it occurs in a variable or regular (fixed-spaced) manner. The fact extended training is required has led us to suggest that abstracting stimulus regularity involves a form of learning (Baumbauer et al., 2009).

At a behavioral level, we have shown that an initial bout of fixed spaced shock (360) lays down a kind of temporal memory that lasts at least 24 h and transforms how subjects respond to a subsequent bout of 360 shocks (processing the latter as fixed spaced; Lee et al., 2013). At a physiological level, we have shown that learning about temporal regularity depends upon a form of NMDAR-mediated plasticity and protein synthesis (Baumbauer et al., 2009). Further, training with fixed spaced stimulation has a

restorative effect analogous to that produced by experience with controllable stimulation. For example, fixed spaced stimulation can both prevent, and reverse, the learning impairment induced by VIS (Baumbauer et al., 2009). An extended exposure to fixed spaced shock also blocks, and reverses, the learning impairment and EMR induced by peripheral inflammation (Baumbauer and Grau, 2011; Baumbauer et al., 2012). And like the other effects described above, fixed spaced stimulation has a general effect that blocks the induction of the learning impairment independent of whether subjects are challenged by stimulation at the same, or a remote, dermatome (Baumbauer et al., 2009).

SPINAL LEARNING: SUMMARY AND IMPLICATIONS

Taken together, we have discovered that environmental events can engage two alternative processes that have a diffuse effect on spinal cord plasticity (**Figure 3**; for reviews see: Grau et al., 2006, 2012; Ferguson et al., 2012a). Exposure to VIS, that is both uncontrollable and unpredictable, inhibits instrumental learning and produces EMR (Grau et al., 1998; Baumbauer et al., 2008), and peripheral inflammation has the same effect (Hook et al., 2008). An equivalent exposure to intermittent stimulation given in a controllable manner has no adverse effect and engages a process that enables learning and counters the adverse effects of both VIS and inflammation (Crown and Grau, 2001; Crown et al., 2002a). Likewise, an extended exposure to fixed spaced shock engages a protective mechanism that counters the adverse effects of VIS (Baumbauer et al., 2009, 2012; Baumbauer and Grau, 2011). These effects are lasting (24 h or longer), involve a form of NMDAR-mediated plasticity, and require protein synthesis (Joynes et al., 2004; Patton et al., 2004; Baumbauer et al., 2006, 2009; Ferguson et al., 2006). Moreover, in all cases the phenomena have a general effect that impacts how stimuli applied at other dermatomes are processed.

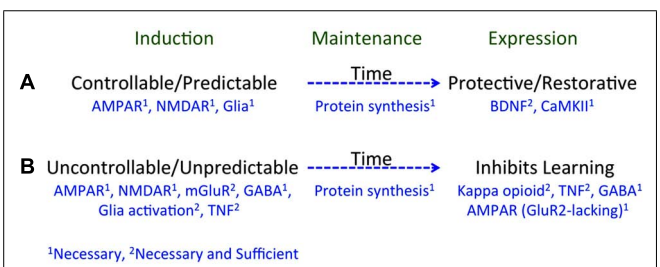


FIGURE 3 | A summary of how training experience affects learning potential. (A) Learning that an environmental event is controllable or predictable depends upon a form of NMDAR-mediated plasticity and is disrupted if glia function is inhibited. Processes initiated during learning (induction) engage a protein synthesis dependent mechanism that maintains the effect over time (memory). Prior experience with controllable/predictable stimulation has a long-term protective/restorative effect that enables instrumental learning and blocks both the learning impairment and EMR induced by VIS. These effects depend upon BDNF and CaMKII. **(B)** Exposure to uncontrollable/unpredictable shock initiates a process that depends upon the NMDAR, group 1 mGluR, GABA, glia activation, and TNF. These processes induce a protein synthesis dependent mechanism that maintains the effect over time. Prior experience with uncontrollable/unpredictable stimulation inhibits instrumental learning through a process that involves a kappa opioid, TNF, GABA, and the trafficking of GluR2-lacking AMPA receptors. Superscripts indicate whether a neurobiological mechanism is necessary (1) or necessary and sufficient (2).

We have seen that learning can both alter a particular response and impact the capacity to learn when faced with new environmental challenges (Grau et al., 1998; Crown et al., 2002a). Our focus here is on the latter phenomena – on how learning can foster (positive transfer), or inhibit (negative transfer), the capacity for future learning (**Figure 1B**). In assuming that learning involves a form of neural plasticity, the question we ask focuses on the plasticity of plasticity: How does a training experience impact the future capacity to learn? We suggest that this reflects a form of metaplasticity.

In the sections that follow, we outline what we have discovered about the neurobiological mechanisms that mediate these metaplastic effects. While we will reference electrophysiological observations, our discussion will lean towards an analysis of behavioral indices of spinal function. We will also remain agnostic regarding the relation of our effects to the phenomena of LTP and LTD. We take this position because we have yet to elucidate the relative role of these phenomena and because we assume that neural plasticity may be mediated by a host of mechanisms.

THE BIOLOGY OF SPINALLY MEDIATED METAPLASTICITY

LINKING METAPLASTICITY TO MECHANISM

Our central concern is with processes that have a lasting effect and, in this way, involve a form of memory. It is assumed here that acute changes in neural function are mediated by pre-existing components and that long-term modifications depend upon protein synthesis (Dudai, 2004; Abraham and Williams, 2008). This holds for our examples of spinally mediated metaplasticity. Supporting this, administration of a protein synthesis inhibitor soon after exposure to VIS blocks the induction of the learning impairment (Patton et al., 2004; Baumbauer et al., 2006). Likewise, administration of a protein synthesis inhibitor after a fixed spaced shock blocks its long-term protective effect (Baumbauer et al., 2009).

Because these metaplastic effects involve a form of memory, we can address the process from a number of perspectives (**Figure 1**). Specifically, we can ask: (1) What processes underlie the *induction* of the phenomenon; (2) What mediates the *maintenance* of the alteration (the memory) over time; and (3) What mediates the *expression* of these phenomena (i.e., how do they affect the capacity to learn)? We address question 3 by blocking a particular process (necessity) and then showing that administration of an agent that should engage the process has a similar effect on learning (sufficiency). The interpretation of sufficiency must, though, be treated with some caution because engaging other (unrelated) processes could yield a similar outcome. For the second issue, the question typically concerns the identification of the neurobiological system that preserves the effect over time. To study the induction of the process (question 1), we can again assay the effect of blocking a particular process, seeking evidence that it plays an essential (necessary) role. For evidence of sufficiency, we can test whether artificially engaging the system effectively substitutes for our experimental treatment. Again, some caution is needed because a similar outcome may be produced in

a variety of ways. Further, the induction of most phenomena is tied to multiple processes. In this case, to discover a substitute for a behavioral training regime, we need to know all of the essential components and how they are sequenced over time.

The link between the learning impairment and central sensitization has provided a rich source of concepts regarding the neurobiological mechanisms that may be involved, implicating opioid peptides, glutamatergic transmission (AMPAR, NMDAR, and mGluR), non-neuronal cells and TNF. Identifying the factors that promote adaptive plasticity has proven more difficult. We have, however, discovered that BDNF, and downstream signal pathways (e.g., CaMKII), play an important role.

ROLE OF THE NMDAR AND mGluR IN THE INDUCTION OF THE LEARNING IMPAIRMENT

We noted above that both instrumental learning and the metaplastic effects of training depend upon the NMDAR. Supporting this, pretreatment with a NMDAR antagonist disrupts instrumental learning, the long-term protective effect of fixed spaced stimulation, and the induction of the learning impairment (Joynes et al., 2004; Ferguson et al., 2006; Baumbauer et al., 2009). We have also examined whether pretreatment with NMDA has a long-term effect on learning. While a high dose of NMDA (6 mM, 15 μL i.t.) induced a lasting learning impairment (Ferguson et al., 2012b), moderate doses (e.g., 0.06–0.6 mM) that are within the range that foster locomotor behavior have no long-term effect (Strain et al., 2013). Because NMDA was only effective at a high concentration, it possible that it impaired plasticity because it induced a non-reversible state. Before we conclude that NMDA is sufficient to induce a VIS-like learning impairment, we need to address this issue. For now, we can conclude only that the NMDAR plays a necessary role. This is true for a wide range of spinal learning phenomena, including sensitization, Pavlovian conditioning, instrumental learning, and the metaplastic effects of training discussed here (Durkovic and Prokowich, 1998; Willis, 2001; Ji et al., 2003; Joynes et al., 2004; Ferguson et al., 2006).

Evidence suggests that glutamate within the hippocampus can induce a metaplastic effect by engaging the mGluR (Cohen et al., 1999). Of particular interest, activation of group I mGluRs has been shown to facilitate both the induction and persistence of LTP within area CA1 (Abraham, 2008). This effect appears to be mediated by a number of mechanisms, including the trafficking of AMPARs to the synaptic membrane and the amplification of NMDAR-mediated currents (**Figure 4**; O'Connor et al., 1994; MacDonald et al., 2007). It has also been suggested that activating mGluRs can engage a "molecular switch" that enhances the persistence of LTP through a process that depends on group 1 mGluRs and PKC (Bortolotto et al., 1994). Within the spinal cord, group 1 mGluR antagonists have been shown to attenuate inflammation-induced EMR (Stanfa and Dickenson, 1998; Neugebauer et al., 1999; Karim et al., 2001; Zhang et al., 2002) and group 1 mGluR activity has been implicated in the development of neuropathic pain and tissue loss after spinal cord injury (SCI; Mukhin et al., 1996; Agrawal et al., 1998; Mills and Hulsebosch, 2002; Mills et al., 2002). Given these observations, we hypothesized that group 1 mGluR activity contributes to the induction of the learning impairment (Ferguson et al., 2008). Recognizing that two group 1 mGluR subtypes (mGluR1 and mGluR5) have been shown to impact hippocampal plasticity, we evaluated the effects of both CPCCOEt (a mGluR1 antagonist) and MPEP (a mGluR5 antagonist). After intrathecal application of the drug, subjects received 6 min of VIS and instrumental learning was tested 24 h later. We found that both drugs blocked the induction of the learning impairment in a dose-dependent manner (**Figure 5A**). We also examined whether either drug disrupted learning. Neither did and, if anything, CPCCOEt facilitated learning. These findings suggest that activation of group 1 mGluRs is necessary to the induction of the learning impairment. Finally, we asked whether mGluR activation in the absence of VIS is sufficient to induce a learning impairment. Subjects received the group 1 mGluR agonist DHPG and were tested 24 h later. We found that pretreatment with DHPG induced a lasting learning impairment.

Other work suggests that group 1 mGluRs can impact synaptic function through a PKC-mediated signal cascade (Aniksztejn et al., 1992; Skeberdis et al., 2001). We assessed PKC activation and observed enhanced activity one hour after treatment (Ferguson et al., 2008). Further, pretreatment with two structurally distinct PKC inhibitors (BIM; chelerythrine) blocked the learning impairment induced by VIS and DHPG (**Figure 5B**). Importantly, BIM had no effect on instrumental learning. Taken together, the findings suggest that the long-term metaplastic effect of VIS on spinal plasticity involves mGluR activation and PKC.

KAPPA OPIOIDS MEDIATE THE EXPRESSION OF THE LEARNING IMPAIRMENT

Prior work has shown that intact subjects exposed to uncontrollable stimulation exhibit a learning/performance deficit in instrumental learning tasks, a phenomenon known as learned helplessness (Maier and Seligman, 1976). Evidence suggests that the performance deficit is mediated, in part, by the release of an endogenous opioid (Maier, 1986). Supporting this, administration of an opioid antagonist (naltrexone) prior to testing attenuates the behavioral impairment observed in a shuttle avoidance task (Blustein et al., 1992). Likewise, we found that intrathecal administration of naltrexone attenuates the learning impairment observed in spinally transected rats that had received VIS (Joynes and Grau, 2004). We further showed that naltrexone is effective when given prior to testing, but has no effect when given the day before uncontrollable stimulation. This implies that a ligand that acts on a naltrexone-sensitive receptor plays an essential role in the expression of the learning impairment, but is not involved in its induction.

Because naltrexone is a relatively non-selective opioid antagonist, we also assessed the impact of drugs that bind to the mu (CTOP), delta (naltrindole), and kappa (nor-BNI) opioid receptors (Washburn et al., 2008). Using intrathecal administration of equal molar concentrations we showed that the expression of the learning impairment is blocked by a kappa receptor antagonist, but not a mu or delta antagonist. Conversely, intrathecal administration of a kappa-2 agonist (GR89696) impairs learning, whereas a mu (DAMGO) or a delta (DPDPE) agonist has no effect. Interestingly, a kappa-1 agonist (U69593) also had no effect on

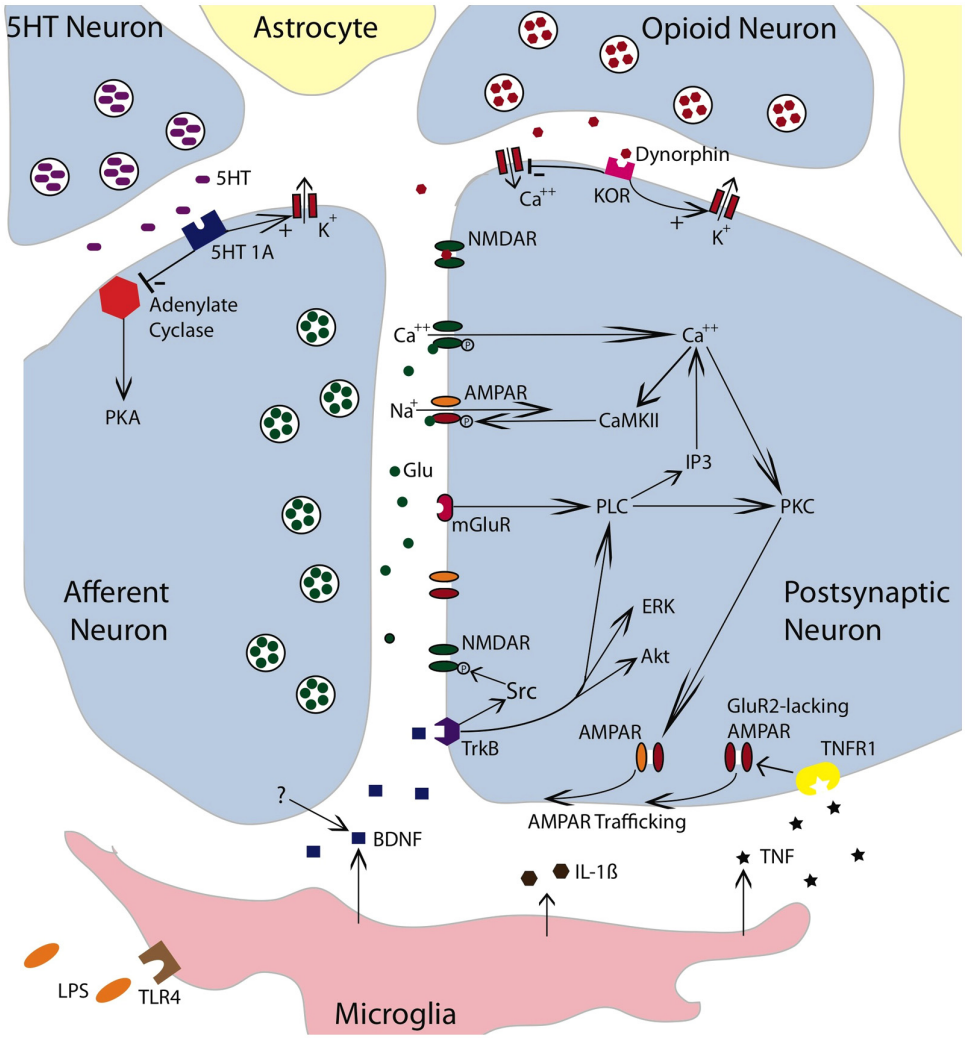


FIGURE 4 | Neurochemical mechanisms involved in spinally mediated learning and metaplasticity. The figure depicts a tripartite synapse involving an afferent neuron, a postsynaptic neuron, and the surrounding astrocytes. The afferent neuron is glutamatergic. Released glutamate (Glu) can engage the NMDAR, AMPAR, or mGluR receptors on the postsynaptic neuron. Activating the NMDAR in conjunction with a strong depolarization allows Ca^{++} to enter the postsynaptic cell, which engages intracellular signals such as Ca^{++}/calmodulin-dependent protein kinase (CaMKII) and protein kinase C (PKC). CaMKII activates the AMPAR and thereby promotes the entry of Na^{+}. Engaging the mGluR activates phospholipase C (PLC), which engages inositol triphosphate (IP3) and PKC. IP3 initiates the release of intracellular Ca^{++}. PKC promotes the trafficking of AMPARs to the active region of the synaptic membrane. To illustrate the relevant pathways, cells that exert a modulatory effect are also indicated. These include a descending serotonergic (5HT) neuron, an kappa opioid neuron, and a microglia. Both the 5HT neuron and opioid neuron would exert an inhibitory effect that could act on either the presynaptic or postsynaptic neuron. Dynorphin released from the opioid neuron would engage the kappa opioid receptor (KOR), which would inhibit neural excitation by facilitating the flow of K^{+} out of the cell and inhibiting the inward flow of Ca^{++}. Dynorphin can also bind to the NMDAR in its closed state and thereby inhibit NMDAR function. Engaging the 5HT 1A receptor would inhibit adynylate cyclase. This reduces the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) and down-regulates cAMP-dependent processes [e.g., protein kinase A (PKA)]. Promoting the outward flow of K^{+} would reduce neural excitability. Lipopolysaccharide (LPS) can engage the toll-like receptor 4 (TLR4) and activate microglia. Microglia have been shown to release TNF, IL-1β, and BDNF. BDNF may also be released from neurons. BDNF can foster NMDAR function through Src kinase. It also promote plasticity by engaging extracellular signal regulated kinase (ERK), serine-threonine-specific protein kinase (Akt) and PLC. By engaging the TNFR1, TNF fosters the trafficking of GluR2-lacking AMPARs to the synaptic membrane. The simplified drawing omits details (e.g., glutamatergic channels on microglia) that could contribute to spinally mediated metaplasticity. Adapted from Ji et al. (2003, 2013), Grau et al. (2006), and Cunha et al. (2010).

learning. Finally, we tested whether pretreatment with a kappa-2 agonist could substitute for VIS and induce a long-term learning impairment. It did not.

These observations suggest that the expression of the learning impairment is mediated by a ligand that acts at the kappa opioid receptor (**Figure 4**), possibly due to a kappa-2 mediated inhibition of NMDAR-mediated synaptic plasticity (Wagner et al., 1993; Caudle et al., 1994, 1997; Ho et al., 1997). Alternatively, kappa-2 opioid activity may "lock" the system in its current state, reducing plastic potential (Washburn et al., 2008). We noted above that the

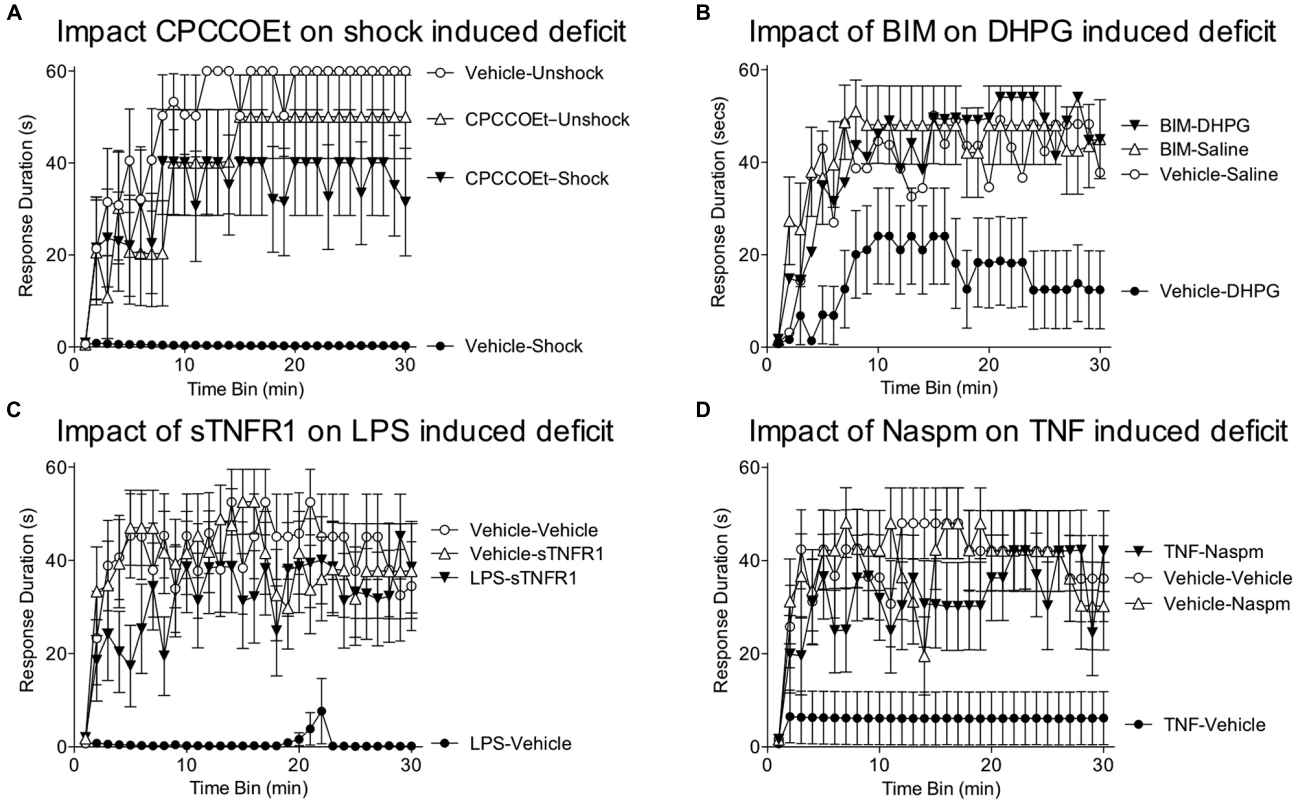


FIGURE 5 | Role of group 1 mGluR, glia, and TNF in the learning deficit. **(A)** Spinally transected rats received the group 1 mGluR1 antagonist CPCCOEt (i.t.; 100 nmol), or its vehicle, prior to VIS (Shock). Instrumental learning was tested 24 h later. Prior exposure to shock inhibited learning (Vehicle-Shock). Pretreatment with CPCCOEt blocked the induction of this learning impairment (CPCCOEt-Shock). **(B)** Rats were pretreated with the PKC inhibitor BIM (i.t.; 0.023 nmol), or its vehicle, and then received an injection of the group 1 mGluR agonist DHPG (100 nmol) or saline. Administration of DHPG alone (Vehicle-DHPG) impaired learning and this effect was blocked by pretreatment with BIM (BIM-DHPG). **(C)** Subjects received an intrathecal injection of LPS (100 μg), or its vehicle. The next day, a TNF inhibitor (sTNFR1; 700 ng) or vehicle was given intrathecal and subjects were tested in the instrumental learning paradigm. Prior treatment with LPS impaired learning (LPS-Vehicle). Administration of sTNFR1 prior to testing eliminated the learning deficit (LPS-sTNFR1). **(D)** Rats received an intrathcal injection of TNF (6000 pg) or its vehicle. The next day, they were given the GluR2 antagonist Naspm (i.e., 10 mM) or vehicle and tested in the instrumental learning paradigm. Rats that had previously received just TNF (TNF-Vehicle) failed to learn. This learning impairment was blocked by Naspm (TNF-Naspm). Adapted from Ferguson et al. (2008) and Huie et al. (2012a).

induction of the learning impairment is blocked by pretreatment with an NMDAR antagonist. If kappa opioids inhibit NMDAR-mediated plasticity, administration of a kappa agonist prior to VIS should interfere with the induction of the learning impairment. Washburn et al. (2008) found that GR89696 had this effect.

We suggested above that exposure to uncontrollable stimulation, or peripheral inflammation, may inhibit instrumental learning because these manipulations diffusely saturate NMDAR-mediated plasticity (Ferguson et al., 2006). If that alone was the cause of the learning impairment, there would be little reason to expect an opioid antagonist to block the expression of the learning impairment. Opioid reversibility implies that NMDAR-mediated plasticity remains functional, because as soon as the opioid brake is removed, learning can proceed. At a minimum, the observation requires a more sophisticated view of the factors that limit neural plasticity, that goes beyond the trafficking of AMPAR's, because it seems unlikely that an opioid antagonist could undo this effect within minutes of administration. These observations are also important for our claim that the learning impairment reflects a form of metaplasticity because the best examples of this phenomena involves cases wherein the underlying plasticity remains functional (Abraham, 2008).

GLIA AND TNF CONTRIBUTE TO SPINAL LEARNING IMPAIRMENTS

Throughout the nervous system, glia regulate synaptic efficacy, leading some to suggest the concept of a tripartite synapse (**Figure 4**; Araque et al., 1999; Haydon, 2001). In the spinal cord, glial activation plays an essential role in the development of inflammation-induced EMR (Meller et al., 1994; Watkins et al., 1997). Glia can be activated by administration of lipopolysaccharide (LPS) and, when applied intrathecally, this induces EMR (Reeve et al., 2000).

To examine whether glial activation is essential to spinal learning, we tested the effect of fluorocitrate. Fluorocitrate inhibits aconitase, an essential component of the tricarboxylic acid cycle within glia, and thereby disrupts energy-dependent transmitter up-take and release (Paulsen et al., 1987). If glia are essential to spinal plasticity, intrathecal fluorocitrate should inhibit

instrumental learning. We found that fluorocitrate does so in a dose-dependent manner (Vichaya et al., 2009). Next, we administered fluorocitrate prior to VIS and tested subjects 24 h later. We found that drug treatment blocked the induction of the learning impairment. Further, intrathecal application of LPS substituted for VIS and interfered with instrumental learning when subjects were tested 24 h later. This long-term effect of LPS was blocked by pretreatment with fluorocitrate. These findings provide further evidence that glia regulate spinal plasticity. More importantly, the results show that glia activation contributes to the long-term consequences of shock treatment; that glial activation is necessary, and sufficient, to the induction of a lasting inhibition of neural plasticity.

Glia can regulate synaptic plasticity through the release of cytokines, such as TNF and interleukin-1. TNF is of particular interest because it is known to modulate synaptic plasticity in hippocampal sections (Stellwagen and Malenka, 2006) and plays an essential role in the development of central sensitization (Czeschik et al., 2008; Park et al., 2011). TNF could interfere with learning by increasing the trafficking of AMPARs to the postsynaptic membrane (Beattie et al., 2002), an effect that has been linked to an up-regulation of Ca^{++} permeable GluR2-lacking AMPARs that increase postsynaptic excitability. If driven too far, this could potentially lead to excitotoxicity enhanced cell death after spinal injury (Ferguson et al., 2008). To explore whether TNF contributes to the learning impairment, we administered the soluble TNF receptor (sTNFR1), which inhibits TNF function by binding free TNF (Huie et al., 2012a). sTNFR1 was given intrathecal prior to VIS (induction phase) or 24 h later prior to testing (expression). sTNFR1 blocked both the induction and the expression of the learning impairment. Next, we asked whether administration of TNF would substitute for VIS treatment. We found that intrathecal TNF impaired learning when subjects were tested 24 h later. Mirroring the long-term effect of VIS treatment, the expression of the TNF-induced learning deficit was blocked by sTNFR1. sTNFR1 also blocked the expression of the learning deficit induced by LPS (**Figure 5C**). Likewise, inhibiting glial activation (with fluorocitrate) prior to TNF treatment blocked the induction of the learning impairment. These observations suggest that TNF has a long-term effect by activating glia and that this in turn enhances subsequent TNF release (Kuno et al., 2005). Cellular assays verified that TNF protein expression was increased 24 h after treatment with VIS (Huie et al., 2012a).

TNF could over-drive neural excitability by increasing the proportion of GluR2-lacking AMPARs. If this is how TNF interferes with learning, administering an antagonist (Naspm) that blocks these AMPARs should reinstate the capacity to learn. To test this, we induced a learning impairment with VIS or intrathecal TNF. The next day, subjects were given Naspm, or its vehicle, and tested in our instrumental learning paradigm. As expected, both TNF and VIS impaired learning. In both cases, treatment with Naspm reinstated the capacity to learn (**Figure 5D**). On-going studies are examining whether VIS reduces the proportion of synaptic AMPARs that contain the GluA2 subunit (Stuck et al., 2012).

In summary, our finding suggests that spinal plasticity depends on glia. Further, VIS appears to induce a lasting learning impairment by engaging glia and up-regulating the release of TNF. We suggest that TNF impairs learning, perhaps by increasing the proportion of Ca^{++} permeable (GluR2-lacking) AMPARs. This could induce a state of over-excitation that interferes with learning, contributes to EMR, and promotes cell death after injury.

BDNF MEDIATES THE BENEFICIAL EFFECT OF TRAINING

We now understand a great deal about how VIS has a lasting effect on spinal plasticity, with evidence implicating the mGluR, glia, and TNF (Ferguson et al., 2008; Vichaya et al., 2009; Huie et al., 2012b). As discussed below, these observations have clinical implications. But of potentially greater long-term value is the discovery of how controllable and/or regular stimulation induces a lasting beneficial effect that can prevent, and restore, adaptive plasticity and attenuate the development of EMR. We began to study this issue in collaboration with Gómez-Pinilla et al. (2007). Others had shown that LTP induces the expression of BDNF (Patterson et al., 1992), that mice with a BDNF deletion fail to exhibit LTP and that exogenous BDNF restores LTP (Patterson et al., 1996; Linnarsson et al., 1997). Other evidence indicated that BDNF can promote synaptic plasticity within the spinal cord. For example, intermittent hypoxia induces an adaptive modification within the cervical spinal cord known as phrenic long-term facilitation (Dale-Nagle et al., 2010). Local application of BDNF has a similar effect and the effect of intermittent hypoxia on neural function is blocked by a BDNF inhibitor (Baker-Herman et al., 2004). BDNF has also been shown to promote locomotor behavior after spinal injury (Boyce et al., 2007, 2012) and the beneficial effect of treadmill training on locomotor performance has been linked to an up-regulation of endogenous BDNF (Gómez-Pinilla et al., 2001). Finally, evidence suggests that TNF and BDNF impact synaptic scaling in opposite ways (Turrigiano, 2008).

Given these observations, we explored whether instrumental training affects BDNF expression in spinally transected rats. Subjects underwent training with controllable (Master) or uncontrollable (Yoked) shock and tissue was collected at the end of training. Relative to both unshocked and yoked groups, training with controllable stimulation up-regulated BDNF expression (**Figure 6A**; Gómez-Pinilla et al., 2007). In contrast, uncontrollable stimulation down-regulated expression. mRNA expression in master rats was well-correlated with an index of instrumental learning (**Figure 6B**). An identical pattern was observed for CaMKII and CREB mRNA expression. These genes were of interest because they are regulated by BDNF, have been implicated in other models of neural plasticity, and have been characterized as molecular memory switches (Yin et al., 1995; Yin and Tully, 1996; Blanquet and Lamour, 1997; Tully, 1997; Finkbeiner, 2000; Lisman et al., 2002). Using *in situ* hybridization, we showed that training with controllable shock induces an increase in BDNF mRNA expression within both the dorsal and ventral horn (Huie et al., 2012b). Western blotting showed BDNF protein was increased within the L3–L5 segments. Training also increased protein expression of the BDNF receptor TrkB [both truncated (TrkB 95) and full length (TrkB 145)]. Immunohistochemical analyses revealed increased TrkB protein expression within the dorsal horn and double labeling showed that most TrkB expression was localized to neurons.

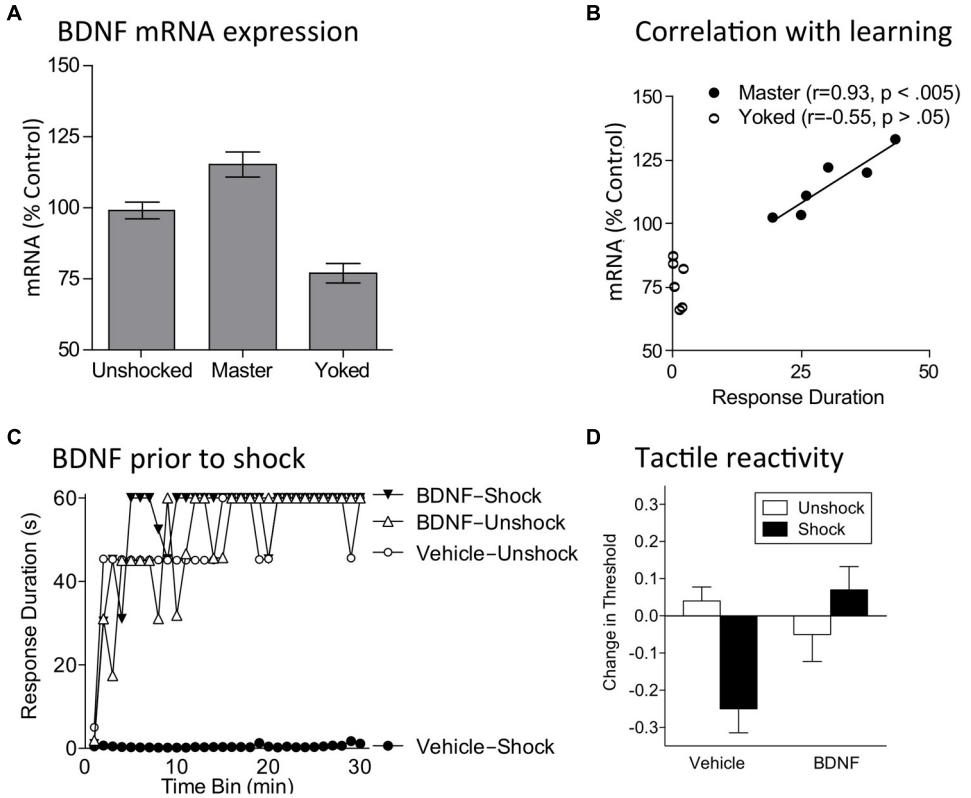


FIGURE 6 | BDNF mediates the beneficial effect of instrumental training. (A) Training with controllable shock (Master) produces an increase in BDNF mRNA expression whereas exposure to uncontrollable shock (Yoked) down-regulates expression. **(B)** In trained subjects (Master), BDNF mRNA expression is highly correlated with a measure of learning (mean response duration during the first 10 min of training). **(C)** Subjects were given BDNF (i.t.; 0.4 μg) or its vehicle and then VIS (Shock) or nothing (Unshock). The next day subjects were tested in the instrumental learning paradigm. Prior exposure to shock impaired learning (Vehicle-Shock). Pretreatment with BDNF (BDNF-Shock) blocked the induction of this learning impairment. **(D)** Spinally transected rats received BDNF (i.t.; 0.4 μg), or its vehicle, followed by VIS (Shock) or nothing (Unshock). Tactile reactivity was tested bilaterally using von Frey stimuli applied to the plantar surface of each hind paw. Because similar results were observed across legs, the data were collapsed across this variable. Vehicle treated rats that received shock exhibited EMR. Pretreatment with BDNF blocked this effect. Adapted from Gómez-Pinilla et al. (2007) and Huie et al. (2012b).

Next, we assessed the impact of inhibiting BDNF function using the sequestering antibody TrkB-IgG. TrkB-IgG did not have a significant effect on instrumental learning (Gómez-Pinilla et al., 2007; Huie et al., 2012b). It did, however, block the facilitation of learning when subjects were tested at a higher response criterion (Gómez-Pinilla et al., 2007). Inhibiting the downstream signal CaMKII with AIP had the same effect. If training fosters learning because it up-regulates BDNF release, exogenous application of BDNF should promote learning. As predicted, intrathecal BDNF facilitated learning in untrained rats tested with a high response criterion (Gómez-Pinilla et al., 2007). This pattern of results implies that training induces a lasting modification that up-regulates BDNF expression, which promotes learning about new environmental relations and alters the capacity for future learning.

If controllable/predictable shock induces a protective effect because it up-regulates BDNF expression and release, then BDNF should substitute for training and block the induction of the VIS induced learning impairment. To test this, subjects received intrathecal BDNF followed by VIS. As usual, rats given VIS exhibited a learning impairment when tested 24 h later with controllable stimulation (Huie et al., 2012b). Pretreatment with BDNF blocked the induction of this learning deficit (**Figure 6C**).

As discussed above, the learning impairment observed after VIS can be eliminated by training rats with controllable stimulation [in conjunction with a drug (naltrexone) that blocks the expression of the learning deficit; Crown and Grau, 2001]. To examine whether this therapeutic effect of training depends upon BDNF, subjects were given VIS followed by instrumental training in the presence of naltrexone (Huie et al., 2012b). Prior to instrumental training, rats received TrkB-IgG or its vehicle. The next day, subjects were tested in our instrumental learning paradigm. As usual, training eliminated the VIS-induced learning impairment. This restorative effect was not observed in subjects given TrkB-IgG prior to instrumental training. Recognizing that TrkB-IgG could have blocked the beneficial effect of training, in part, by interfering with instrumental learning, we examined whether TrkB-IgG would be effective if given immediately after instrumental training. Again, rats received variable shock followed by instrumental training in compound

with naltrexone. At the end of training, half the subjects received TrkB-IgG. We found that blocking BDNF *after* instrumental training attenuated its restorative effect. This suggests that the effect of TrkB-IgG is not due to a disruption of instrumental learning and implies that training induces a prolonged increase in BDNF release that contributes to the restoration of learning.

Having shown that BDNF is essential to the restorative effect of instrumental training, we asked whether exogenous BDNF could substitute for training and restore the capacity to learn in rats that had previously received VIS (Huie et al., 2012b). Subjects received VIS or nothing followed by intrathecal BDNF or vehicle. When tested in our instrumental paradigm 24 h later, subjects that had received VIS exhibited a learning impairment. BDNF given after VIS restored the capacity to learn. Further work revealed that BDNF given 24 h after VIS, immediately before testing, also eliminates the learning impairment.

VIS also induces EMR (Ferguson et al., 2006). Our results imply that BDNF may mediate this effect too. We examined this possibility by administering BDNF prior to 6 min of VIS applied to one hindlimb in spinally transected rats. Tactile reactivity was assessed using von Frey stimuli applied to the plantar surface of each hind paw. Our usual dose of BDNF (0.4 μg) had no effect on baseline tactile reactivity. Exposure to VIS induced EMR and this effect was blocked by BDNF (**Figure 6D**). More recently, we have shown that this same dose of BDNF counters inflammation-induced EMR in spinally transected rats and down-regulates a cellular marker of nociceptive sensitization (Erk phosphorylation; Lee et al., 2014).

In summary, we found that BDNF generally counters maladaptive plasticity, reinstating the capacity for learning and attenuating EMR in spinally transected rats. Our results further show that instrumental training and exposure to fixed spaced stimulation have a beneficial effect because they up-regulate BDNF expression (Gómez-Pinilla et al., 2007; Huie et al., 2012b). These finding complement other data demonstrating that locomotor training, exercise, and intermittent hypoxia, can promote adaptive plasticity through a BDNF-dependent process (Gómez-Pinilla et al., 2001; Baker-Herman et al., 2004).

How BDNF affects spinal function appears to be modulated by spinal injury. Specifically, in spinally injured rats BDNF attenuates EMR (Cejas et al., 2000; Huie et al., 2012b; Lee et al., 2014) whereas it often enhances pain in uninjured subjects (Merighi et al., 2008). As we discuss below, these differences may be related to the regulation of intracellular Cl^- concentrations, which can alter GABA function. Other important factors may include the BDNF source (neural or glial) and BDNF concentration (cf Miki et al., 2000; Cunha et al., 2010).

We found that training induced a rapid increase in BDNF protein, which was evident when tissue was collected immediately after 30 min of training. Likewise, intermittent hypoxia has been shown to increase BDNF protein within 60 min (Baker-Herman et al., 2004). These findings may reflect the local dendritic cleavage of the pro-form of BDNF into the mature form. This mechanism, which is mediated by tissue plasminogen activator (tPA), can be rapidly engaged in an activity-dependent manner (Waterhouse and Xu, 2009). Interestingly, in the absence of cleavage, pro-BDNF can have an opponent-like effect through its action at the P75 neurotrophin receptor (P75NTR; Bothwell, 1996; Lu et al., 2005; Cunha et al., 2010). For example, while BDNF fosters the development of LTP, proBDNF favors the induction of LTD. This suggests the intriguing possibility that training may influence BDNF function, in part, by regulating cleavage of proBDNF.

Our work suggests that BDNF plays a major role in mediating the restorative effect of behavioral training; that it is both necessary and sufficient to its expression. However, we have found no evidence that BDNF is required for the induction, or maintenance, of these training effects. Further, while BDNF can substitute for training to enable learning, and counter the adverse effect of uncontrollable/unpredictable stimulation, its effect appears to wane within a few hours (Zhang et al., 2014).

METAPLASTICITY AND SPINAL CORD INJURY

UNCONTROLLABLE STIMULATION IMPAIRS RECOVERY AND ENHANCES PAIN IN CONTUSED RATS

We have begun to explore the implications of our results for recovery after a contusion injury. Our work was motivated by both our studies in spinally transected rats and the clinical observation that spinal injuries are often accompanied by other tissue damage that provide a source of nociceptive input and peripheral inflammation. Our hypothesis was that afferent nociceptive signals could induce a state of over-excitation that enhances secondary damage and undermines recovery. Because we have a good understanding of how VIS induced nociceptive activity affects spinal function after a transection, and because this type of stimulation is readily controlled and produces (at the intensities used) no secondary peripheral effects that extend beyond the period of stimulation, we began by exploring the impact of VIS.

To assess the impact of stimulation on recovery, we used a moderate (12.5 mm) contusion injury at T12 produced with the MASCIS device (Grau et al., 2004). A day after injury, we assessed locomotor performance and then exposed rats to VIS. We found that VIS produced a profound disruption in recovery (**Figure 7A**). This effect was evident within 3 days and was maintained over the next 6 weeks. Further work showed that shock treatment was most effective when given within 4 days of injury. Most importantly, nociceptive stimulation only had an adverse effect on recovery when shock was given in an uncontrollable manner; subjects that received the same amount of shock, but could control its occurrence (by exhibiting a flexion response) exhibited normal recovery. Shock treatment also enhanced mortality, led to greater weight loss, slowed the recovery of bladder function, and led to a higher incidence of spasticity. Histological analyses revealed that uncontrollable intermittent shock enhanced tissue loss (white and gray matter) at the site of injury and increased damage caudal to injury (Grau et al., 2004; Hook et al., 2007).

More recently, we have examined whether VIS affects the development of EMR in contused subjects (Garraway et al., 2012). As others have reported, contused rats exhibited EMR relative to sham-operated subjects from 7 to 28 days after injury. Contused rats that received 6 min of VIS exhibited an EMR that emerged more rapidly (within 24 h of shock treatment) and remained more robust (7–28 days after injury).

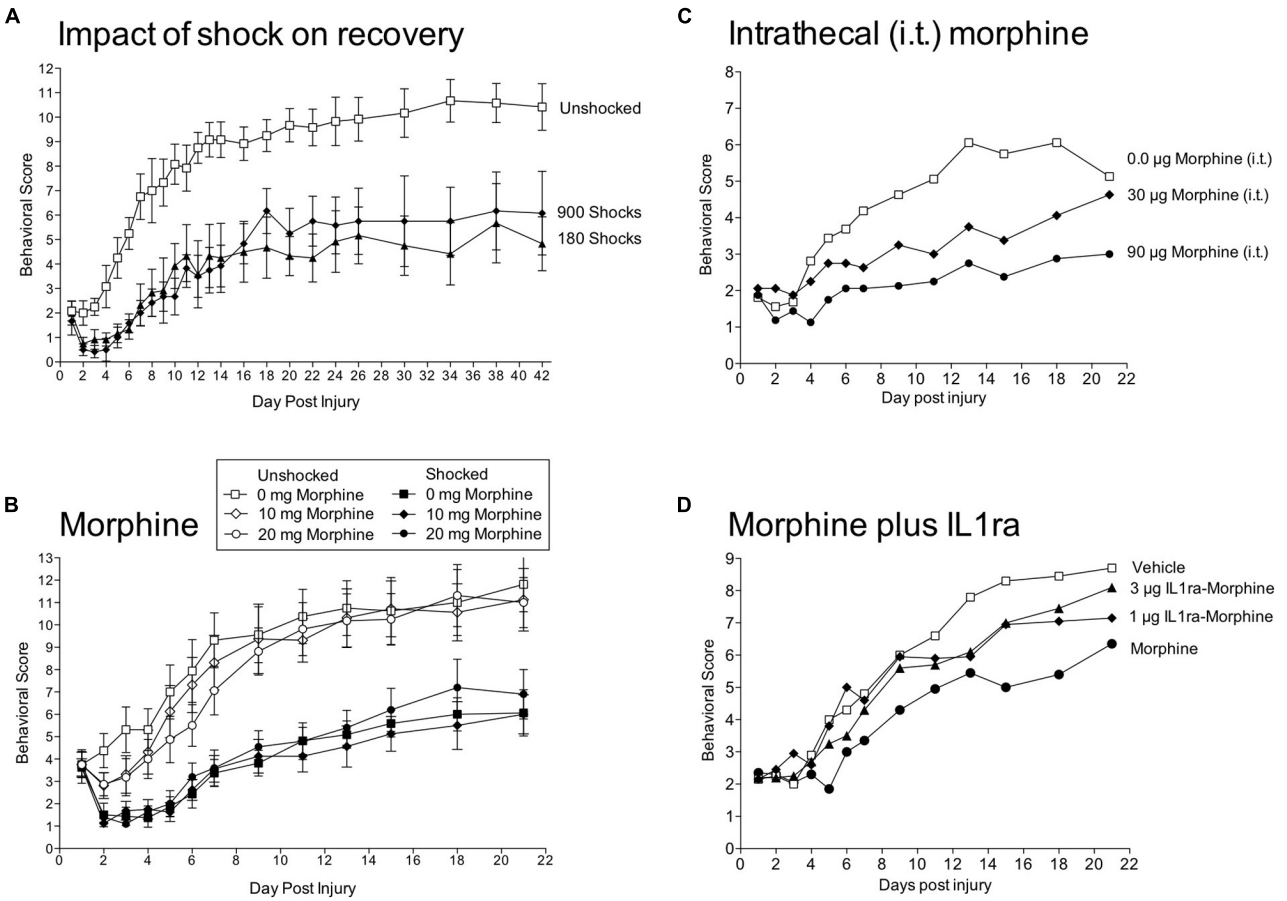


FIGURE 7 | Impact of shock and morphine treatment on recovery after a contusion injury. (A) Rats received a moderate contusion injury and 24 h later VIS (180 or 900 shocks) or nothing (Unshocked). Locomotor recovery was scored over the next six weeks by experimenters that were blind to the subjects experimental treatment using the BBB scale (Basso et al., 1995). Scores were adjusted using the transformation derived by Ferguson et al. (2004), which improves the metric properties of the scale. Shock treatment impaired the recovery of locomotor function. **(B)** Contused rats were given morphine [0, 10, or 20 mg, intraperitoneal (i.p.)] followed by VIS (Shocked) or nothing (Unshocked). Behavioral observations confirmed that the highest dose of morphine blocked shock-elicited movement and vocalization. Morphine treatment did not block the adverse effect of shock treatment on locomotor recovery. **(C)** Intrathecal (i.t.) administration of morphine 24 h after a contusion injury produces a dose-dependent impairment in locomotor recovery. **(D)** A day after a contusion injury, rats were pretreated with the IL-1 receptor antagonist (IL1ra; 1 or 3 µg, i.t.) or its vehicle and then given morphine (90 µg, i.t.) or vehicle. Morphine alone impaired locomotor recovery and this effect was attenuated by pretreatment with IL1ra. Adapted from Grau et al. (2004) and Hook et al. (2008, 2007, 2009).

OPIOIDS DO NOT BLOCK THE EFFECT OF NOCICEPTIVE STIMULATION AND IMPAIR RECOVERY

Having shown that nociceptive stimulation impairs recovery after a contusion injury, we reasoned that inhibiting nociceptive transmission could have a protective effect. We first verified that an injection of morphine (20 mg/kg, i.p.) induced a robust antinociception on the tail-flick test in contused rats (Hook et al., 2007). Importantly, morphine also inhibited shock-elicit movements and brain-dependent responses to pain (e.g., vocalization). In morphine treated contused rats, VIS induced little movement or pain, but nonetheless impaired recovery (**Figure 7B**). Morphine not only failed to have a protective effect, it interacted with nociceptive stimulation and enhanced mortality. Indeed, half the subjects (8 out of 16) given both VIS and 20 mg/Kg of morphine died. Oddly, subjects typically died days after morphine treatment (mean = 4.6).

Systemic morphine could affect recovery by directly impacting a spinal process or by engaging a brain system that indirectly affects spinal function. We hypothesized that the drug effect was due to a direct mode of action. To show this, we tested the impact of intrathecal morphine given 24 h after a contusion injury (Hook et al., 2009). Again, we confirmed that drug treatment induced a robust antinociception. Intrathecal morphine (90 µg) impaired the recovery of locomotor function (**Figure 7C**), led to greater weight loss, increased tissue loss at the site of injury, and enhanced rear paw-directed grooming/chewing (autophagia), a potential index of neuropathic pain.

Morphine has been shown to up-regulate proinflammatory cytokines [e.g., interleukin-1β (IL-1β), interleukin-6 (IL-6), TNF; Song and Zhao, 2001; Johnston et al., 2004]. Consistent with this work, systemic morphine (20 mg/kg) a day after a contusion injury increased expression of IL-1β and IL-6 24 h after

drug treatment (Hook et al., 2011). Intrathecal morphine had a similar effect and increased IL-1β within 30 min of drug treatment. To explore whether the release of IL-1β was causally related to the adverse effect of morphine treatment, we administered a IL-1 receptor antagonist (IL-1ra) prior to intrathecal morphine (90 μg). Morphine impaired locomotor recovery and this effect was blocked by IL-1ra (**Figure 7D**). Three weeks after injury, morphine treated rats also showed increased vocalization to tactile stimulation applied to the girdle region, an indication of increased at-level pain. This effect too was blocked by IL-1ra. While these results are promising, we also found that IL-1ra treatment led to greater issue loss at the site of injury, presumably because it blocked a beneficial effect of injury-induced IL-1β expression.

Current research is exploring the site of opioid action. As described above, the kappa-2 agonist GR89696 inhibits adaptive plasticity in transected rats. This same drug also impairs recovery after a contusion injury (Aceves and Hook, 2013). This is consistent with early studies that linked contusion-induced damage to kappa opioid activity (Faden, 1990). Other work suggests that opioids can also engage glia, and promote cytokine release, by engaging non-classic receptors [e.g., the toll-like receptor 4 (TLR4); Hutchinson et al., 2007, 2010; Watkins et al., 2007]. It seems likely that the adverse effects of morphine on spinal function are due to its action at multiple sites, including TLR4.

While morphine did not block the adverse effect of nociceptive stimulation, the data yielded an important discovery—opioid treatment after a contusion injury impairs the recovery of locomotor function, enhances pain, and leads to greater tissue loss (Hook et al., 2007, 2009). Further, when combined with nociceptive stimulation, morphine enhanced mortality. These results are especially troubling given the widespread use of opioids to treat pain after SCI (Warms et al., 2002; Widerstrom-Noga and Turk, 2003).

The results also have implications regarding the mechanisms that underlie the adverse effect of VIS on recovery. For example, it could be argued that this effect is secondary to brain-mediated pain or VIS-induced movement. Morphine treatment blocked both behavioral signs of pain and VIS-induced movement, but did not attenuate the effect of VIS on recovery. Further, if brain systems exert a protective effect by inhibiting spinal nociceptive transmission, our results imply that this antinociception is mediated by a nonopioid process (Meagher et al., 1993). Finally, the data indicate that a kappa-2 opioid dependent process, that we have shown inhibits adaptive plasticity in transected rats (Washburn et al., 2008), can substitute for VIS treatment (i.e., is sufficient) and impair recovery after a contusion injury (Aceves and Hook, 2013).

UNCONTROLLABLE STIMULATION INCREASES TNF AND REDUCES BDNF IN CONTUSED RATS

Earlier we described how the learning impairment induced by VIS in transected rats depends upon TNF (Huie et al., 2012a). Given this, we examined whether VIS induces TNF expression in contused subjects (Garraway et al., 2012). We found that nociceptive stimulation a day after a contusion injury increased TNF mRNA and protein expression from 1 to 7 days after VIS treatment. Interestingly, stimulation also increased protein levels of caspase 3 and 8, two indices of programmed cell death (apoptosis; Beattie et al., 2000; Duprez et al., 2009). Immunofluorescent labeling revealed that caspase 3 was co-labeled with OX-42 (microglia) and NeuN (neurons), but not GFAP (astrocytes). These observations parallel the results found with the transection paradigm and suggest that TNF release may foster secondary damage by promoting apoptotic cell death.

We have also examined the impact of nociceptive stimulation on BDNF/TrkB expression after a contusion injury (Garraway et al., 2011). Our hypothesis was that uncontrollable nociceptive stimulation impairs recovery, in part, by down-regulating BDNF expression. To test this, subjects were given a moderate contusion injury and were exposed to VIS or nothing the next day. A contusion injury, *per se*, down-regulated BDNF mRNA and protein expression (**Figure 8A**). Exposure to VIS a day after injury further down-regulated BDNF mRNA and protein expression and this effect was most evident a day after shock treatment (48 h after injury). In the dorsal horn, VIS induced a lasting reduction

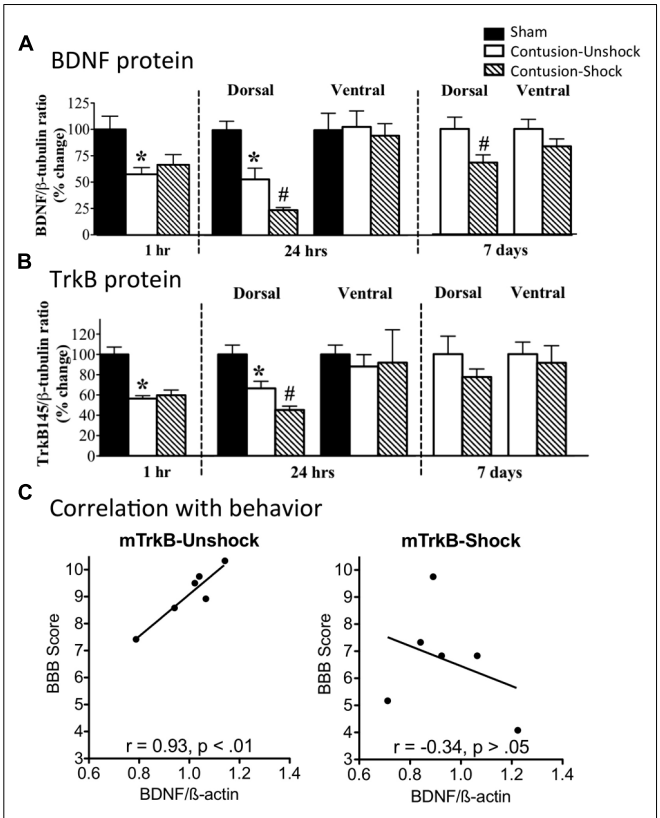


FIGURE 8 | Impact of VIS on BDNF and TrkB expression in contused rats. (A) Rats received a moderate contusion injury or a sham surgery. The next day, contused rats received VIS (Contused-Shock) or nothing (Contused-Unshock). BDNF protein was assayed 1 h, 24 h, and 7 days after treatment. A contusion injury produced a significant decrease (*) in BDNF expression at 1 and 24 h (25 and 48 h after surgery). Shock treatment further down-regulated BDNF expression (#) at 24 h and 7 days. **(B)** TrkB protein expression was also down regulated by a contusion injury at 1 and 24 h (*). Shock treatment produced a further decrease at 24 h (#). **(C)** Locomotor performance on days 2–7 was highly correlated with mTRKB expression in untreated (Unshock) contused rats (left panel), but not in rats that received shock (right panel). Adapted from Garraway et al. (2011).

in BDNF protein that was evident a week after shock treatment. A contusion injury and VIS treatment had a similar effect on TrkB mRNA and protein expression (**Figure 8B**), reducing expression during the first 48 h of recovery. TrkB immunolabeling showed that it was co-expressed with NeuN, but not GFAP or OX-42. Correlational analyses revealed that locomotor recovery was highly related with TrkB mRNA expression in unshocked, but not shocked, subjects (**Figure 8C**). A similar pattern was observed for BDNF. This suggests that improved recovery is normally associated with enhanced TrkB expression and that shock treatment may adversely affect recovery by dysregulating this process.

In summary, the results obtained to date generally parallel the findings obtained in our transection paradigm. In both cases, exposure to VIS has a maladaptive effect that induces EMR and disrupts adaptive plasticity, impairing both instrumental learning and recovery after a contusion injury (Grau et al., 1998, 2004; Joynes et al., 2003; Ferguson et al., 2008; Garraway et al., 2011, 2012). As observed in transected rats, VIS induces an increase in TNF expression and down-regulates BDNF in contused subjects (Gómez-Pinilla et al., 2007; Garraway et al., 2011, 2012; Huie et al., 2012a). The contusion paradigm also showed that nociceptive stimulation engages markers of apoptotic cell death and leads to enhanced tissue loss. These adverse effects may explain, in part, why other types of nociceptive stimulation (e.g., from stretching; Caudle et al., 2011) impair the recovery process.

SPINAL PROCESSES ARE REGULATED BY THE BRAIN

ANESTHESIA BLOCKS THE BRAIN-DEPENDENT INHIBITION OF MALADAPTIVE PLASTICITY

We have shown that VIS inhibits spinal plasticity in transected animals and impairs recovery after a contusion injury (Grau et al., 1998, 2004). Does this effect impact spinal function in the absence of injury? The answer appears to be a qualified no. We explored this issue by applying VIS before or after a spinal transection (Crown and Grau, 2005). The next day we tested subjects in our instrumental paradigm. As usual, VIS given after a spinal transection induced a learning impairment (**Figure 9A**). When given before, it had no effect, which suggests that brain-dependent processes exert a modulatory effect that counters the development of the learning impairment. This is consistent with other studies showing that the induction of spinal LTP is inhibited by descending pathways (Sandkühler and Liu, 1998; Sandkühler, 2000). The results suggest that the brain normally acts to quell over-excitation within the spinal cord and thereby helps to maintain neural homeostasis. We would also expect this process to counter the development of central sensitization. Supporting this, we recently found that capsaicin-induced EMR is weaker in intact subjects (relative to spinally transected; Huang et al., 2014).

A caveat to our description of brain-dependent regulation is that the protective role of brain processes can be disrupted by surgical anesthesia. This issue was explored by Washburn et al. (2007), who tested whether VIS induced a learning impairment in pentobarbital anesthetized rats. Others have suggested that pentobarbital anesthesia induces a physiological state within the spinal cord that resembles the consequences of a spinal transection (Mori et al., 1981). Given this, she predicted that VIS applied to the tail would have its usual effect in intact anesthetized rats. To test this, intact subjects received anesthetic dose of pentobarbital or its vehicle followed by VIS. The subjects were then transected and a day later, tested for instrumental learning. As expected, VIS did not induce a learning impairment in unanesthetized subjects (**Figure 9B**). Rats that received pentobarbital prior to VIS failed to learn, implying that pentobarbital anesthesia eliminates the brain-dependent protection of spinal circuitry. This finding is important because it suggests that nociceptive input during general anesthesia could have an unanticipated effect that sensitizes spinal nociceptive systems and promotes the development of neuropathic pain.

As noted earlier, VIS does not induce antinociception in spinally transected rats. However, in intact rats, VIS induces a robust antinociception (**Figure 9C**). This effect too was blocked by pentobarbital anesthesia (Washburn et al., 2007). The finding implies that brain-dependent processes may protect spinal systems by inhibiting nociceptive transmission. Because pretreatment with an opioid does not have a protective effect (Hook and Grau, 2007), we posit that brain systems inhibit the development of central sensitization through a non-opioid process (e.g., serotonin).

DESCENDING SEROTONERGIC FIBERS MEDIATE THE INHIBITION OF MALADAPTIVE PLASTICITY

Prior work suggested that the inhibition of the VIS-induced learning impairment could be mediated by serotonergic fibers that descend through the dorsal lateral funiculus (DLF; Davies et al., 1983; Watkins et al., 1984). If this is true, we should be able to eliminate the brain-dependent protection of spinal circuits by lesioning the DLF. Crown and Grau (2005) examined this issue by bilaterally lesioning the DLF at T2. Relative to the sham operated controls, DLF lesions had little effect on sensory/motor function. Subjects then received VIS and 2 h later the spinal cord was transected at T8. The capacity for instrumental learning was assessed the next day. As expected, in the absence of DLF lesions, VIS had no effect on spinal function. However, in DLF lesioned rats VIS induced a learning impairment (**Figure 10A**).

Because a large proportion of the fibers within the DLF are serotonergic (Davies et al., 1983), we hypothesized that the brain-mediated protective effect depends upon serotonin (5HT). This is consistent with other work that has linked the development of EMR after spinal injury to the loss of 5HT. Further, 5HT has been shown to attenuate EMR in injured rats and this effect has been linked to an action at the 5HT 1A receptor (e.g., Eaton et al., 1997; Bardin et al., 2000; Gjerstad et al., 2001; Hains et al., 2001a,b, 2003). If this same system mediates the brain-dependent inhibition of the learning impairment, engaging the 5HT-1A receptor should have a protective effect in spinally transected rats. To test this, rats were spinally transected and administered the agonist 5HT or 8-OH DPAT (5HT 1A/7) intrathecal prior to VIS. Instrumental learning was tested the next day. Crown and Grau (2005) found that both drugs block the VIS-induced learning impairment (**Figure 10B**). Drugs that acted at other 5HT receptors [DOI (5HT 2) and quipazine (5HT 2/3)] had no effect. Recognizing that some fibers within the DLF are noradrenergic (Davies et al., 1983), Crown and Grau

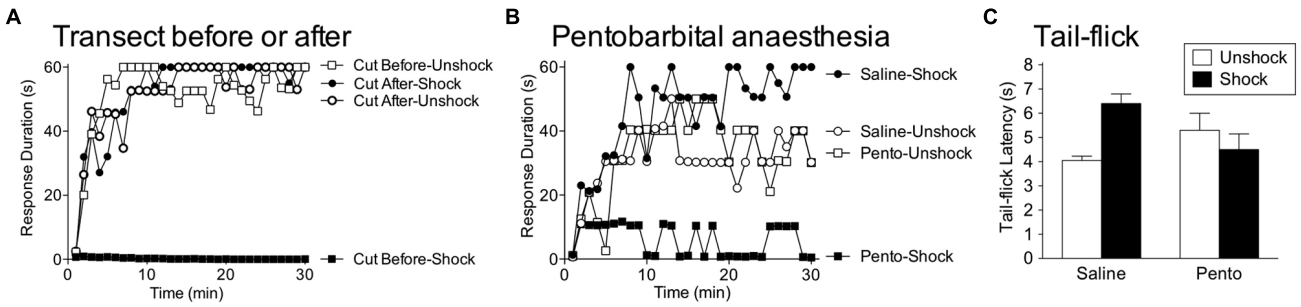


FIGURE 9 | Brain systems transform how shock affects spinal function. **(A)** Rats received VIS (Shock) or nothing (Unshock) before or after a spinal transection (Cut) and were tested for instrumental learning the next day. When the spinal cord was cut prior to treatment, shock induced a learning impairment (Cut Before-Shock). When the spinal cord was cut after shock treatment (Cut After-Shock), no impairment was observed. **(B)** Intact rats were anesthetized with pentobarbital (50 mg/kg, i.p.) or given saline. Subjects then received VIS (Shock) or nothing (Unshock), followed by a spinal transection and instrumental testing. Shock induced a learning impairment in anesthetized (Pento-Shock) but not awake (Saline-Shock) rats. **(C)** Intact rats received saline or pentobarbital (50 mg/kg, i.p.) followed by VIS (Shock) or nothing (Unshock). Nociceptive reactivity was tested by applying a noxious thermal stimulus to the tail and measuring the latency to exhibit a tail-withdrawal (tail-flick). In awake rats (Saline), shock treatment induced antinociception. Shock had no effect on tail-flick latencies in anesthetized (Pento) rats. Adapted from Crown and Grau (2005) and Washburn et al. (2007).

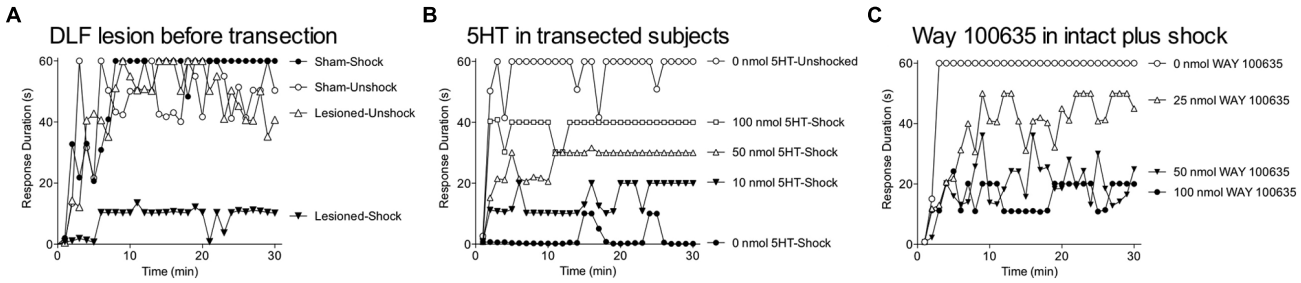


FIGURE 10 | Descending serotonergic systems modulate the induction of the shock-induced learning impairment. **(A)** Subjects underwent bilateral lesions of the DLF at T2 or a sham surgery. Two hours later the spinal cord was transected at T8 and instrumental learning was assessed the next day. Shock treatment induced a learning impairment in DLF lesioned rats (Lesioned-Shock) but not in intact sham operated rats (Sham-Shock). **(B)** Spinally transected rats received serotonin (i.t.; 0, 10, 50, or 100 nmol) followed by VIS (Shock) or nothing (Unshock). Instrumental learning was tested the next day. Shock treatment impaired learning in vehicle treated rats (0 nmol 5HT-Shock). This learning impairment was blocked by pretreatment with 5HT in a dose-dependent manner. **(C)** Intact rats received the 5HT 1A antagonist WAY 100635 (i.t.; 0, 25, 50, or 100 nmol) prior to VIS. The spinal cord was then transected and instrumental learning was assessed the next day. As expected, intact rats that received shock and the vehicle (0 nmol WAY 100635) did not exhibit a learning impairment. Shock induced a learning impairment in subjects given the 5HT 1A antagonist and this effect was dose-dependent. Adapted from Crown and Grau (2005).

(2005) also assessed the impact of the α-2 noradrenergic agonist clonidine. While clonidine had a protective effect, its action was blocked by a 5HT 1A antagonist (WAY 100635), implying that the positive effect was due to cross-reactivity with the 5HT 1A receptor (Newman-Tancredi et al., 1998; Shannon and Lutz, 2000).

These results suggest that activation of the 5HT 1A receptor can substitute for the brain-dependent process in transected rats to inhibit the development of maladaptive plasticity. To test the necessity of this process, Crown blocked the 5HT 1A receptor in intact rats (using i.t. WAY 100635) and administered VIS. The spinal cord was then transected and subjects were tested for instrumental learning. When the 5HT 1A receptor was blocked, VIS induced a learning impairment in intact rats (**Figure 10C**). We conclude that descending serotonergic fibers counter the development of maladaptive plasticity by engaging the 5HT 1A receptor. The corollary to this is that damage to this tract will remove this protective effect and set the stage for maladaptive plasticity in response to uncontrollable nociceptive input and peripheral inflammation.

SPINAL INJURY ALTERS GABAERGIC FUNCTION WITHIN THE SPINAL CORD

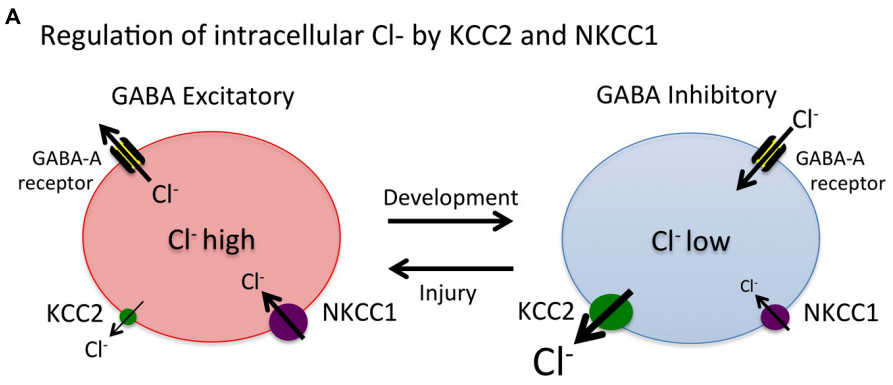
Our results imply that the loss of brain input can fundamentally alter how spinal systems operate. We suggest that this may explain why some treatments affect spinal systems in opposite ways depending whether brain input is intact or absent. An especially revealing example of this emerged from our work examining the role of GABAergic systems (Ferguson et al., 2003). We had shown that administration of the GABA-A antagonist bicuculline blocks the expression of the VIS-induced learning impairment. VIS also induces EMR (Ferguson et al., 2006). If the learning impairment and the EMR are mediated by a common mechanism, the EMR should also be blocked by bicuculline. This seemed paradoxical because GABA is typically viewed as having an inhibitory effect that should counter neural excitation and EMR. Indeed, in intact

rats, blocking GABA with bicuculline generally enhances reactivity to nociceptive and mechanical stimuli (Roberts et al., 1986; Millan, 2002). However, in spinally transected rats, we found that intrathecal bicuculline blocks VIS-induced EMR (Huang et al., 2011). More surprising, treatment with bicuculline also blocked capsaicin-induced EMR, and cellular indices of central sensitization (e.g., ERK phosphorylation), in transected rats (Huang et al., 2014). Thus, when communication with the brain was disrupted, blocking GABAergic activity seemingly quelled, rather than enhanced, nociceptive sensitization.

The fault in our reasoning likely lies with the assumption that GABA uniformly has an inhibitory effect. The impact of GABA on neural excitation is regulated by the concentration of intracellular Cl^-, which is controlled by K^+-Cl^- cotransporter 2 (KCC2) and Na^+-K^+-Cl^- cotransporter 1 (NKCC1; **Figure 11A**). As the nervous system develops, there is an increase in KCC2 expression, which lowers the concentration of intracellular Cl^- (Ben-Ari, 2002). As a result, engaging the GABA-A receptor causes Cl^- to flow into the cell, which produces neural inhibition. Spinal injury, however, can cause a reduction in KCC2 expression, which leads to an increase in intracellular Cl^- (Ben-Ari et al., 2012). Consequently, engaging the GABA-A receptor will allow Cl^- to flow out of the cell, which has a depolarizing (excitatory) effect that may contribute to the development of EMR (Cramer et al., 2008; Hasbargen et al., 2010). Recently, we confirmed that a spinal transection reduces the ratio of membrane bound KCC2 (relative to the cytosolic fraction) in the lumbosacral spinal cord within 24 h (Huang et al., 2014).

In the uninjured system, GABAergic transmission would have a homeostatic effect that would act to counter nociception-induced over-excitation. But if KCC2 levels are low, engaging this process would promote over-excitation and the development of central sensitization. Under these conditions, pretreatment with a GABA-A antagonist should counter the development of central sensitization and EMR, and our data suggest that this is true (Huang et al., 2013). We would further predict that in intact animals, intrathecal bicuculline should have the opposite effect. In the intact system, plasmalemmal KCC2 levels should be high. Now, engaging the GABA-A receptor would promote the inward flow of Cl^-, producing neural inhibition. Supporting this, we found that in intact animals, bicuculline enhanced capsaicin-induced EMR and ERK phosphorylation (Huang et al., 2014).

Changes in GABAergic function may also help to explain another paradoxical effect. Earlier, we described how intrathecal administration of BDNF has a beneficial effect in spinally transected rats, countering both the learning impairment and EMR induced by VIS (Huie et al., 2012b). More recently, we showed that



	Uninjured	Transected
Capsaicin alone	↑ EMR, ↑ ERK act.	⬆ EMR, ⬆ ERK act.
Capsaicin + BDNF	⬆ EMR, ⬆ ERK act.	↑ EMR, ↑ ERK act.
Capsaicin + Bicuculline	⬆ EMR, ⬆ ERK act.	↑ EMR, ↑ ERK act.

FIGURE 11 | Injury increases intracellular Cl^- in GABA-responsive cells, causing GABA to have an excitatory effect and transforming how experimental manipulations impact spinal function. (A) Schematic of the processes that regulate intracellular Cl^-. NKCC1 regulates the inward flow of Cl^- whereas KCC2 controls its outward flow. Early in development, membrane-bound (plasmalemmal) KCC2 levels are low, and as result, the intracellular concentration of Cl^- remains high. Under these conditions, engaging the GABA-A receptor allows Cl^- to exit the cell, causing it depolarize, which promotes neural excitation. With development, there is an up-regulation of KCC2, which drives down the intracellular concentration of Cl^-. This causes a shift in polarity because engaging the GABA-A receptor now causes Cl^- to flow into the cell, inducing hyperpolization and neural inhibition. Recent work has revealed that spinal injury can cause a regression to the immature state, by down-regulating KCC2 expression (Ben-Ari et al., 2012). This would reduce the hyperpolarizing effect of GABA and enhance the degree to which it induces neural excitation. There is also a complimentary change in NKCC1 function, which is regulated through phosphorylation (Flemmer et al., 2002). **(B)** Capsaicin produces a stronger EMR, and greater ERK activation, in spinally transected rats. After a spinal injury, pretreatment with BDNF or bicuculline attenuates capsaicin-induced EMR and ERK activation. In uninjured rats, BDNF and bicuculline enhance capsaicin-induced EMR and ERK activation. Adapted from Kahle et al. (2014).

BDNF also attenuates capsaicin-induced EMR in transected subjects (Lee et al., 2012). These effects on EMR run counter to other studies demonstrating that BDNF in intact subjects can foster the development of central sensitization and enhance pain (Merighi et al., 2008). In intact subjects, BDNF reduces plasmalemmal KCC2 within the lumbosacral spinal cord, which would reduce GABAergic inhibition and explain, in part, why BDNF promotes the development of central sensitization (Coull et al., 2005; Valencia-de Ita et al., 2006; Lu et al., 2009; Biggs et al., 2010). Interestingly, after spinal injury, BDNF appears to have the opposite effect on KCC2 expression (Boulenguez et al., 2010), which would re-establish the inhibitory action of GABA. This suggests that BDNF should attenuate inflammation-induced EMR in transected rats, which is what we observed (Lee et al., 2012). It further suggests that BDNF should have the opposite effect on capsaicin-induced EMR in intact rats. Lee et al. (2014) recently confirmed that this too is true using both behavioral and cellular indices of central sensitization.

In summary, our results suggest that spinal injury removes a brain-dependent protective effect that is mediated by descending serotonergic fibers and the 5HT-1A receptor. A loss of brain communication also leads to a shift in KCC2 that transforms how GABA affects spinal circuits, causing it to have an excitatory effect that we suggest contributes to the development of central sensitization. BDNF may have a protective effect, in part, by promoting KCC2 plasmalemmal expression. This would re-establish GABA-mediated inhibition (Boulenguez et al., 2010), which could counter the development of neural excitation. In the absence of injury, GABAergic inhibition would be blocked by bicuculline and reduced by BDNF (through a down-regulation of KCC2), and in both cases, this would promote nociceptive activity and the development of EMR (**Figure 11B**). These conclusions are consistent with electrophysiological data indicating that BDNF facilitates AMPA and NMDA mediated currents in intact, but not spinally transected, subjects (Garraway et al., 2003, 2005; Garraway and Mendell, 2007). These data suggest that BDNF is pronociceptive in intact subjects, but not after spinal injury.

CONCLUSION

Studies of brain plasticity have uncovered processes that have a lasting impact on plastic potential, and we have suggested that this concept of metaplasticity has relevance to spinal function. We have shown that uncontrollable/unpredictable stimulation, and peripheral inflammation, induce a process that has a lasting inhibitory effect (Grau et al., 1998; Hook et al., 2008; Baumbauer et al., 2009). We related this process to the development of central sensitization and EMR, and characterized these effects as examples of maladaptive plasticity. We showed that predictable/controllable stimulation engages an opponent process, that fosters a form of adaptive plasticity (instrumental learning) and that counters the adverse effects of VIS and peripheral inflammation (Crown and Grau, 2001; Crown et al., 2002a). The beneficial effects of training appear related to an up-regulation of BDNF (Huie et al., 2012b). The adverse effects were tied to multiple processes: The expression of the deficit depends on kappa opioid activity (Washburn et al., 2008); its induction is coupled to the mGluR, non-neuronal cells, and the cytokine TNF (Ferguson et al., 2008; Vichaya et al., 2009; Huie et al., 2012a). We also showed that VIS disrupts recovery after a contusion injury and that this adverse effect was related to an up-regulation of TNF and a down-regulation of BDNF (Garraway et al., 2011, 2012).

Spinal injury appears to set the stage for damage and EMR by causing a loss of serotonergic fibers, which in the uninjured system, counter the development of maladaptive plasticity by acting at the 5HT-1A receptor (Crown and Grau, 2005). Disconnected from the brain, GABAergic systems revert to an immature state, due to a down-regulation of plasmalemmal KCC2 (Hasbargen et al., 2010; Ben-Ari et al., 2012). This causes GABA to have an excitatory effect that we posit contributes to the development of maladaptive plasticity.

We have linked the learning impairment to the release of TNF from microglia. The beneficial effects of training were tied to the release of BDNF. While we have not identified the relevant source of BDNF, it too could be expressed by microglia. If so, activity within microglia would determine whether an earlier experience engages a metaplastic effect that promotes (BDNF) or interferes (TNF) with adaptive plasticity. A potentially more intriguing question is whether microglia support a kind of biological switch that maintains enhanced expression. Such a process could be initiated by the profile of extracellular signals secreted by neurons during training. For example, the relative release of adenosine triphosphate (ATP), glutamate, and matrix metalloprotein-9 (MMP9), may vary depending upon whether the afferent signals are predictable/controllable versus unpredictable/uncontrollable. This could initiate alternative processes in microglia and astrocytes that are preserved over time and relayed to remote sites (Hansen et al., 2013).

It has long been recognized that the core (primary) injury affects the surrounding tissue to promote tissue loss (secondary injury; Beattie and Bresnahan, 2000). Our work shows that how this process unfolds is affected by peripheral stimulation. This is clinically important because SCI is often accompanied by other tissue damage (polytrauma; Chu et al., 2009; Putz et al., 2011), which provides a source of nociceptive input that can fuel nociceptive sensitization and promote cell death. Such a view anticipates that inhibiting neural excitation (e.g., by local cooling or administration of a Na^{++} channel blocker), or microglia activation (e.g., using minocycline), should reduce secondary damage and attenuate chronic pain (Baptiste and Fehlings, 2006; Batchelor et al., 2013; Hansebout and Hansebout, 2014).

In considering alternative treatments, the focus is on spinally mediated nociception, not conscious pain. Surgical anesthesia blocks the experience of pain, but does not protect spinal circuits. Rather, it allows nociceptive stimulation to induce a maladaptive effect in the absence of spinal injury (Washburn et al., 2007). Likewise, systemic morphine eliminates behavioral signs of pain, but does not counter the adverse effect peripheral stimulation has on spinal function and, worst yet, increases the extent of secondary injury (Hook et al., 2007, 2009).

Peripheral stimulation can also have an adverse effect during the chronic phase of injury, by inducing a form of maladaptive plasticity that inhibits learning and promotes nociceptive sensitization. Potential sources of nociceptive input include

peripheral inflammation (e.g., from bed sores), stretching (Caudle et al., 2011), and electrical stimulation to induce muscle activity (Creasey et al., 2004). While pharmacological treatments that attenuate neural excitation can lessen the development of maladaptive plasticity, they will also inhibit adaptive plasticity and undermine the effectiveness of physical therapy. A better approach may involve training with predictable/controllable forms of stimulation, because these should both inhibit nociceptive sensitization and promote adaptive plasticity. Further, the link to metaplasticity implies that effective training can have a long-term benefit. Behavioral control is also relevant to the design of robotic systems (e.g., Del-Ama et al., 2014; McGie et al., 2014). These observations fit well with studies demonstrating long-term benefits of locomotor training (Edgerton et al., 2004). The work also implies that encouraging active behavioral control can enhance the beneficial effects of treatments that enable spinal function [e.g., epidural stimulation (Harkema et al., 2011; Angeli et al., 2014)].

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REFERENCES

Abraham, W. C. (2008). Metaplasticity: tuning synapses and networks for plasticity. *Nat. Rev. Neurosci.* 9:387. doi: 10.1038/nrn2356

Abraham, W. C., and Bear, M. F. (1996). Metaplasticity: the plasticity of synaptic plasticity. *Trends Neurosci.* 19, 126–130. doi: 10.1016/S0166-2236(96)80018-X

Abraham, W. C., and Williams, J. M. (2008). LTP maintenance and its protein synthesis-dependence. *Neurobiol. Learn. Mem.* 89, 260–268. doi: 10.1016/j.nlm.2007.10.001

Aceves, M., and Hook, M. A. (2013). Morphine binding to KORs and nonclassic ORs undermines recovery following SCI. *Abstract in 2013 Neuroscience Meeting Planner* (San Diego, CA: Society for Neuroscience).

Agrawal, S. K., Theriault, E., and Fehlings, M. G. (1998). Role of group I metabotropic glutamate receptors in traumatic spinal cord white matter injury. *J. Neurotrauma* 15, 929–941. doi: 10.1089/neu.1998.15.929

Angeli, C. A., Edgerton, V. R., Gerasimenko, Y. P., and Harkema, S. J. (2014). Altering spinal cord excitability enables voluntary movements after chronic complete paralysis in humans. *Brain* 137, 1277–1280. doi: 10.1093/brain/awu038

Aniksztejn, L., Otani, S., and Ben-Ari, Y. (1992). Quisqualate metabotropic receptors modulate NMDA currents and facilitate induction of long-term potentiation through protein kinase C. *Eur. J. Neurosci.* 4, 500–505. doi: 10.1111/j.1460-9568.1992.tb00900.x

Araque, A., Parpura, V., Sanzgiri, R. P., and Haydon, P. G. (1999). Tripartite synapses: glia, the unacknowledged partner. *Trends Neurosci.* 22, 208–215. doi: 10.1016/S0166-2236(98)01349-6

Baker-Herman, T. L., Fuller, D. D., Bavis, R. W., Zabka, A. G., Golder, F. J., and Doperalski, N. J., (2004). BDNF is necessary and sufficient for spinal respiratory plasticity following intermittent hypoxia. *Nat. Neurosci.* 7, 48–55. doi: 10.1038/nn1166

Baptiste, D. C., and Fehlings, M. G. (2006). Pharmacological approaches to repair the injured spinal cord. *J. Neurotrauma* 23, 318–334. doi: 10.1089/neu.2006.23.318

Bardin, L., Schmidt, J., Alloui, A., and Eschalier, A. (2000). Effect of intrathecal administration of serotonin in chronic pain models in rats. *Eur. J. Pharmacol.* 409, 37–43. doi: 10.1016/S0014-2999(00)00796-2

Basso, D. M., Beattie, M. S., and Bresnahan, J. C. (1995). A sensitive and reliable locomotive rating scale for open field testing in rats. *J. Neurotrauma* 12, 1–21. doi: 10.1089/neu.1995.12.1

Batchelor, P. E., Skeers, P., Antonic, A., Wills, T. E., Howells, D. W., Macleod, M. R., et al. (2013). Systematic review and meta-analysis of therapeutic hypothermia in animal models of spinal cord injury. *PLoS ONE* 8:e71317. doi: 10.1371/journal.pone.0071317

Baumbauer, K. M., and Grau, J. W. (2011). Timing in the absence of supraspinal input III: regularly spaced cutaneous stimulation prevents and reverses the spinal learning deficit produced by peripheral inflammation. *Behav. Neurosci.* 125, 37–45. doi: 10.1037/a0022009

Baumbauer, K. M., Hoy, K. C., Huie, J. R., Hughes, A. J., Woller, S., Puga, D. A., et al. (2008). Timing in the absence of supraspinal input I: variable, but not fixed, spaced stimulation of the sciatic nerve undermines spinally-mediated instrumental learning. *Neuroscience* 155, 1030–1047. doi: 10.1016/j.neuroscience.2008.07.003

Baumbauer, K. M., Huie, J. R., Hughes, A. J., and Grau, J. W. (2009). Timing in the absence of supraspinal input II: regular spaced stimulation induces a lasting alteration in spinal function that depends on the NMDA receptor, BDNF release, and protein synthesis. *J. Neurosci.* 29, 14383–14393. doi: 10.1523/JNEUROSCI.3583-09.2009

Baumbauer, K. M., Puga, D. A., Lee, K. H., Woller, S. A., Hughes, A. J., and Grau, J. W. (2012). Temporal regularity determines the impact of electrical stimulation on tactile reactivity and response to capsaicin in spinally transected rats. *Neuroscience* 227, 119–133. doi: 10.1016/j.neuroscience.2012.09.054

Baumbauer, K. M., Young, E. E., Hoy, K. C., and Joynes, R. L. (2006). Intrathecal infuions of anisomycin impact the learning deficit but not learning effect observed in spinal rats that received instrumental training. *Behav. Brain Res.* 173, 299–309. doi: 10.1016/j.bbr.2006.06.041

Bear, M. F. (2003). Bidirectional synaptic plasticity: from theory to reality. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 358, 649–655. doi: 10.1098/rstb.2002.1255

Beattie, E. C., Stellwagen, D., Morishita, W., Bresnahan, J. C., and Ha, B. K. (2002). Control of synaptic strength by glial TNFalpha. *Science* 295, 2282–2285. doi: 10.1126/science.1067859

Beattie, M. S., and Bresnahan, J. C. (2000). "Cell death, repair, and recovery of function after spinal cord contusion injuries in rats," in *Neurobiology of Spinal Cord Injury*, eds R. G. Kalb and S. M. Strittmatter (Totowa, NJ: Humana Press), 1–21.

Beattie, M. S., Farooqui, A. A., and Bresnahan, J. C. (2000). Review of current evidence for apoptosis after spinal cord injury. *J. Neurotrauma* 17, 915–925. doi: 10.1089/neu.2000.17.915

Ben-Ari, Y. (2002). Excitatory actions of GABA during development: the nature of the nurture. *Nat. Rev. Neurosci.* 3, 728–739. doi: 10.1038/nrn920

Ben-Ari, Y., Khalilov, I., Kahle, K. T., and Cherubini, E. (2012). The GABA excitatory/inhibitory shift in brain maturation and neurological disorders. *Neuroscientist* 18, 467–486. doi: 10.1177/1073858412438697

Biggs, J. E., Lu, V. B., Stebbing, M. J., Balasubramanyan, S., and Smith, P. A. (2010). Is BDNF sufficient for information transfer between microglia and dorsal horn neurons during the onset of central sensitization? *Mol. Pain* 6:44. doi: 10.1186/1744-8069-6-44

Blanquet, R., and Lamour, Y. (1997). Brain-derived neurotrophic factor in- creases Ca^{2+}/calmodulin-dependent protein kinase 2 activity in hippocampus. *J. Biol. Chem.* 272, 24133–24136. doi: 10.1074/jbc.272.39.24133

Blustein, J. E., Whitehouse, W. G., Calcagnetti, D. J., Troisi, J. R., Margules, D. L., and Bersh, P. J. (1992). Reduction of learned helplessness by central administration of quaternary naltrexone. *Physiol. Behav.* 51, 1075–1078. doi: 10.1016/0031-9384(92)90095-J

Bortolotto, Z. A., Bashir, Z. I., Davies, C. H., and Collingridge, G. L. (1994). A molecular switch activated by metabotropic glutamate receptors regulates induction of long-term potentiation. *Nature* 368, 740–743. doi: 10.1038/368740a0

Bothwell, M. (1996). p75NTR: a receptor after all. *Science* 272, 506–507. doi: 10.1126/science.272.5261.506

Boulenguez, P., Liabeuf, S., Bos, R., Bras, H., Jean-Xavier, C., Brocard, C., et al. (2010). Down-regulation of the potassium-chloride cotransporter KCC2 contributes to spasticity after spinal cord injury. *Nat. Med.* 16, 302–307. doi: 10.1038/nm.2107

Boyce, V. S., Park, J., Gage, F. H., and Mendell, L. M. (2012). Differential effects of brain derived neurotrophic factor and neutrophin-3 on hindlimb function in paraplegic rats. *Eur. J. Neurosci.* 35, 221–232. doi: 10.1111/j.1460-9568.2011.07950.x

Boyce, V. S., Tumolo, M., Fischer, I., Murray, M., and Lemay, M. A. (2007). Neurotrophic factors promote and enhance locomotor recovery in untrained spinalized cats. *J. Neurophysiol.* 98, 1988–1996. doi: 10.1152/jn.00391.2007

Caudle, K. L., Brown, E. H., Shum-Siu, A., Burke, D. A., Magnuson, T. S., Voor, M. J., et al. (2011). Hindlimb immobilization in a wheelchair alters functional recovery following contusive spinal cord injury in the adult rat. *Neurorehabil. Neural Repair* 25, 729–739. doi: 10.1177/1545968311407519

Caudle, R. M., Chavkin, C., and Dubner, R. (1994). Kappa 2 opioid receptors inhibit NMDA receptor-mediated synaptic currents in guinea pig CA3 pyramidal cells. *J. Neurosci.* 14, 5580–5589.

Caudle, R. M., Mannes, A. J., and Iadarola, M. J. (1997). GR89,696 is a kappa-2 opioid receptor agonist and a kappa-1 opioid receptor antagonist in the guinea pig hippocampus. *J. Pharmacol. Exp. Ther.* 283, 1342–1349.

Cejas, P. J., Martinez, M., Karmally, S., McKillop, M., McKillop, J., Plunkett, J. A., et al. (2000). Lumbar transplant of neurons genetically modified to secrete brain-derived neurotrophic factor attenuates allodynia and hyperalgesia after sciatic nerve constriction. *Pain* 86, 195–210. doi: 10.1016/S0304-3959(00)00245-1

Chu, D., Lee, Y.-H., Lin, C.-H., Chou, P., and Yang, N.-P. (2009). Prevalence of associated injuries of spinal trauma and their effect on medical utilization among hospitalized adult subjects – a nationwide data-based study. *BMC Health Serv. Res.* 9:137. doi: 10.1186/1472-6963-9-137

Cohen, A. S., Coussens, C. M., Raymond, C. R., and Abraham, W. C. (1999). Long-lasting increase in cellular excitability associated with the priming of LTP induction in rat hippocampus. *J. Neurophysiol.* 82, 3139–3148.

Collingridge, G. L., and Bliss T. V. P. (1987). NMDA receptors and their role in long-term potentiation. *Trends Neurosci.* 10, 288–293. doi: 10.1016/0166-2236(87)90175-5

Coull, J. A., Beggs, S., Boudreau, D., Boivin, D., Tsuda, M., Inoue, K., et al. (2005). BDNF from microglia causes the shift in neuronal anion gradient underlying neuropathic pain [see comment]. *Nature* 438, 1017–1021. doi: 10.1038/nature04223

Cramer, S. W., Baggott, C., Cain, J., Tilghman, J., Allcock, B., Miranpuri, G., et al. (2008). The role of cation-dependent chloride transporters in neuropathic pain following spinal cord injury. *Mol. Pain* 4, 36–44. doi: 10.1186/1744-8069-4-36

Creasey, G. H., Ho, C. H., Triolo, R. J., Gater, D. R., DiMarco, A. F., Bogie, K. M., et al. (2004). Clinical applications of electrical stimulation after spinal cord injury. *J. Spinal Cord Med.* 27, 365–375.

Crown, E. D., Ferguson, A. R., Joynes, R. L., and Grau, J. W. (2002a). Instrumental learning within the spinal cord: II. Evidence for central mediation. *Physiol. Behav.* 77, 259–267. doi: 10.1016/S0031-9384(02)00859-4

Crown, E. D., Joynes, R. L., Ferguson, A. R., and Grau, J. W. (2002b). Instrumental learning within the spinal cord: IV. Induction and retention of the behavioral deficit observed after noncontingent shock. *Behav. Neurosci.* 116, 1032–1051. doi: 10.1037/0735-7044.116.6.1032

Crown, E. D., and Grau, J. W. (2001). Preserving and restoring behavioral potential within the spinal cord using an instrumental training paradigm. *J. Neurophysiol.* 86, 845–855.

Crown, E. D., and Grau, J. W. (2005). Evidence that descending systems protect behavioral plasticity against the disruptive effect of nociceptive stimulation. *Exp. Neurol.* 196, 164–176. doi: 10.1016/j.expneurol.2005.07.016

Cunha, C., Brambilla, R., and Thomas, K. L. (2010). A simple role for BDNF in learning and memory. *Front. Mol. Neurosci.* 3:1. doi: 10.3389/neuro.02.001.2010

Czeschik, J. C., Hagenacker, T., Schafers, M., and Busselberg, D. (2008). TNF-alpha differentially modulates ion channels of nociceptive neurons. *Neurosci. Lett.* 434, 293–298. doi: 10.1016/j.neulet.2008.01.070

Dale-Nagle, E. A., Hoffman, M. S., MacFarlane, P. M., Satriotomo, I., Lovett-Barr, M. R., Vinit, S., et al. (2010). Spinal plasticity following intermittent hypoxia: implications for spinal injury. *Ann. N. Y. Acad. Sci.* 1198, 252–259. doi: 10.1111/j.1749-6632.2010.05499.x

Davies, J. E., Marsden, C. A., and Roberts, M. H. T. (1983). Hyperalgesia and reduction of monoamines resulting from lesions of the dorsolateral funiculus. *Brain Res.* 261, 59–68. doi: 10.1016/0006-8993(83)91283-0

Del-Ama, A. J., Gil-Agudo, A., Pons, J. L., and Moreno, J. C. (2014). Hybrid FES-robot cooperative control of ambulatory gait rehabilitation exoskeleton. *J. Neuroeng. Rehabil.* 11:27. doi: 10.1186/1743-0003-11-27

Dickenson, A. H., and Sullivan, A. F. (1987). Evidence for a role of the NMDA receptor in the frequency dependent potentiation of deep rat dorsal horn nociceptive neurons following C fibre stimulation. *Neuropharmacology* 26, 1235–1238. doi: 10.1016/0028-3908(87)90275-9

Domjan, M. (2015). *Principles of Learning and Behavior*, 7th Edn. Belmont, CA: Wadsworth.

Dudai, Y. (1989). *The Neurobiology of Memory: Concepts, Findings, Trends.* Oxford: Oxford University Press.

Dudai, Y. (2004). The neurobiology of consolidations, or, how stable is the engram? *Annu. Rev. Psychol.* 55, 51–86. doi: 10.1146/annurev.psych.55.090902.142050

Duprez, L., Wirawan, E., Berghe, T. V., and Vandenabeele, P. (2009). Major cell death pathways at a glance. *Microbes Infect.* 11, 1050–1062. doi: 10.1016/j.micinf.2009.08.013

Durkovic, R. G., and Prokowich, L. J. (1998). D-2-amino-5-phosphonovalerate, and NMDA receptor antagonist, blocks induction of associative longterm potentiation of the flexion reflex in spinal cat. *Neurosci. Lett.* 257, 162–164. doi: 10.1016/S0304-3940(98)00820-9

Eaton, M. J., Santiago, D. I., Dancausse, H. A., and Whittemore, S. R. (1997). Lumbar transplants of immortalized serotonergic neurons alleviate chronic neuropathic pain. *Pain* 72, 59–69. doi: 10.1016/S0304-3959(97)00015-8

Edgerton, V. R., Tillakartne, N. J., Bigbee, A. J., De Leon, R. D., and Roy, R. R. (2004). Plasticity of the spinal neural circuitry after injury. *Annu. Rev. Neurosci.* 27, 145–167. doi: 10.1146/annurev.neuro.27.070203.144308

Faden, A. I. (1990). Opioid and nonopioid mechanisms may contribute to dynorphin's pathophysiological actions in spinal cord injury. *Ann. Neurol.* 27, 67–74. doi: 10.1002/ana.410270111

Ferguson, A. R., Bolding, K. A., Huie, J. R., Hook, M. A., Santillano, D. R., Miranda, R. C., et al. (2008). Group I metabotropic glutamate receptors control metaplasticity of spinal cord learning through a PKC-dependent mechanism. *J. Neurosci.* 28, 11939–11949. doi: 10.1523/JNEUROSCI.3098-08.2008

Ferguson, A. R., Crown, E. D., and Grau, J. W. (2006). Nociceptive plasticity inhibits adaptive learning in the spinal cord. *Neuroscience* 141, 421–431. doi: 10.1016/j.neuroscience.2006.03.029

Ferguson, A. R., Hook, M. A., Garcia, G., Bresnahan, J. C., Beattie, M. S., and Grau, J. W. (2004). A simple transformation that improves the metric properties of the BBB scale. *J. Neurotrauma* 21, 1601–1613. doi: 10.1089/neu.2004.21.1601

Ferguson, A. R., Huie, J. R., Crown, E. D., Baumbauer, K. M., Hook, M. A., Garraway, S. M., et al. (2012a). Maladaptive spinal plasticity opposes adaptive spinal learning after spinal cord injury. *Front. Physiol.* 3:399. doi: 10.3389/fphys.2012.00399

Ferguson, A. R., Huie, J. R., Crown, E. D., and Grau, J. W. (2012b). Central nociceptive sensitization vs. spinal cord training: opposing forms of plasticity that dictate function after complete spinal cord injury. *Front. Physiol.* 3:396. doi: 10.3389/fphys.2012.00396

Ferguson, A. R., Washburn, S. N., Crown, E. D., and Grau, J. W. (2003). GABAA receptor activation is involved in non-contingent shock inhibition of instrumental conditioning in spinal rat. *Behav. Neurosci.* 117, 799–812. doi: 10.1037/0735-7044.117.4.799

Fields, H. L. (2000). Pain modulation: expectation, opioid analgesia and virtual pain. *Prog. Brain Res.* 122, 245–253. doi: 10.1016/S0079-6123(08)62143-3

Finkbeiner, S. (2000). CREB couples neurotrophin signals to survival messages. *Neuron* 25, 11–14. doi: 10.1016/S0896-6273(00)80866-1

Flemmer, A. W., Gimenez, I., Dowd, B. F., and Darman, R. B. (2002). Forbush antibodyB. Activation of the Na–K–Cl cotransporter NKCCl detected with a phospho-specific antibody. *J. Biol. Chem.* 277, 37551–37558. doi: 10.1074/jbc.M206294200

Garraway, S. M., Anderson, A. J., and Mendell, L. M. (2005). BDNF-induced facilitation of afferent-evoked responses in lamina II neurons is reduced after neonatal spinal cord contusion injury. *J. Neurophysiol.* 94, 1798–1804. doi: 10.1152/jn.00179.2005

Garraway, S. M., and Mendell, L. M. (2007). Spinal cord transection enhances afferent-evoked inhibition in lamina II neurons and abolishes BDNF-induced facilitation of their sensory input. *J. Neurotrauma* 24, 379–390. doi: 10.1089/neu.2006.0115

Garraway, S. M., Petruska, J. C., and Mendell, L. M. (2003). BDNF sensitizes the response of lamina II neurons to high threshold primary afferent inputs. *Eur. J. Neurosci.* 18, 2467–2476. doi: 10.1046/j.1460-9568.2003.02982.x

Garraway, S. M., Turtle, J. D., Huie, J. R., Lee, K. H., Hook, M. A., Woller, S. A., et al. (2011). Intermittent noxious stimulation following spinal cord contusion injury impairs locomotor recovery and reduces spinal BDNF-TrkB signaling in adult rats. *Neuroscience* 199, 86–102. doi: 10.1016/j.neuroscience.2011.10.007

Garraway, S. M., Woller, S. A., Huie, J. R., Hook, M. A., Huang, Y.-Y., Hartman, J. J., et al. (2012). Peripheral noxious stimulation following contusion spinal cord injury increases the incidence of mechanical allodynia and the expression of TNF alpha in the spinal cord. *Abstract in 2012 Neuroscience Meeting Planner* (New Orleans, LA: Society for Neuroscience).

Gjerstad, J., Tjolsen, A., and Hole, K. (2001). Induction of long-term potentiation of single wide dynamic range neurones in the dorsal horn is inhibited by descending pathways. *Pain* 91, 263–268. doi: 10.1016/S0304-3959(00)00448-6

Gómez-Pinilla, F., Huie, J. R., Ying, Z., Ferguson, A., Crown, E. D., Baumbauer, K. M., et al. (2007). BDNF and learning: evidence that instrumental training promotes learning within the spinal cord by up-regulating BDNF expression. *Neuroscience* 148, 893–906. doi: 10.1016/j.neuroscience.2007.05.051

Gómez-Pinilla, F., Ying, Z., Opazo, P., Roy, R. R., and Edgerton, V. R. (2001). Differential regulation by exercise of BDNF and NT-3 in rat spinal cord and skeletal muscle. *Eur. J. Neurosci.* 13, 1078–1084. doi: 10.1046/j.0953-816x.2001.01484.x

Grau, J. W. (1987). The central representation of an aversive event maintains the opioid and nonopioid forms of analgesia. *Behav. Neurosci.* 101, 272–288. doi: 10.1037/0735-7044.101.2.272

Grau, J. W. (2010). "Instrumental conditioning," in *Corsini Encyclopedia of Psychology*, 4th Edn, eds I. B. Weiner and C. B. Nemeroff (New York: John Wiley and Sons).

Grau, J. W. (2014). Learning from the spinal cord: how the study of spinal cord plasticity informs our view of learning. *Neurobiol. Learn. Mem.* 108, 155–171. doi: 10.1016/j.nlm.2013.08.003

Grau, J. W., Barstow, D. G., and Joynes, R. L. (1998). Instrumental learning within the spinal cord: I. Behavioral properties. *Behav. Neurosci.* 112, 1366–1386. doi: 10.1037/0735-7044.112.6.1366

Grau, J. W., Crown, E. D., Ferguson, A. R., Washburn, S. N., Hook, M. A., and Miranda, R. C. (2006). Instrumental learning within the spinal cord: underlying mechanisms and implications for recovery after injury. *Behav. Cogn. Neurosci. Rev.* 5, 191–239. doi: 10.1177/1534582306289738

Grau, J. W., Huie, J. R., Garraway, S. M., Hook, M. A., Crown, E. D., Baumbauer, K. M., et al. (2012). Impact of behavioral control on the processing of nociceptive stimulation. *Front. Physiol.* 3:262. doi: 10.3389/fphys.2012.00262

Grau, J. W., Washburn, S. N., Hook, M. A., Ferguson, A. R., Crown, E. D., Garcia, G., et al. (2004). Uncontrollable nociceptive stimulation undermines recovery after spinal cord injury. *J. Neurotrauma* 21, 1795–1817. doi: 10.1089/neu.2004.21.1795

Hains, B. C., Fullwood, S. D., Eaton, M. J., and Hulsebosch, C. E. (2001a). Subdural engraftment of serotonergic neurons following spinal hemisection restores spinal serotonin, downregulates serotonin transporter, and increases BDNF tissue content in rat. *Brain Res.* 913, 35–46. doi: 10.1016/S0006-8993(01)02749-4

Hains, B. C., Johnson, K. M., McAdoo, D. J., Eaton, M. J., and Hulsebosch, C. E. (2001b). Engraftment of serotonergic precursors enhances locomotor function and attenuates chronic central pain behavior following spinal hemisection injury in the rat. *Exp. Neurol.* 171, 361–378. doi: 10.1006/exnr.2001.7751

Hains, B. C., Willis, W. D., and Hulsebosch, C. E. (2003). Serotonin receptors 5-HT1A and 5-HT3 reduce hyperexcitability of dorsal horn neurons after chronic spinal cord hemisection injury in rat. *Exp. Brain Res.* 149, 174–186.

Hansen, C. N., Kerr, S., Fisher, L., Deibert, R., Wohleb, E. S., Williams, J., et al. (2013). Thoracic SCI produces remote neuroinflammation that prevents plasticity in locomotor networks. *Abstract in 2013 Neuroscience Meeting Planner* (San Diego, CA: Society for Neuroscience).

Harkema, S., Gerasimenko, Y., Hodes, J., Burdick, J., Angeli, C., Chen, Y., et al. (2011). Effect of epidural stimulation of the lumbosacral spinal cord on voluntary movement, standing, and assisted stepping after motor complete paraplegia: a case study. *Lancet* 377, 1938–1947. doi: 10.1016/S0140-6736(11)60547-3

Hansebout, R. R., and Hansebout, C. R. (2014). Local cooling for traumatic spinal cord injury: outcomes in 20 patients and review of the literature. *J. Neurosurg. Spine* 20, 550–561. doi: 10.3171/2014.2.SPINE13318

Hasbargen, T., Ahmed, M. M., Miranpuri, G., Li, L., Kahle, K. T., Resnick, D., et al. (2010). Role of NKCC1 and KCC2 in the development of chronic neuropathic pain following spinal cord injury. *Ann. N. Y. Acad. Sci.* 1198, 168–172. doi: 10.1111/j.1749-6632.2010.05462.x

Haydon, P. G. (2001). Glia: listening and talking to the synapse. *Nat. Rev. Neurosci.* 2, 185–193. doi: 10.1038/35058528

Ho, J., Mannes, A. J., Dubner, R., and Caudle, R. M. (1997). Putative kappa-2 opioid agonists are antihyperalgesic in a rat model of inflammation. *J. Pharm. Exp. Ther.* 281, 136–141.

Hook, M. A., and Grau, J. W. (2007). An animal model of functional electrical stimulation: evidence that the central nervous system modulates the consequences of training. *Spinal Cord* 45, 702–712. doi: 10.1038/sj.sc.3102096

Hook, M. A., Huie, J. R., and Grau, J. W. (2008). Peripheral inflammation undermines the plasticity of the isolated spinal cord. *Behav. Neurosci.* 122, 233–249. doi: 10.1037/0735-7044.122.1.233

Hook, M. A., Liu, G. T., Washburn, S. N., Ferguson, A. R., Bopp, A. C., Huie, J. R., et al. (2007). The impact of morphine after a spinal cord injury. *Behav. Brain Res.* 179, 281–293. doi: 10.1016/j.bbr.2007.02.035

Hook, M. A., Moreno, G., Woller, S., Puga, D., Hoy, K., Baldyn, R., et al. (2009). Intrathecal morphine attenuates recovery of function after a spinal cord injury. *J. Neurotrauma* 26, 741–752. doi: 10.1089/neu.2008.0710

Hook, M. A., Washburn, S. N., Moreno, G., Woller, S., Lee, K., and Grau, J. W. (2011). An IL1 receptor antagonist blocks a morphine-induced attenuation of locomotor recovery after spinal cord injury. *Brain Behav. Immun.* 25, 349–359. doi: 10.1016/j.bbi.2010.10.018

Hoy, K. C., Huie, J. R., and Grau, J. W. (2013). AMPA receptor mediated behavioral plasticity in the isolated rat spinal cord. *Behav. Brain Res.* 236, 319–326. doi: 10.1016/j.bbr.2012.09.007

Huang, Y.-J., Garraway, S. M., and Grau, J. W. (2011). GABA-A antagonist bicuculline blocks the allodynia induced by noncontingent stimulation in spinal transected rats. *Paper Presented, at 2011 Neuroscience Meeting Planner* (Washington, DC: Society for Neuroscience).

Huang, Y.-J., Lee, K. H., Garraway, S. M., and Grau, J. W. (2013). SCI alters the effect of GABAergic system on nociception: different effect of GABA-A antagonist bicuculline on capsaicin-indued allodynia in sham vs. spinally transected rats. *Abstract in 2013 Neuroscience Meeting Planner* (San Diego, CA: Society for Neuroscience).

Huang, Y.-J., Lee, K. H., and Grau, J. W. (2014). Spinal injury transforms how GABAergic system regulate nociceptive systems within the spinal cord: Role of KCC2. *Abstract in 2014 Neuroscience Meeting Planner* (Washington, DC: Society for Neuroscience).

Huie, J. R., Baumbauer, K. M., Lee, K. H., Beattie, M. S., Bresnahan, J. C., Ferguson, A. R., et al. (2012a). Glial tumor necrosis factor alpha (TNFα) generates metaplastic inhibition of spinal learning. *PLoS ONE* 7:e39751. doi: 10.1371/journal.pone.0039751

Huie, J. R., Garraway, S. M., Hoy, K. C., and Grau, J. W. (2012b). Learning in the spinal cord: BDNF mediates the beneficial effects of instrumental training. *Neuroscience* 200, 74–90. doi: 10.1016/j.neuroscience.2011.10.028

Hutchinson, M. R., Bland, S. T., Johnson, K. W., Rice, K. C., Maier, S. F., and Watkins, L. R. (2007). Opioid-induced glial activation: mechanisms of activation and implications for opioid analgesia, dependence, and reward. *Sci. World J.* 7, 98–111. doi: 10.1100/tsw.2007.230

Hutchinson, M. R., Zhang, Y., Shridhar, M., Evans, J. H., Buchanan, M. M., Zhao, T. X., et al. (2010). Evidence that opioids may have toll-like receptor 4 and MD-2 effects. *Brain Behav. Immun.* 24, 83–95. doi: 10.1016/j.bbi.2009.08.004

Ji, R. R., Berta, T., and Nedergaard, M. (2013). Glia and pain: is chronic pain a gliopathy. *Pain* 6:22.

Ji, R.-R., Kohno, T., Moore, K. A., and Woolf, C. J. (2003). Central sensitization and LTP: do pain and memory share similar mechanisms. *Trends Neurosci.* 26, 696–705. doi: 10.1016/j.tins.2003.09.017

Johnston, I. N., Milligan, E. D., Wieseler-Frank, J., Frank, M. G., Zapata, V., Campisi, J., et al. (2004). A role for proinflammatory cytokines and fractalkine in analgesia, tolerance, and subsequent pain facilitation induced by chronic intrathecal morphine. *J. Neurosci.* 24, 7353–7365. doi: 10.1523/JNEUROSCI.1850-04.2004

Joynes, R. L., Ferguson, A. R., Crown, E. D., Patton, B. C., and Grau, J. W. (2003). Instrumental learning within the spinal cord: V. Evidence the behavioral deficit observed after noncontingent nociceptive stimulation reflects an intraspinal modification. *Behav. Brain Res.* 141, 159–170. doi: 10.1016/S0166-4328(02)00372-8

Joynes, R. L., and Grau, J. W. (2004). Instrumental learning within the spinal cord: III. Prior exposure to noncontingent shock induces a behavioral deficit that is blocked by an opioid antagonist. *Neurobiol. Learn. Mem.* 82, 35–51. doi: 10.1016/j.nlm.2004.04.001

Joynes, R. L., Janjua, K. R., and Grau, J. W. (2004). Instrumental learning within the spinal cord: VI. Disruption of learning by the NMDA antagonist APV. *Behav. Brain Res.* 154, 431–438. doi: 10.1016/j.bbr.2004.03.030

Kahle, K. T., Khanna, A., Clapham, D. E., and Woolf, C. J. (2014). Therapeutic restoration of spinal inhibition via druggable enhancement of potassium-chloride cotransporter KCC2-mediated chloride extrusion in peripheral neuropathic pain. *JAMA Neurol.* 71, 640–645. doi: 10.1001/jamaneurol.2014.21

Karim, F., Wang, C. C., and Gereau, R. W. IV. (2001). Metabotropic glutamate recep- tor subtypes 1 and 5 are activators of extracellular signal-regulated kinase signaling required for inflammatory pain in mice. *J. Neurosci.* 21, 3771–3779.

Kuno, R., Wang, J., Kawanokuchi, J., Takeuchi, H., and Mizuno, T. (2005). Autocrine activation of microglia by tumor necrosis factor-alpha. *J. Neuroimmunol.* 162, 89–96. doi: 10.1016/j.jneuroim.2005.01.015

Latremoliere, A., and Woolf, C. J. (2009). Central sensitization: a generator of pain hypersensitivity by central neural plasticity. *J. Pain* 10, 895–926. doi: 10.1016/j.jpain.2009.06.012

Lee, K. H., Baumbauer, K. M., and Grau, J. W. (2014). Brain-derived neurotrophic factor reduces inflammation-induced allodynia. *Abstract in 2014 Neuroscience Meeting Planner* (Washington, DC: Society for Neuroscience).

Lee, K. H., Baumbauer, K. M., Horton, J. B., Munro, E., and Grau, J. W. (2012). Brain-derived neurotrophic factor reduces inflammation-induced allodynia in spinally transected rats. *Paper Presented, at 2012 Neuroscience Meeting Planner* (New Orleans, LA: Society for Neuroscience).

Lee, K. H., Strain, M. M., Huan, Y.-J., and Grau, J. (2013). *Timing* in the absence of supraspinal input: integration of fixed spaced stimulation. *Paper Presented at 2013 Neuroscience Meeting Planner* (San Diego, CA: Society for Neuroscience).

Linnarsson, S., Bjorklund, A., and Ernfors, P. (1997). Learning deficit in BDNF mutant mice. *Eur. J. Neurosci.* 9, 2581–2587. doi: 10.1111/j.1460-9568.1997.tb01687.x

Lisman, J., Schulman, H., and Cline, H. (2002). The molecular basis of CaMKII function in synaptic and behavioural memory. *Nat. Rev. Neurosci.* 3, 175–190. doi: 10.1038/nrn753

Lu, B., Pang, P. T., and Woo, N. H. (2005). The yin and yang of neurotrophin action. *Nat. Rev. Neurosci.* 6, 603–614. doi: 10.1038/nrn1726

Lu, V. B., Biggs, J. E., Stebbing, M. J., Balasubramanyan, S., Todd, K. G., Lai, A. Y., et al. (2009). Brian-derived neurotrophic factor drives the changes in excitatory synaptic transmission in the rat superficial dorsal horn that follow sciatic nerve injury. *J. Physiol.* 587, 1013–1032. doi: 10.1113/jphysiol.2008.166306

MacDonald, J. F., Jackson, M. F., and Beazely, M. A. (2007). G protein-coupled receptors control NMDARs and metaplasticity in the hippocampus. *Biochim. Biophys. Acta* 1768, 941–951. doi: 10.1016/j.bbamem.2006.12.006

Maier, S. F. (1986). Stressor controllability and stress-induced analgesia. *Ann. N. Y. Acad. Sci.* 467, 55–72. doi: 10.1111/j.1749-6632.1986.tb14618.x

Maier, S. F., and Seligman, M. E. P. (1976). Learned helplessness: theory and evidence. *J. Exp. Psychol. Gen.* 105, 3–46. doi: 10.1037/0096-3445.105.1.3

Maier, S. F., and Watkins, L. R. (2005). Stressor controllability and learned helplessness: the roles of the dorsal raphe nucleus, serotonin, and corticotropin-releasing factor. *Neurosci. Biobehav. Rev.* 29, 829–841. doi: 10.1016/j.neubiorev.2005.03.021

McGie, S. C., Zariffa, J., Popovic, M. R., and Nagai, M. K. (2014). Short-term neuroplastic effects of brain-controlled and muscle-controlled electrical stimulation. *Neuromodulation*. doi: 10.1111/ner.12185 [Epub ahead of print].

McNally, G. P., Johansen, J. P., and Blair, H. T. (2011). Placing prediction into the fear circuit. *Trends Neurosci.* 34, 283–292. doi: 10.1016/j.tins.2011.03.005

Meagher, M. W., Chen, P., Salinas, J. A., and Grau, J. W. (1993). Activation of the opioid and nonopioid hypoalgesic systems at the level of the brainstem and spinal cord: does a coulometric relation predict the emergence or form of environmentally-induced hypoalgesia? *Behav. Neurosci.* 107, 493–505. doi: 10.1037/0735-7044.107.3.493

Meller, S. T., Dykstra, C., Grzybycki, D., Murphy, S., and Gebhart, G. F. (1994). The possible role of glia in nociceptive processing and hyperalgesia in the spinal cord of the rat. *Neuropharmacology* 33, 1471–1478. doi: 10.1016/0028-3908(94)90051-5

Merighi, A., Salio, C., Ghirri, A., Lossi, L., Ferrini, F., Betelli, C., et al. (2008). BDNF as a pain modulator. *Prog. Neurobiol.* 85, 297–317. doi: 10.1016/j.pneurobio.2008.04.004

Miki, K., Fukuoka, T., Tokunaga, A., Kondo, E., Dai, Y., and Noguchi, K. (2000). Differential effect of brain-derived neurotrophic factor on high-threshold mechanosensitivity in a rat neuropathic pain model. *Neurosci. Lett.* 278, 85–88. doi: 10.1016/S0304-3940(99)00908-8

Millan, M. J. (2002). Descending control of pain. *Prog. Neurobiol.* 66, 355–474. doi: 10.1016/S0301-0082(02)00009-6

Mills, C. D., and Hulsebosch, C. E. (2002). Increased expression of metabotropic glutamate receptor subtype 1 on spinothalamic tract neurons following spinal cord injury in the rat. *Neurosci. Lett.* 319, 59–62. doi: 10.1016/S0304-3940(01)02551-4

Mills, C. D., Johnson, K. M., and Hulsebosch, C. E. (2002). Group I metabotropic glutamate receptors in spinal cord injury: roles in neuroprotection and the development of chronic central pain. *J. Neurotrauma* 19, 23–42. doi: 10.1089/089771502753460213

Mori, K., Komatsu, T., Tomemori, N., Shingu, K., Urabe, N., Seo, N., et al. (1981). Pentobarbital-anesthetized and decerebrate cats reveal different neurological responses in anesthetic-induced analgesia. *Acta Anaesthesiol. Scand.* 25, 349–354. doi: 10.1111/j.1399-6576.1981.tb01665.x

Morris, R. G. (2013). NMDA receptors and memory encoding. *Neuropharmacology* 74, 32–40. doi: 10.1016/j.neuropharm.2013.04.014

Mukhin, A., Fan, L., and Faden, A. I. (1996). Activation of metabotropic glutamate receptor subtype mGluR1 contributes to post-traumatic neuronal injury. *J. Neurosci.* 16, 6012–6020.

Neugebauer, V., Chen, P. S., and Willis, W. D. (1999). Role of metabotropic glutamate receptor subtype mGluR1 in brief nociception and central sensitization of primate STT cells. *J. Neurophysiol.* 82, 272–282.

Newman-Tancredi, A., Nicolas, J. P., Audinot, V., Gavaudan, S., Verriele, L., Touzard, M., et al. (1998). Actions of alpha2 adrenoceptor ligands at alpha2A and 5-HT1A receptors: the antagonist, atipamezole, and the agonist, dexmedetomidine, are highly selective for alpha2A adrenoceptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 358, 197–206. doi: 10.1007/PL00005243

O'Connor, J. J., Rowan, M. J., and Anwyl, R. (1994). Long-lasting enhancement of NMDA receptor-mediated synaptic transmission by metabotropic glutamate receptor activation. *Nature* 367, 557–559. doi: 10.1038/367557a0

Onifer, S. M., Smith, G. M., and Fouad, K. (2011). Plasticity after spinal cord injury: relevance to recover and approaches to facilitate it. *Neurotherapeutics* 8, 283–293. doi: 10.1007/s13311-011-0034-4

Park, C. K., Lu, N., Xu, Z. Z., Liu, T., and Serhan, C. N. (2011). Resolving TRPV1- and TNF-alpha-mediated spinal cord synaptic plasticity and inflammatory pain with neuroprotectin D1. *J. Neurosci.* 31, 15072–15085. doi: 10.1523/JNEUROSCI.2443-11.2011

Patterson, S. L., Abel, T., Deuel, T. A., Martin, K. C., Rose, J. C., and Kande, E. R. (1996). Recombinant BDNF rescues deficits in basal synaptic transmission and hippocampal LTP in BDNF knockout mice. *Neuron* 16, 1137–1145. doi: 10.1016/S0896-6273(00)80140-3

Patterson, S. L., Grover, L. M., Schwartzkroin, P. A., and Bothwell, M. (1992). Neurotrophin expression in rat hippocampal slices: a stimulus paradigm inducing LTP in CA1 evokes increases in BDNF and NT-3 mRNAs. *Neuron* 9, 1081–1088. doi: 10.1016/0896-6273(92)90067-N

Patton, B. C., Hook, M. A., Crown, E. D., Ferguson, A. R., and Grau, J. W. (2004). The behavioral deficit observed following noncontingent shock in spinalized rats is prevented by the protein synthesis inhibitor cycloheximide. *Behav. Neurosci.* 118, 653–658. doi: 10.1037/0735-7044.118.3.653

Paulsen, R. E., Contestabile, A., Villani, L., and Fonnum, F. (1987). An in vivo model for studying function of brain tissue temporarily devoid of glial cell metabolism: the use of fluorocitrate. *J. Neurochem.* 48, 1377–1385. doi: 10.1111/j.1471-4159.1987.tb05674.x

Putz, C., Schuld, C., Gantz, S., Grieser, T., Akbar, M., Moradi, B., et al. (2011). The effect of polytrauma as a possible confounder in the outcome of monotraumatic vs polytraumatic paraplegic patients: a clinical cohort study. *Spinal Cord* 49, 721–727. doi: 10.1038/sc.2010.181

Reeve, A. J., Patel, S., Fox, A., Walker, K., and Urban, L. (2000). Intrathecally administered endotoxin or cytokines produce allodynia, hyperalgesia, and changes in spinal cord neuronal response to nociceptive stimuli in the rat. *Eur. J. Pain* 4, 247–257. doi: 10.1053/eujp.2000.0177

Rescorla, R. A. (1988). Behavioral studies of Pavlovian conditioning. *Ann. Rev. Neurosci.* 11, 329–352. doi: 10.1146/annurev.ne.11.030188.001553

Roberts, L. A., Beyer, C., and Komisaruk, B. R. (1986). Nociceptive responses to altered GABAergic activity at the spinal cord. *Life Sci.* 39, 1667–1674. doi: 10.1016/0024-3205(86)90164-5

Sandkühler, J. (2000). Learning and memory in pain pathways. *Pain* 88, 113–118. doi: 10.1016/S0304-3959(00)00424-3

Sandkühler, J., and Liu, X. (1998). Induction of long-term potentiation at spinal synapses by noxious stimulation or nerve injury. *Eur. J. Neurosci.* 10, 2476–2480. doi: 10.1046/j.1460-9568.1998.00278.x

Shannon, H. E., and Lutz, E. A. (2000). Yohimbine produces antinociception in the formalin test in rats: involvement of serotonin 1A receptors. *Psychopharmacology* 149, 93–97. doi: 10.1007/s002139900343

Skeberdis, V. A., Lan, J., Opitz, T., Zheng, X., Bennett, M. V., and Zukin, R. S. (2001). mGluR1-mediated potentiation of NMDA receptors involves a rise in intracellular calcium and activation of protein kinase C. *Neuropharmacology* 40, 856–865. doi: 10.1016/S0028-3908(01)00005-3

Song, P., and Zhao, Z. Q. (2001). The involvement of glial cells in the development of morphine tolerance. *Neurosci. Res.* 39, 281–286. doi: 10.1016/S0168-0102(00)00226-1

Squire, L. R., and Wixted, J. T. (2011). The cognitive neuroscience of human memory since H.M. *Annu. Rev. Neurosci.* 34, 259–288. doi: 10.1146/annurev-neuro-061010-113720

Stanfa, L. C., and Dickenson, A. H. (1998). Inflammation alters the effects of mGlu receptor agonists on spinal nociceptive neurones. *Eur. J. Pharmacol.* 347, 165–172. doi: 10.1016/S0014-2999(98)00098-3

Stellwagen, D., and Malenka, R. C. (2006). Synaptic scaling mediated by glial TNF-alpha. *Nature* 440, 1054–1059. doi: 10.1038/nature04671

Strain, M. M., Lee, K. H., Niemerski, A. L., and Grau, J. W. (2013). Treatment with 5HT and NMDA induces stepping in spinally transected adult rats while maintaining spinal cord plasticity. *Abstract in 2013 Neuroscience Meeting Planner* (San Diego, CA: Society for Neuroscience).

Stuck, E. D., Huie, J. R., Lee, K. H., Irvine, K.-A., Bresnahan, J. C., Beattie, M., et al. (2012). Synaptic GluA2-lacking ampa receptors as a biomarker for maladaptive spinal plasticity following spinal cord injury. *Abstract in 2012 Neuroscience Meeting* (New Orleans, LA: Society for Neuroscience).

Thompson, A. K., and Wolpaw, J. R. (2014). Operant conditioning of spinal reflexes: from basic science to clinical therapy. *Front. Integr. Neurosci.* 8:25. doi: 10.3389/fnint.2014.00025

Tully, T. (1997). Regulation of gene expression and its role in long-term memory and synaptic plasticity. *Proc. Natl. Acad. Sci. U.S.A.* 94, 4239–4241. doi: 10.1073/pnas.94.9.4239

Turrigiano, G. G. (2008). The self-tuning neuron: synaptic scaling of excitatory synapses. *Cell* 135, 422–435. doi: 10.1016/j.cell.2008.10.008

Valencia-de Ita, S., Lawand, N. B., Lin, Q., Castaneda-Hernandez, G., and Willis, W. D. (2006). Role of the Na^+-K^+-$2Cl^-$ cotransporter in the development of capsaicin-induced neurogenic inflammation. *J. Neurophysiol.* 95, 3553–3561. doi: 10.1152/jn.01091.2005

Vichaya, E. G., Baumbauer, K. M., Carcoba, L. M., Grau, J. W., and Meagher, M. W. (2009). Spinal glia modulate both adaptive and pathological processes. *Brain Behav. Immun.* 23, 969–976. doi: 10.1016/j.bbi.2009.05.001

Wagner, J. J., Terman, G. W., and Chavkin, C. (1993). Endogenous dynorphins inhibit excitatory neurotransmission and block LTP induction in the hippocampus. *Nature* 363, 451–454. doi: 10.1038/363451a0

Warms, C. A., Turner, J. A., Marshall, H. M., and Cardenas, D. D. (2002). Treatments for chronic pain associated with spinal cord injuries: many are tried, few are helpful. *Clin. J. Pain* 18, 154–163. doi: 10.1097/00002508-200205000-00004

Washburn, S. N., Maultsby, M. L., Puga, D. A., and Grau, J. W. (2008). Opioid regulation of spinal cord plasticity: evidence the kappa-2 opioid receptor agonist GR89686 inhibits learning within the rat spinal cord. *Neurobiol. Learn. Mem.* 89, 1–16. doi: 10.1016/j.nlm.2007.09.009

Washburn, S. N., Patton, B. C., Ferguson, A. R., Hudson, K. L., and Grau, J. W. (2007). Exposure to intermittent nociceptive stimulation under pentobarbital anesthesia disrupts spinal cord function in rats. *Psychopharmacology* 192, 243–252. doi: 10.1007/s00213-007-0707-1

Waterhouse, E. G., and Xu, B. (2009). New insights into the role of brain- derived neurotrophic factor in synaptic plasticity. *Mol. Cell. Neurosci.* 42, 81–89. doi: 10.1016/j.mcn.2009.06.009

Watkins, L. R., Drugan, R., Hyson, R. L., Moye, T. B., Ryan, S. M., Mayer, D. J., et al. (1984). Opiate and non-opiate analgesia induced by inescapable tail-shock: effects of dorsolateral funiculus lesions and decerebration. *Brain Res.* 291, 325–336. doi: 10.1016/0006-8993(84)91265-4

Watkins, L. R., Hutchinson, M. R., Ledeboer, A., Wieseler-Frank, J., Milligan, E. D., and Maier, S. F. (2007). Norman Cousins Lecture Glia as the "bad guys": implications for improving clinical pain control and the clinical utility of opioids. *Brain Behav. Immun.* 21, 131–146. doi: 10.1016/j.bbi.2006.10.011

Watkins, L. R., Martin, D., Ulrich, P., Tracey, K. J., and Maier, S. F. (1997). Evidence for the involvement of spinal cord glia in subcutaneous formalin induced hyperalgesia in the rat. *Pain* 71, 225–235. doi: 10.1016/S0304-3959(97)03369-1

Widerstrom-Noga, E. G., and Turk, D. C. (2003). Types and effectiveness of treatments used by people with chronic pain associated with spinal cord injuries: influence of pain and psychosocial characteristics. *Spinal Cord* 41, 600–609. doi: 10.1038/sj.sc.3101511

Willis, W. D. (2001). "Mechanisms of central sensitization of nociceptive dorsal horn neurons," in *Spinal Cord Plasticity: Alterations in Reflex Function*, eds M. M. Patterson and J. W. Grau (Norwell, MA: Kluwer Academic Publishers).

Wolpaw, J. R. (2010). What can the spinal cord teach us about learning and memory? *Neuroscientist* 16, 532–549. doi: 10.1177/1073858410368314

Wolpaw, J. R., and Carp, J. S. (1990). Memory traces in spinal cord. *Trends Neurosci.* 13, 137–142. doi: 10.1016/0166-2236(90)90005-U

Woolf, C. J. (1983). Evidence for a central component of post-injury pain hypersensitivity. *Nature* 306, 686–688. doi: 10.1038/306686a0

Yin, J. C., Del Vecchio, M., Zhou, H., and Tully, T. (1995). CREB as a memory modulator: induced expression of a dCREB2 activator isoform enhances long-term memory in *Drosophila*. *Cell* 81, 107–115. doi: 10.1016/0092-8674(95)90375-5

Yin, J. C., and Tully, T. (1996). CREB and the formation of long-term memory. *Curr. Opin. Neurobiol.* 6, 264–268. doi: 10.1016/S0959-4388(96)80082-1

Zhang, L., Lu, Y., Chen, Y., and Westlund, K. N. (2002). Group I metabotropic glutamate receptor antagonists block secondary thermal hyperalgesia in rats with knee joint inflammation. *J. Pharmacol. Exp. Ther.* 300, 149–156. doi: 10.1124/jpet.300.1.149

Zhang, Z. Z., Park, J., Grau, J., Lee, K. H., Niemerski, A., and Schmidt, C. E. (2014). Local and sustained delivery of BDNF mediates spinal learning after injury. *Abstract in 2014 Neuroscience Meeting Planner* (Washington, DC: Society for Neuroscience).

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Regulatory effects of intermittent noxious stimulation on spinal cord injury-sensitive microRNAs and their presumptive targets following spinal cord contusion

Eric R. Strickland[1], Sarah A. Woller[2], Sandra M. Garraway[2], Michelle A. Hook[1], James W. Grau[2] and Rajesh C. Miranda[1]*

[1] *Department of Neuroscience and Experimental Therapeutics, College of Medicine, Texas A&M Health Science Center, Bryan, TX, USA*
[2] *Department of Psychology, Texas A&M University, College Station, TX, USA*

Edited by:
Brian R. Noga, University of Miami, USA

Reviewed by:
Deborah Baro, Georgia State University, USA
Jeffrey C. Petruska, University of Louisville, USA

***Correspondence:**
Rajesh C. Miranda, Department of Neuroscience and Experimental Therapeutics, College of Medicine, Texas A&M Health Science Center, Medical Research and Education Building, 8447 State Highway 47, Bryan, TX 77807-3260, USA
e-mail: miranda@medicine.tamhsc.edu

Uncontrollable nociceptive stimulation adversely affects recovery in spinally contused rats. Spinal cord injury (SCI) results in altered microRNA (miRNA) expression both at, and distal to the lesion site. We hypothesized that uncontrollable nociception further influences SCI-sensitive miRNAs and associated gene targets, potentially explaining the progression of maladaptive plasticity. Our data validated previously described sensitivity of miRNAs to SCI alone. Moreover, following SCI, intermittent noxious stimulation decreased expression of miR124 in dorsal spinal cord 24 h after stimulation and increased expression of miR129-2 in dorsal, and miR1 in ventral spinal cord at 7 days. We also found that brain-derived neurotrophic factor (BDNF) mRNA expression was significantly down-regulated 1 day after SCI alone, and significantly more so, after SCI followed by tailshock. Insulin-like growth factor-1 (IGF-1) mRNA expression was significantly increased at both 1 and 7 days post-SCI, and significantly more so, 7 days post-SCI with shock. MiR1 expression was positively and significantly correlated with IGF-1, but not BDNF mRNA expression. Further, stepwise linear regression analysis indicated that a significant proportion of the changes in BDNF and IGF-1 mRNA expression were explained by variance in two groups of miRNAs, implying co-regulation. Collectively, these data show that uncontrollable nociception which activates sensorimotor circuits distal to the injury site, influences SCI-miRNAs and target mRNAs within the lesion site. SCI-sensitive miRNAs may well mediate adverse consequences of uncontrolled sensorimotor activation on functional recovery. However, their sensitivity to distal sensory input also implicates these miRNAs as candidate targets for the management of SCI and neuropathic pain.

Keywords: spinal cord injury, microRNA, uncontrollable nociception, BDNF, IGF

INTRODUCTION

Significant attention has been given to investigating the central molecular changes that modulate locomotor recovery and pain following spinal cord injury (SCI; Boyce and Mendell, 2014; Lerch et al., 2014; Schwab and Strittmatter, 2014; Silva et al., 2014). However, surprisingly, there is relatively little information on the role of afferent peripheral signals in the development of SCI-related pain or attenuation of locomotor function. Given that SCI rarely occurs in isolation, but is often accompanied by peripheral tissue damage and trauma, examining the effects of afferent nociceptive signaling seems warranted. This is further underscored by previous research in our laboratory suggesting that functional recovery after SCI is attenuated by peripheral nociceptive stimulation applied below the level of the spinal injury (Grau et al., 2004). Intermittent noxious stimulation decreased the recovery of locomotor function, delayed reinstatement of bladder function, led to greater mortality and spasticity, and increased tissue loss at the injury site in adult rats (Grau et al., 2004). Further, we found that nociceptive stimulation rapidly resulted in decreased brain-derived neurotrophic factor (BDNF) – tropomyosin-receptor kinase B signaling (Gomez-Pinilla et al., 2007; Garraway et al., 2011). This signaling pathway has been shown to play a key role in modulating neuronal plasticity, promoting axonal regeneration, and improving functional recovery following SCI (Xu et al., 1995; Patterson et al., 1996; McTigue et al., 1998; Kerr et al., 1999; Garraway et al., 2003; Boyce et al., 2007; Huie et al., 2012b).

The present study focused on identifying intracellular regulatory molecules that, when activated by nociceptive input, might influence neurotrophic signaling pathways and functional recovery. Specifically, we hypothesized that miRNAs may be sensitive to nociceptive stimulation, and activate potentially maladaptive gene networks to further undermine recovery after SCI. We, and others, have previously shown that SCI leads to significant alterations in miRNA expression (Liu et al., 2009; Nakanishi et al., 2010; Strickland et al., 2011). Our previous study showed that miR1, miR124, and miR129 were significantly down-regulated following a spinal cord contusion, while miR146a and miR21 were transiently induced (Strickland et al., 2011), and that these miRNAs were sensitive to opioid analgesics like morphine (Strickland et al., 2014). These miRNAs

play important roles in regulating critical gene networks that have been implicated in the development of hypersensitivity and may contribute to impaired functional recovery. For example, though the expression of miR124 is often associated with early neuronal differentiation (Visvanathan et al., 2007), and neurite outgrowth (Yu et al., 2008a), miR124 is also important for promoting neuronal survival in the adult (Doeppner et al., 2013). Moreover, miR124 may play an important role in neuropathic pain by inhibiting neuro-inflammation (Ponomarev et al., 2011; Kynast et al., 2013) and inflammatory hyperalgesia (Willemen et al., 2012) in the adult. Similarly, miR129-2 promotes cell cycle arrest, and dysregulation of miR129-2 results in neuronal death (Di Giovanni et al., 2003; Byrnes et al., 2007; Herrup and Yang, 2007; Wu et al., 2010). Interestingly, miR1, a member of the miR1/miR206 family has been shown to regulate important growth-promoting neurotrophic factors like BDNF (Lewis et al., 2003; Lee et al., 2012). Modulation of miR1 activity may play a significant role in regulating BDNF signaling after SCI and, importantly for this study, in mediating the effects of intermittent noxious stimulation on recovery after SCI. To assess this possibility, the current study investigated the relationship between SCI-sensitive miRNA expression and intermittent noxious stimulation in tissue collected 1 h, 24 h, and 7 days after stimulation. We also assessed the relationship between the expression of miRNAs sensitive to intermittent noxious stimulation and that of their mRNA targets.

MATERIALS AND METHODS

SUBJECTS

The subjects were male Sprague–Dawley rats (*Rattus norvegicus*) obtained from Harlan (Houston, TX, USA). The rats were approximately 90–110 days old (350–400 g), were individually housed in Plexiglas bins [45.7 (length) × 23.5 (width) × 20.3 (height) cm] with food and water continuously available, and were maintained on a 12-h light/dark cycle. All behavioral testing was performed during the light cycle. To facilitate access to the food and water, extra bedding was added to the bins after surgery and long mouse sipper tubes were used so that the rats could reach the water without rearing. All of the experiments were reviewed and approved by the Institutional Animal Care and Use Committee at Texas A&M University and all NIH guidelines for the care and use of animal subjects were followed.

SURGERY

Subjects were anesthetized with isoflurane (5%, gas). Once a stable level of anesthesia was achieved, the concentration of isoflurane was lowered to 2–3%. An area extending approximately 4.5 cm above and below the injury site was shaved and disinfected with iodine, and a 7.0 cm incision was made over the spinal cord. Next, two incisions were made on either side of the vertebral column (approximately 4–5 mm depth), extending about 3 cm rostral and caudal to the T12 segment. The dorsal spinous processes at T12 vertebral level were removed (laminectomy), and the spinal tissue exposed. The dura remained intact. For the contusion injury, the vertebral column was fixed within the MASCIS device (Gruner, 1992; Constantini and Young, 1994), and a moderate injury was produced by allowing the 10 g impactor (outfitted with a 2.5 mm tip) to drop 12.5 mm. Sham controls received a laminectomy, but the cord was not contused with the MASCIS device. Following surgery, the wound was closed with Michel clips. T12 vertebral level contusion models have been routinely used by members of our group to define spinal cord learning circuits and molecular mechanisms involved with recovery of function (Ferguson et al., 2008; Brown et al., 2011; Hook et al., 2011). Lesions at this level result in well-defined and replicable sensory-motor deficits, and we therefore chose to utilize contusion at this level to also examine changes in miRNA expression.

To help prevent infection, subjects were treated with 100,000 units/kg Pfizerpen (penicillin G potassium) immediately after surgery and again 2 days later. For the first 24 h after surgery, rats were placed in a recovery room maintained at 26.6°C. To compensate for fluid loss, subjects were given 2.5 ml of saline after surgery. Bladders were manually expressed twice daily (morning and evening) until the animals had empty bladders for three consecutive days, at the times of expression.

UNCONTROLLABLE INTERMITTENT NOXIOUS STIMULATION

Intermittent shock was applied 24 h after surgery while subjects were loosely restrained in Plexiglas tubes as previously described (Crown et al., 2002; Grau et al., 2004). Electrical stimulation was applied through cutaneous electrodes coated with electrode gel (Harvard Apparatus, Holliston, MA, USA) and attached 2 cm from the tip of the tail with Orthaleic tape. Leads from the electrodes were attached to a shock generator (BRS/LVE, Model SG-903, Laurel, MD, USA), and intermittent constant current 1.5 mA, electrical stimulation was applied through the electrodes. Rats treated with uncontrollable intermittent stimulation received 180, 80-ms tail shocks on a variable time schedule with a mean inter-stimulus interval of 2 s (range 0.2–3.8 s). Unshocked subjects (contused and sham operated) were placed in the restraining tubes for the same amount of time as shock subjects. The unshocked subjects had the tail electrodes attached, but did not receive the electrical stimuli. The number of subjects within each experimental condition is given **Table 1**.

RNA EXTRACTION AND qRT-PCR

Animals [SCI/unshocked ($SCI_{unshock}$) or SCI/shock-exposed (SCI_{shock})] were sacrificed at 1 h, 1 day, or 7 days following exposure to intermittent shock or control treatment (i.e., 25 h, 48 h, and 8 days following SCI). All subjects were deeply anesthetized with pentobarbital (50 mg/kg), and 1 cm of spinal cord around the lesion epicenter was rapidly removed. To determine the spatial

Table 1 | Number of subjects (*n*) per condition.

Treatment	1 h	1 day	7 days
Sham operated	4	4 (d and v)	4 (d and v)
Contused-unshocked	6	6 (d and v)	6 (d and v)
Contused-shocked	6	6 (d and v)	6 (d and v)

Tissue collected at 1 and 7 days postsurgery was subdivided into dorsal (d) and ventral (v) portions and separately analyzed.

(dorsal–ventral) changes in the expression of miRNAs and genes of interest, the spinal cord tissue was further subdivided to yield dorsal and ventral regions in the 1 and 7 days after treatment groups. The cord was processed for the extraction of total RNA (RNeasy Mini Kit; Qiagen, Valencia, CA, USA) and subsequently quantified using a NanoDrop 2000 Spectrophotometer (Thermo Scientific; Wilmington, DE, USA), and stored at −80°C.

MiRNA expression data was measured with quantitative reverse transcription (qRT)-PCR for miRNAs, based on the protocol of the miRCURY^{TM} LNA microRNA Universal RT-PCR system (EXIQON; Woburn, MA, USA). RNA samples were converted to cDNA, and qRT-PCR was performed using a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Validated commercially available forward and reverse primers (EXIQON; **Table 2**) for hsa-miR124, hsa-miR1, hsa-miR21, hsa-miR129-2, and hsa-miR146a were used for PCR amplification, and real time data was normalized to U6 small nuclear RNA ($U6_{SNR}$). Similarly, mRNA expression of BDNF and IGF-1 was measured using qRT-PCR for mRNAs, based on the protocol for PerfeCTa® SYBR® Green SuperMix with ROX^{TM} (Quanta Biosciences; Gaithersburg, MD, USA). RNA samples were converted to cDNA using qScript^{TM} cDNA SuperMix (Quanta Biosciences; Gaithersburg, MD, USA), and qRT-PCR was performed using a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Forward and reverse primers (Integrated DNA Technologies; Coralville, IA, USA) for BDNF and IGF-1 were used for PCR amplification (**Table 2**), and real time data was normalized using glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Three technical replicates were averaged per sample. Selection criteria for non-commercially provided mRNA primer pairs were the identification of a single amplicon and efficiencies based on serial cDNA dilution according to our previously published protocols (Camarillo and Miranda, 2008). Efficiency for mRNA primers was 100.4% for BDNF, 99.71% for IGF-1 and 100.06% for GAPDH. Normalization controls were utilized as reported previously (Strickland et al., 2011, 2014), and there were no statistical differences in the expression of either $U6_{SNR}$ or GAPDH with treatment condition or spinal cord region. Relative miRNA and mRNA expression was determined by calculating the mean difference between cycle threshold of either the miRNA from the $U6_{SNR}$ normalization control, or the BDNF/IGF-1 mRNA from the GAPDH normalization control for each sample (Δ cycle threshold (ΔCT)) within each sample group (samples with same miRNA ID, time, and condition parameters) and expressed as −ΔCT for relative change in expression. Sample means that were greater than ±2 SDs from the mean ΔCT were considered outliers and removed from the analysis. As indicated in **Table 1**, the experimental design yielded 80 samples and for each, seven miRNAs were assessed, yielding a total of 560 data points. Our criteria identified just 30 data points for exclusion (approximately 5.4%), and no more than one data point in any individual experimental group was excluded. Fold change in miRNA/mRNA expression was determined by calculating the difference between the mean ΔCT for the sham control and individual sample ΔCTs of sham, SCI/unshocked ($SCI_{unshock}$), and SCI/shock-exposed (SCI_{shock}) sample groups at the same time point (−ΔΔCT), and expressed as mean fold-change ($2^{-\Delta\Delta CT}$; Livak and Schmittgen, 2001).

DATA ANALYSIS

All data were analyzed using SPSS software version 18 (SPSS; Chicago, IL, USA). MicroRNA expression, verified by qRT-PCR, was analyzed by multivariate analysis of variance (ANOVA) using Pillai's trace statistic, and further analyzed using *post hoc* univariate ANOVA and Fisher's least significant difference (LSD) test. Other data were analyzed using ANOVAs followed by *post hoc* f-LSD using planned comparisons to limit the number of *post hoc* comparisons. In all cases, the *a priori* α value was set at 0.05. Data were expressed as mean ± SEM, as indicated in the figure legends.

Correlations between expression of miRNAs, and between miRNA and either BDNF or IGF-1 mRNA expression, were determined by Pearson's product–moment correlation using –ΔCT values of either the miRNAs or BDNF/IGF-1 as separate independent variables. The *a priori* α value was set at 0.05, and data were expressed as the mean difference in cycle threshold change of either each miRNA relative to the cycle threshold of U6 controls ($-\Delta CT = CT_{U6} - CT_{miRNA}$), or BDNF or IGF-1 expression relative to the cycle threshold of GAPDH controls ($-\Delta CT = CT_{GAPDH} - CT_{mRNA}$). Additionally, stepwise linear regression analyses were performed on Pearson's correlation data

Table 2 | Primer sequences.

mRNA Primers	Forward	Reverse
BDNF	TGGACATATCCATGACCAGAAA	CACAATTAAAGCAGCATGCAAT
IGF-1	CCGCTGAAGCCTACAAAGTC	GGGAGGCTCCTCCTACATTC
GAPDH	AGTATGTCGTGGAGTCTACTG	TGGCAGCACCAGTGGATGCAG
miRNA Primers/cat#	**Target Sequence**	**Sequence reference**
hsa-miR-1/#204344	UGGAAUGUAAAGAAGUAUGUAU	MIMAT0000416
hsa-miR-21-5p/#204230	UAGCUUAUCAGACUGAUGUUGA	MIMAT0000076
hsa-miR-124-3p/#204319	UAAGGCACGCGGUGAAUGCC	MIMAT0000422
hsa-miR-129-2-3p/# 204026	AAGCCCUUACCCCAAAAAGCAU	MIMAT0004605
hsa-miR-146a-5p/# 204688	UGAGAACUGAAUUCCAUGGGUU	MIMAT0000449
U6 snRNA/# 203907		

between miRNA and either BDNF or IGF-1 mRNA expression to determine which miRNAs contributed to the significant correlations. BDNF or IGF-1 mRNA expression was the dependent variable, while miRNA expression was the independent variable, and the *a priori* α value was set at 0.05 for model significance.

RESULTS

QUANTIFICATION OF SHOCK-INDUCED CHANGES IN miRNA EXPRESSION

Contused rats exhibited increased miR21 and miR146a expression 1 h after shock treatment

We previously reported that miR1, miR21, miR124, miR129-2, and miR146a were significantly affected by a spinal cord contusion (Strickland et al., 2011). To determine whether uncontrollable intermittent noxious stimulation affects these SCI-sensitive miRNAs, their expression was determined by qRT-PCR in sham controls and in contused animals following either no shock exposure ($SCI_{unshock}$) or uncontrollable intermittent tailshock (SCI_{shock}). Initially, we analyzed miRNA expression within the whole spinal cord segment (combined dorsal and ventral spinal cord) at the lesion site at 1 h following intermittent noxious stimulation (25 h after contusion surgery). Both miR21 [$F_{(2,13)} = 15.4$, $p < 0.0003$] and miR146a [$F_{(2,13)} = 6.2$, $p < 0.01$] were significantly increased following SCI (all *post hoc* comparisons relative to sham control, $p < 0.02$), and exposure to intermittent tail shock did not result in further alterations of miRNA expression (**Figure 1**). MiR1, miR124, and miR129-2 were not significantly altered at the lesion site, either by contusion or by intermittent tail-shock at 1 h poststimulation.

A contusion injury down-regulated expression of miR1, miR124, mi129-2, and up-regulated miR21, 1–7 days after treatment

We microdissected the 1 cm of spinal cord tissue bracketing the injury segment to yield dorsal/sensory and ventral/motor regions in the 1 and 7 days treatment groups. Microdissection of the cord provides additional information on the spatial (dorsal–ventral) and functional relevance (sensory-motor) of changes in SCI-sensitive miRNA expression. We hypothesized that SCI-induced expression changes of some trauma-sensitive miRNAs would exhibit spatial and functional specificity, and that uncontrollable nociception might exacerbate these changes. miRNA expression in contused rats was significantly altered by exposure to shock and the pattern of expression depended upon both time and region (dorsal versus ventral). Preliminary analyses using a multivariate ANOVA of qRT-PCR data confirmed that there was a significant main effect of time [Pillai's Trace Statistic, $F_{(5,33)} = 20.89$; $p < 0.001$], treatment [$F_{(10,68)} = 6.44$; $p < 0.001$], and spinal region (dorsal/ventral) [$F_{(5,33)} = 8.31$; $p < 0.001$], as well as a three-way statistically significant interaction between time, treatment, and spinal region [$F_{(10,68)} = 2.59$; $p < 0.01$]. *Post hoc* univariate ANOVAs indicated a main effect of time on miR1, miR21, miR124, and miR146a, a main effect of treatment on miR1, miR21, miR124, and miR129-2, and a main effect of spinal region on miR1 and miR146a (all $Fs > 9.14$, $p < 0.005$). There was also a significant interaction effect of time and treatment on miR124 expression, and of time, treatment, and spinal region on miR1, miR129-2, and miR146a (all $Fs > 3.68$, $p < 0.05$). An effect of time emerged because expression generally declined across days. miR1 and miR146a exhibited an overall difference across spinal regions because expression was somewhat less in the ventral portion.

Post hoc LSD *t*-tests were used to further analyze the effect of treatment. We found that miR1, miR124, and miR129-2 expression was significantly decreased following spinal cord trauma, irrespective of exposure to uncontrollable intermittent tailshock ($p_{miR1} < 0.001$, $p_{miR124} < 0.001$, and $p_{miR129-2} < 0.001$; **Figures 2** and **3**). In contrast, miR21 expression was up-regulated in contused subjects ($p_{miR21} < 0.001$), and there was no change in the expression of miR146a.

Intermittent tailshock produces a selective increase in mR1/miR129-2 and a decrease in miR124 in contused rats

The effect of shock treatment also depended upon region and time (**Figures 2** and **3**). The SCI_{shock} rats alone exhibited a significant down-regulation of miR1 and miR124 in the dorsal region of the spinal cord at 7 days posttreatment. *Post hoc* planned comparisons also indicated that the contusion injury *per se* decreased the expression of miR1 in the ventral/motor tissue, but this effect was significantly attenuated by uncontrollable shock; expression of miR1 was increased in the SCI_{shock} group compared with $SCI_{unshock}$ treatment. Similarly, there was a significant increase in the expression of miR129-2 in dorsal/sensory tissue at 7 days following SCI_{shock} treatment relative to $SCI_{unshock}$ treatment ($p < 0.05$; **Figure 3**), and a significant decrease in the expression of miR124 in dorsal/sensory tissue at 1 day following SCI_{shock} relative to $SCI_{unshock}$ treatment ($p < 0.01$; **Figure 2**).

Correlation analyses indicated significant relationships between expression changes within distinct groups of SCI-sensitive miRNAs

Given miRNA dysregulation following both contusion injury and uncontrollable nociception, we hypothesized that statistical relationships might exist between expression changes of different SCI-sensitive miRNAs, indicative of possible co-regulation. Pearson's product–moment correlations indicated three groups of significant correlations between miRNAs (**Figures 4A,B**; **Table 3**). In combined analysis of data obtained from both dorsal and ventral spinal cord, and at both 1 and 7 days, there were significant correlations between miR1 and miR21, miR124, miR129-2, and miR146a (**Figure 4A**), between miR124 and miR1, miR129-2, and miR146a, and between miR146a and miR1, miR21, and miR124 (for all Pearson's *r*s, $p < 0.05$, see **Table 3**; **Figure 4B**).

INTERMITTENT SHOCK SENSITIVITY OF miRNA TARGET GENES IN CONTUSED RATS

Contused rats exhibited a decrease in IGF-1 expression at 1 h

As miR1 is further modified by uncontrollable intermittent tailshock following contusion, we assessed the extent to which changes in miR1 expression corresponded to modulation of potential neurotrophin and growth factor mRNA targets. Based on prior work (Lewis et al., 2003; Yu et al., 2008b; Lee et al., 2012; Li et al., 2012), we assayed BDNF and IGF-1 mRNA expression by

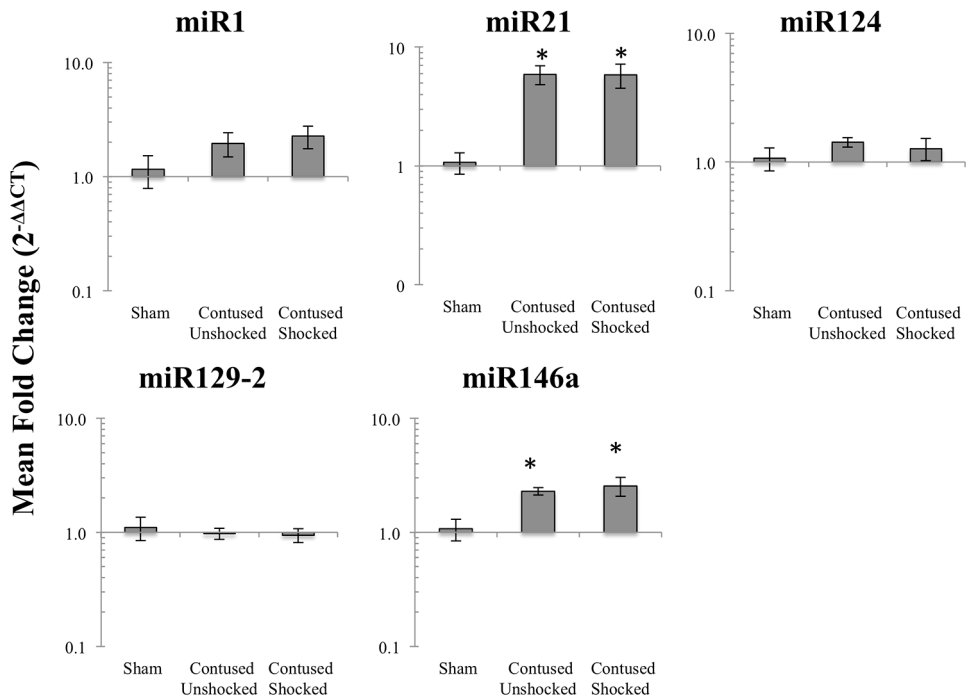

FIGURE 1 | Bar graphs depicting qRT-PCR analysis of miRNA expression of miR1, miR21, miR124, miR129-2, and miR146a at the lesion site for sham animals and after unshocked or shock treatment in contused animals at 1 h following tailshock treatment. The *x*-axis denotes treatment condition, and the *y*-axis denotes the log of mean fold expression change of miRNA. Data are normalized relative to U6 small nuclear RNA internal gene expression and expressed as the mean of all fold changes of individual samples within a treatment group relative to the mean of the treatment group, with asterisks indicating significance (LSD) compared with sham controls; *p* values are as indicated in the text. The error bars indicate the standard error of the mean (SEM).

qRT-PCR in sham controls and in response to either no shock exposure or uncontrollable intermittent tailshock following injury. As with the miRNA expression analysis, we first analyzed mRNA expression in full segments (dorsal + ventral) at 1 h following treatment. Planned comparisons indicated that there was a significant decrease in expression of IGF-1 following the contusion injury, irrespective of uncontrollable intermittent tailshock (Student's two-tailed *t*-test, $p_{\text{unshock}} < 0.01$ and $p_{\text{shock}} < 0.005$; **Figure 5A**).

A contusion injury down-regulated expression of BDNF and up-regulated IGF-1 mRNA expression

Subsequently, spatial expression (dorsal/sensory–ventral/motor) of BDNF and IGF-1 mRNA was assessed at 1 and 7 days following $SCI_{unshock}$ or SCI_{shock} treatment. A multivariate ANOVA of the qRT-PCR data indicated a significant main effect of time [Pillai's Trace Statistic, $F_{(2,51)} = 5.20$; $p < 0.01$], treatment [$F_{(4,104)} = 11.66$; $p < 0.001$], and spinal region (dorsal/ventral) [$F_{(2,51)} = 17.97$; $p < 0.001$], and a significant interaction effect between both time and treatment [$F_{(4,104)} = 6.29$; $p < 0.001$], and treatment and spinal region [$F_{(4,104)} = 2.56$; $p < 0.05$]. *Post hoc* univariate ANOVAs indicated a main effect of time on IGF-1 mRNA, a main effect of treatment on both BDNF and IGF-1 mRNAs, and a main effect of spinal region on BDNF mRNA (all Fs > 9.49, $p < 0.005$). For IGF-1 and BDNF mRNA expression, the effect of treatment depended on time (both Fs > 7.95; $p < 0.001$). In addition, for BDNF the effect of treatment depended upon spinal region as well as time (all Fs > 4.03; $p < 0.05$).

Post hoc LSD *t*-tests indicated that BDNF mRNA was significantly decreased, while IGF-1 mRNA was significantly increased following spinal cord trauma, ($p < 0.001$; **Figures 5B,C**).

Intermittent tailshock reduces BDNF expression in the dorsal region a day after treatment and increases IGF-1 expression at 7 days following treatment

Post hoc planned comparisons indicated that there was a significant decrease in expression of BDNF in dorsal/sensory tissue of SCI_{shock} subjects relative to $SCI_{unshock}$ at 1 day following tailshock ($p < 0.05$; **Figure 5B**). In contrast, shock treatment increased expression of IGF-1 mRNA in ventral/motor tissue at 7 days following $SCI_{unshock}$ or SCI_{shock} treatment ($p < 0.05$; **Figure 5C**). *Post hoc* planned comparisons also indicated significant spatial changes (dorsal/sensory relative to ventral/motor) in mRNA expression, including increased relative dorsal/sensory expression of BDNF in sham, $SCI_{unshock}$, and SCI_{shock} treated subjects at both 1 day ($p < 0.01$, $p < 0.001$, and $p < 0.05$, respectively) and 7 days

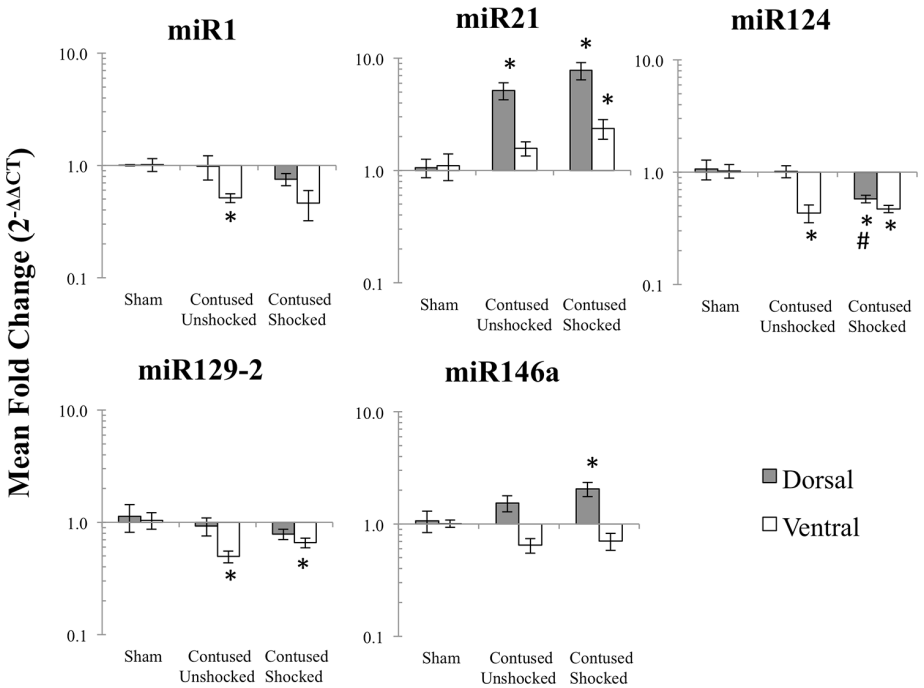

FIGURE 2 | Bar graphs depicting qRT-PCR analysis of miRNA expression of miR1, miR21, miR124, miR129-2, and miR146a at the lesion site for sham animals and after unshocked or shock treatment in contused animals at 1 day following tailshock treatment. The *x*-axis denotes treatment condition, and the *y*-axis denotes the log of mean fold expression change of miRNA. Data are normalized relative to U6 small nuclear RNA internal gene expression and expressed as the mean of all fold changes of individual samples within a treatment group relative to the mean of the treatment group (±SEM), with asterisks indicating significance (LSD) compared with sham controls and hash tags indicating significance (LSD) compared with contused unshocked controls; *p* values are as indicated in the text.

following $SCI_{unshock}$ or SCI_{shock} treatment ($p < 0.01$, $p < 0.01$, and $p < 0.001$, respectively), and increased expression of IGF-1 in SCI_{shock} subjects at 7 days following $SCI_{unshock}$ or SCI_{shock} treatment ($p < 0.005$; **Figures 5B,C**).

Correlation analyses indicated that expression changes of multiple SCI-sensitive miRNAs were associated with changes in BDNF and IGF-1 expression

As BDNF and IGF-1 can be mRNA targets of miR1, we hypothesized that a statistical relationship would exist between expression changes in miR1 and that of BDNF and IGF-1. Pearson's product–moment correlations (combining data for dorsal and ventral spinal cord and for day 1 and 7 post-shock) indicated two groups of significant correlations between SCI-sensitive miRNAs, BDNF, and IGF-1. There were significant correlations between BDNF and both miR21 and miR124, and between IGF-1 and miR1, miR124, and miR129-2 (**Table 4**). Additionally, BDNF and IGF-1 were also significantly, but inversely correlated with each other (Pearson's $r = -0.31$, $p < 0.05$).

We also used multivariate analyses to discover which miRNAs accounted for a significant proportion of the variance in IGF-1 and BDNF mRNA expression. For IGF-1, the analyses revealed that miR124, miR21, and miR146a (in that order) each accounted for an independent proportion of the variance (all Fs > 6.36, $p < 0.05$), and together explained 42.3% of the variance. BDNF mRNA expression was largely explained by miR21 and miR124 expression (both Fs > 7.57, $p < 0.001$), which together explained 24.8% of the variance. For both mRNAs, no other miRNA independently explained a significant proportion of variance.

DISCUSSION

One of the current clinical challenges with mitigating the short- and long-term effects of spinal cord injury is managing the uncontrollable nociception that results from concomitant peripheral tissue damage. It is critical for future efforts to develop therapeutic strategies to attenuate glial activation, cell death, and sensitization of spinal neurons associated with intermittent noxious stimulation in order to inhibit maladaptive spinal plasticity, improve functional recovery, and suppress pain hypersensitivity following SCI with associated peripheral injuries (Liu et al., 1999; Frei et al., 2000; Beattie et al., 2002; Hains et al., 2003; Xu et al., 2004; Hains and Waxman, 2006; Lampert et al., 2006; Detloff et al., 2008; Kuzhandaivel et al., 2011). Uncontrollable nociception clearly inhibits functional recovery (Grau et al., 2004; Garraway et al., 2011; Huie et al., 2012a). It is possible that nociceptive stimuli may interfere with functional recovery

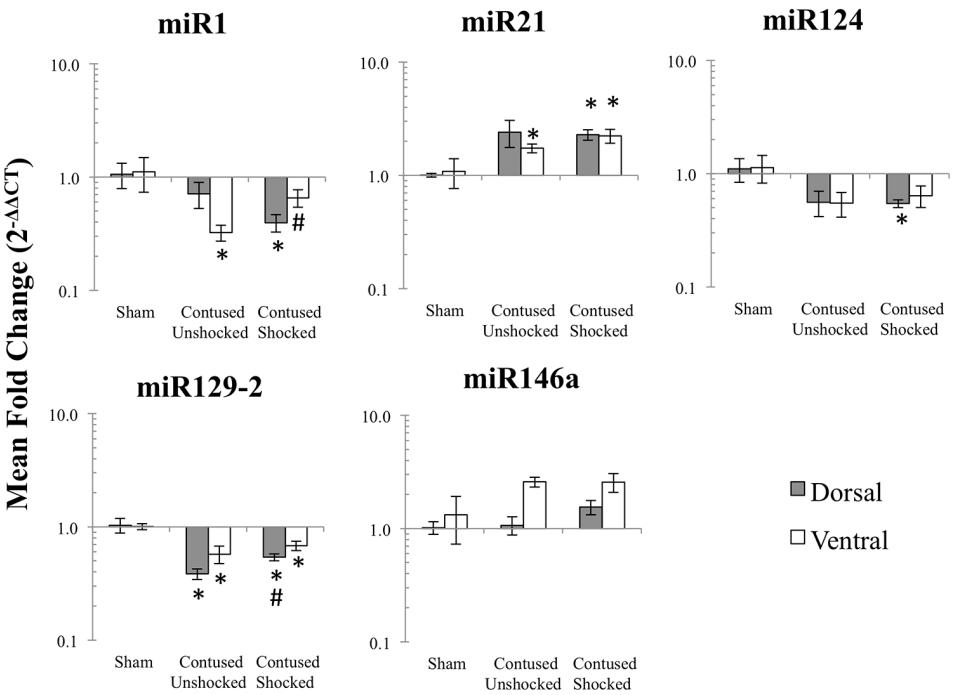

FIGURE 3 | Bar graphs depicting qRT-PCR analysis of miRNA expression of miR1, miR21, miR124, miR129-2, and miR146a at the lesion site for sham animals and after unshocked or shock treatment in contused animals at 7 days following tailshock treatment. The *x*-axis denotes treatment condition, and the *y*-axis denotes the log of mean fold expression change of miRNA. Data are normalized relative to U6 small nuclear RNA internal gene expression and expressed as the mean of all fold changes of individual samples within a treatment group relative to the mean of the treatment group (±SEM), with asterisks indicating significance (LSD) compared with sham controls and hash tags indicating significance (LSD) compared with contused unshocked controls; *p* values are as indicated in the text.

in part, by directly altering biology within damaged tissue at the injury site. Specifically, it is possible that miRNAs, given their dysregulation following SCI (Liu et al., 2009; Nakanishi et al., 2010; Strickland et al., 2011, 2014; Bhalala et al., 2012) and ability to coordinate the expression of large gene networks, are involved in the modulation of nociceptive circuitry and prevention of functional recovery resulting from uncontrollable nociception. Therapeutic manipulation of these miRNAs could alleviate the maladaptive effects of noxious stimulation in both the acute and chronic phases of SCI by suppressing activation of glia-mediated inflammation and inhibiting the synaptic remodeling of nociceptive circuitry that results in pain hypersensitivity, increased spasticity, and exacerbation of behavioral deficits.

The current study investigated the regulation of SCI-sensitive miRNAs at the site of an injury in rats administered uncontrollable shock (SCI_{shock}) or nothing ($SCI_{unshock}$) 1 h, 1 day, or 7 days after shock treatment. To examine the effect of a contusion injury *per se*, we included a sham-operated unshocked control as a comparison group. We found that the contusion injury increased expression of miR21 and miR146a at 1 h relative to the sham control. In the unshocked rats, miR21 remained elevated in the dorsal (at 1 day) and ventral (at 7 days) regions. In contused shocked rats, miR21 remained elevated in both the dorsal and ventral region across days (1 and 7). The change in miR146 appeared, in contrast, to fade over days, with the only significant increase observed in the dorsal region of shocked rats at 1 day. At 1 day, the contusion injury down-regulated miR1, miR124, and miR129-2 in the ventral region of unshocked subjects. miR1 remained down-regulated in the ventral region at 7 days and miR129-2 was down-regulated in both the dorsal and ventral regions. Shocked rats also showed a down-regulation of miR124 (dorsal and ventral) and miR129-2 (ventral) at 1 day. At 7 days, only shocked-contused rats exhibited a significant down-regulation of miR1 and miR124 in the dorsal region, whereas miR129-2 was down-regulated in both regions. This overall pattern replicates key components of our earlier study (Strickland et al., 2011), which showed that a contusion injury down-regulates miR1, miR129, and miR124, and up-regulates miR21 and miR146a.

When we examined the effect of shock treatment on miRNA expression in contused rats, we found that shock significantly decreased expression of miR124 in dorsal/sensory tissue at 1 day. At 7 days, shock treatment significantly increased the expression of miR1 (ventral region) and miR129-2 (dorsal region), relative to injured, unshocked controls. This is the first evidence that miRNA networks at the site of a contusion injury are affected

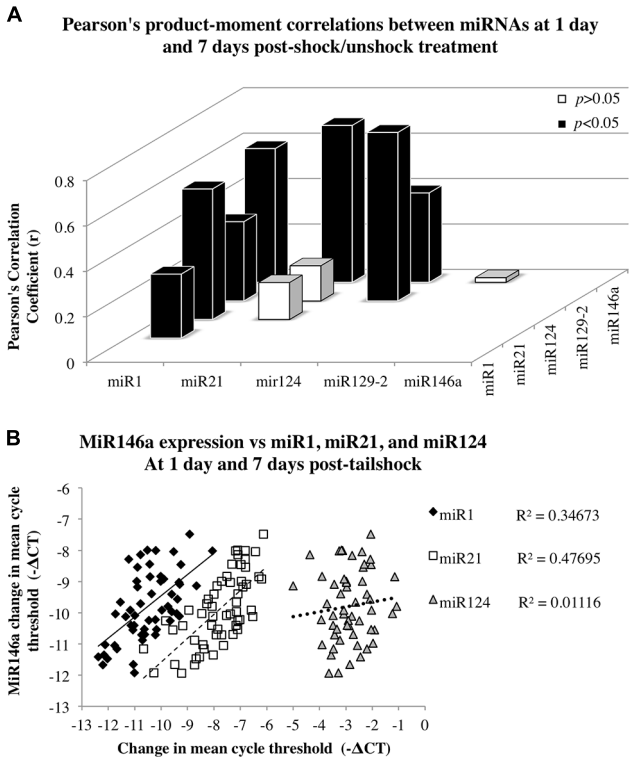

FIGURE 4 | Correlation analyses to assess the relationship between miR146a miRNA expression and expression of miR1, miR21, and miR124 following SCI. Data for dorsal and ventral spinal cord and for day 1 and 7 post-shock were combined for analysis. **(A)** The *x*-axis and *z*-axis represent the miRNAs of interest, and the *y*-axis depicts the magnitude of the Pearson's product–moment correlation coefficient, *r*, between the corresponding miRNAs indicated on the *x*- and *z*-axes. Pearson's correlations indicated seven statistically significant relationships ($p < 0.05$; black bars) and three non-significant relationships ($p > 0.05$; white bars). **(B)** The *x*-axis represents the mean difference in cycle threshold change ($-\Delta$CT) between miRNA expression at both dorsal and ventral spinal cord and at 1 and 7 days following tailshock treatment and U6 controls, and the y-axis depicts the mean difference in cycle threshold change ($-\Delta$CT) between miR146a expression in dorsal and ventral spinal cord and at both 1 and 7 days following tailshock treatment and U6 controls. Pearson's correlations indicated a significant correlation between miR1 (black diamonds), miR21 (white squares), and miR124 (gray triangles), and miR146a (Pearson's $r = 0.59$, $P < 0.001$, Pearson's $r = 0.69$, $P < 0.001$, and Pearson's $r = 0.39$, $P < 0.005$, respectively). Data are represented as the mean change in cycle threshold of miRNA expression relative to U6 controls ($-\Delta CT = CT_{U6} - CT_{miRNA}$).

Table 3 | Pearson's product–moment correlations for miRNA expression.

	miR1	miR21	miR124	miR129-2	miR146a
miR1					
Pearson correlation		0.282*	0.575**	0.349**	0.589**
Sig. (two-tailed)		*0.034*	*0.001*	*0.01*	*0.001*
N		*57*	*54*	*54*	*54*
miR21					
Pearson correlation			0.163	−0.155	0.691**
Sig. (two-tailed)			*0.227*	*0.255*	*0.001*
N			*57*	*56*	*57*
miR124					
Pearson correlation				0.742**	0.394**
Sig. (two-tailed)				*0.001*	*0.003*
N				*57*	*56*
miR129-2					
Pearson correlation					0.021
Sig. (2-tailed)					*0.876*
N					*56*

Analyses were based on combined data for both dorsal and ventral spinal cord and at 1 and 7 days following tailshock treatment. For each correlation between miRNAs given by row and column heading, the Pearson's Correlation Coefficient, r, the p value based on Student's two-tailed t-test, and the sample number, N, are listed in order from top to bottom, respectively. Three main clusters of miRNAs correlate significantly together: miR1 correlates with miR21, miR124, miR129-2, and miR146a, miR124 correlates with miR1, miR129-2, and miR146a, and miR146a correlates with miR1, miR21, and miR124.

**Correlation is significant at the 0.05 level (two-tailed).*

***Correlation is significant at the 0.01 level (two-tailed).*

by uncontrollable stimulation to distal spinal segments. Systemic factors like inflammatory stimuli (Huie et al., 2012a) and the activation of glia (Vichaya et al., 2009) may mediate the effects of distal uncontrollable stimulation on miRNA expression. However, the direct involvement of intra-spinal circuitry cannot be eliminated. Previous tract-tracing studies in the rat have described intra-spinal proprioceptive circuits between the sacro-caudal spinal cord that exerts sensorimotor control over the tail and more rostral spinal levels including thoracic segments (Masson et al., 1991). Moreover, spinal cord trauma has also been previously shown to result in extensive rewiring of intra-spinal circuitry (Bareyre et al., 2004) in the rat. While rewired circuitry matures over a period of several weeks following injury (Bareyre et al., 2004), a combination of undamaged and early rewiring intra-spinal circuits may constitute a mechanism whereby distal nociceptive stimuli regulate miRNA expression at the injury site. Interestingly, shock treatment shifted the regional distribution miRNA expression in contused rats, leading to decreased expression of miR124 in the dorsal region and at 7 days, a region-specific up-regulation of miR1 (ventral) and miR129-2 (dorsal) relative to unshocked animals. In addition, differences in expression of miR124 were accounted for by variation in the expression of miR1, miR129-2, and miR146a, suggesting that these miRNAs may be co-regulated. Collectively, these data suggest that uncontrollable nociception modulates SCI-sensitive miRNA networks in a spatially specific manner.

We observed that although there was initially significant down-regulation of IGF-1 following contusion, uncontrolled intermittent shock did not immediately result in additional suppression of Igf-1 at the 1-h time point. In contrast, IGF-1 was significantly increased in response to prior contusion at both 1 and 7 days following shock/unshock exposure, in both dorsal/sensory and ventral/motor tissue. Interestingly uncontrollable intermittent tailshock induced additional significant up-regulation only after a temporal delay, i.e, at 7 days after treatment, and only in the dorsal (i.e., sensory) region of the spinal cord. These data suggest

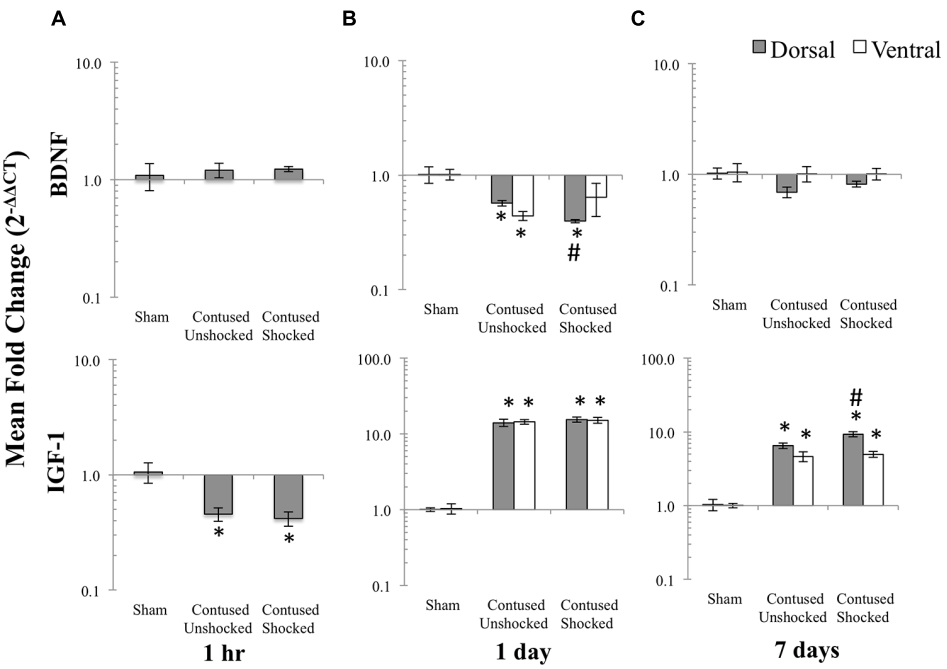

FIGURE 5 | Bar graphs depict qRT-PCR analysis of BDNF and IGF-1 mRNA expression at the lesion site following shock treatment in contused animals, compared to sham lesioned controls and non-shocked (unshock), lesioned animals at 1 h (A), 1 day (B), or 7 days (C) following tailshock treatment. The *x*-axis denotes treatment condition, and the *y*-axis denotes the log of mean fold expression change of mRNA. Data were normalized relative to GAPDH mRNA and expressed as the mean of fold changes of individual samples within a treatment group relative to the mean of the treatment group (±EM). As indicated in the text, there were significant main effects of time and spinal cord region (dorsal versus ventral) on mRNA expression. Within each time group, asterisks indicate significance (LSD) compared with time-group specific sham controls and hash tags indicate significance (LSD) compared with contused unshocked controls; *p* values are as indicated in the text.

that the IGF-1 response of the dorsal spinal cord is more responsive to nociceptive influences compared to the ventral spinal cord.

Table 4 | Pearson's product–moment correlations between miRNA expression and the expression of Igf1 and BDNF mRNA transcripts.

	miR1	miR21	miR124	miR129-2	miR146a
BDNF mRNA					
Pearson correlation	0.069	0.332**	−0.289*	−0.164	0.154
Sig. (two-tailed)	*0.609*	*0.009*	*0.024*	*0.211*	*0.244*
N	*57*	*60*	*61*	*60*	*59*
Igf1 mRNA					
Pearson correlation	0.402**	−0.235	0.469**	0.415**	0.173
Sig. (two-tailed)	*0.002*	*0.071*	*0.001*	*0.001*	*0.189*
N	*57*	*60*	*61*	*60*	*59*

Analysis was based on combined data for dorsal and ventral spinal cord and from 1 and 7 days following tailshock treatment. For each correlation between miRNAs given by row and column heading, the Pearson's correlation coefficient, r, the p value based on Student's two-tailed t-test, and the sample number, N, are listed in order from top to bottom, respectively.

**Correlation is significant at the 0.05 level (two-tailed).*

***Correlation is significant at the 0.01 level (two-tailed).*

Consistent with our previous findings (Garraway et al., 2011), we found that BDNF expression was significantly down-regulated at 1 day, as a function of SCI, but that uncontrollable intermittent tail-shock resulted in additional and significant BDNF suppression, within the dorsal/sensory region of the spinal cord. A step-wise linear regression analysis revealed that variation in miR124, miR21, and miR146 accounted for a significant proportion of the variation in IGF-1 mRNA expression, and that miR21 and miR124 accounted for variation in BDNF mRNA expression, suggesting that both BDNF and IGF-1 regulation involves a network of miRNAs. Alternatively, BDNF and IGF-1 may also influence miRNA expression and the potential role of their downstream signaling cascades in influencing the expression of mR21 and miR124 need further investigation. The correlated expression of miRNA and trophic factor transcripts may also point to the existence of positive feed-forward regulatory pathways. For example, recent research has shown that another member of the neurotrophin family, nerve growth factor (NGF) induces the expression of miR21, and miR21 in turn promoted NGF-induced neuronal differentiation (Montalban et al., 2014). Following SCI such correlated trophic factor-miRNA networks may be important for promoting neuroprotection.

Surprisingly, though miR1 and IGF-1 were both responsive to uncontrollable nociception variation in miR1 did not account

for a significant proportion of the variation in IGF-1 (or BDNF). This surprising finding perhaps reflects physiological differences between dorsal-sensory and ventral-motor spinal cord and their association with nociceptive stimulation. At day 7 following nociceptive/shock stimulation, miR1 expression in dorsal spinal cord in animals that had only received SCI were not different from controls, whereas IGF-1 mRNA expression was significantly increased. Uncontrollable nociception resulted in a significant decrease in miR1 while further increasing IGF-1 mRNA. Therefore, in the dorsal, sensory spinal cord, nociceptive stimulation at least resulted in a prototypic inverse relationship between miRNA and target gene. Since the dorsal spinal cord is likely to be the primary recipient of nociceptive input, the inverse relationship between miR1 and IGF-1 may represent a direct sensory modulation of SCI and SCI-sensitive miRNA function. However, in ventral spinal cord, at 7 days following uncontrollable shock, animals that received SCI alone exhibited a significant decrease in miR1 and an increase in IGF1. While uncontrollable nociceptive stimulation in SCI animals attenuated this decrease in miR1 observed following SCI alone, effectively resulting in increased miRNA expression, the expression of IGF-1 remained unchanged from the shock-only condition. These data suggest a dissociation between miRNA and target gene networks following SCI and intermittent noxious stimulation, as we have previously observed in other models of neural damage (Pappalardo-Carter et al., 2013). While such dissociation may be simply the result of miRNA and mRNAs being expressed in distinct and non-overlapping cell cohorts, this dissociation in ventral spinal cord may be a secondary consequence of activating the dorsal spinal cord. It is also intriguing to hypothesize that this dissociation may indicate the emergence of intervening, compensatory biological mechanisms, including perhaps shifts in miRNA function from translation repression to transcription activation (Place et al., 2008; Liu et al., 2013).

Uncontrollable nociception may have significant adverse consequences for recovery of function, that may be mediated by SCI-sensitive miRNAs. These miRNAs have also been shown to be activated and to influence cellular responses to neuropathic pain. For example, consistent with our own observation showing delayed reduction in miR1, others have reported that peripherally applied nociceptive and inflammatory stimuli also result in long term reduction of miR1 expression in dorsal root ganglia (Kusuda et al., 2011). An accumulating literature suggests that several SCI-sensitive miRNAs control inflammatory and other biological functions that are pertinent to nociception. For example, miR124 suppresses activation of resting microglia and macrophages prior to injury, and both miR21 and miR146a have been shown to negatively regulate astrocyte activation following SCI (Ponomarev et al., 2011; Bhalala et al., 2012; Iyer et al., 2012; Willemen et al., 2012; Kynast et al., 2013). MiR124 is an especially important candidate, as it is involved in the regulation of both BDNF and IGF-1, is sensitive to uncontrollable intermittent tailshock, and has been shown to inhibit nociceptive behavior associated with neuropathic pain (Kynast et al., 2013). While these miRNA changes were assessed within the spinal cord, and presumably directly influenced by activation of neural nociceptive pathways, the role of intermediate physiological and cellular activators cannot be ignored. For example, changes in heart rate, blood pressure, or immune system activation in response to nociceptive stimuli may mediate changes in miRNA expression. In this context, it is important to note that miR1 is also highly expressed in the vascular system and increases the barrier capacity of endothelial cells (Wang et al., 2013). One prediction is that the persistent decrease in miR1 expression following SCI may lead to increased vascular permeability and consequently be permissive for leukocyte infiltration. In the early stages of injury, the suppression of miR1 may well have a protective effect, since the predicted increase in leukocyte infiltration may limit tissue damage and promote clearance of debris (Peruzzotti-Jametti et al., 2014). Uncontrollable nociceptive stimuli may be predicted to prevent or delay that effect.

SCI and nociception-sensitive target genes like IGF-1 also have important consequences for functional recovery. IGF-1 promotes both oligodendrocyte survival after SCI upon up-regulation through leukemia inhibitory factor-mediated activation of microglia (Kerr and Patterson, 2005; Mekhail et al., 2012), and reduces blood–brain barrier permeability and edema through attenuation of nitric oxide synthase up-regulation upon its topical application prior to and following SCI (Sharma et al., 1997; Nyberg and Sharma, 2002). IGF may have general neuroprotective value in a variety of disease models. For example, recent research has shown that IGF-1 protects against ischemic stroke (Selvamani et al., 2012). Intriguingly, in the above report, antisense-mediated suppression of miR1 had the same neuroprotective effect. These data suggest that the decrease in miR1 and increase in IGF-1 mRNA following SCI may represent a neuroprotective adaptation. Conversely, uncontrollable nociceptive stimulation effectively increases miR1 at least in dorsal spinal cord, and may therefore adversely influence functional recovery.

Collectively, these data suggest that SCI-sensitive miRNAs constitute an important component of a response to uncontrollable nociception from peripheral injury. While it remains to be ascertained, these miRNAs and their targets may well engage direct astrocyte- and glial-mediated mechanisms as well as indirect inflammatory pathways. Consequently, these miRNAs may constitute therapeutic targets for attenuating neuropathic pain following SCI.

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REFERENCES

Bareyre, F. M., Kerschensteiner, M., Raineteau, O., Mettenleiter, T. C., Weinmann, O., and Schwab, M. E. (2004). The injured spinal cord spontaneously forms a new intraspinal circuit in adult rats. *Nat. Neurosci.* 7, 269–277. doi: 10.1038/nn1195

Beattie, M. S., Hermann, G. E., Rogers, R. C., and Bresnahan, J. C. (2002). Cell death in models of spinal cord injury. *Prog. Brain Res.* 137, 37–47. doi: 10.1016/S0079-6123(02)37006-7

Bhalala, O. G., Pan, L., Sahni, V., Mcguire, T. L., Gruner, K., Tourtellotte, W. G., et al. (2012). microRNA-21 regulates astrocytic response following spinal cord injury. *J. Neurosci.* 32, 17935–17947. doi: 10.1523/JNEUROSCI.3860-12.2012

Boyce, V. S., and Mendell, L. M. (2014). Neurotrophic factors in spinal cord injury. *Handb. Exp. Pharmacol.* 220, 443–460. doi: 10.1007/978-3-642-45106-5_16

Boyce, V. S., Tumolo, M., Fischer, I., Murray, M., and Lemay, M. A. (2007). Neurotrophic factors promote and enhance locomotor recovery in untrained spinalized cats. *J. Neurophysiol.* 98, 1988–1996. doi: 10.1152/jn.00391.2007

Brown, A. K., Woller, S. A., Moreno, G., Grau, J. W., and Hook, M. A. (2011). Exercise therapy and recovery after SCI: evidence that shows early intervention improves recovery of function. *Spinal Cord* 49, 623–628. doi: 10.1038/sc.2010.167

Byrnes, K. R., Stoica, B. A., Fricke, S., Di Giovanni, S., and Faden, A. I. (2007). Cell cycle activation contributes to post-mitotic cell death and secondary damage after spinal cord injury. *Brain* 130, 2977–2992. doi: 10.1093/brain/awm179

Camarillo, C., and Miranda, R. C. (2008). Ethanol exposure during neurogenesis induces persistent effects on neural maturation: evidence from an ex vivo model of fetal cerebral cortical neuroepithelial progenitor maturation. *Gene Expr.* 14, 159–171.

Constantini, S., and Young, W. (1994). The effects of methylprednisolone and the ganglioside GM1 on acute spinal cord injury in rats. *J. Neurosurg.* 80, 97–111. doi: 10.3171/jns.1994.80.1.0097

Crown, E. D., Ferguson, A. R., Joynes, R. L., and Grau, J. W. (2002). Instrumental learning within the spinal cord. II. Evidence for central mediation. *Physiol. Behav.* 77, 259–267. doi: 10.1016/S0031-9384(02)00859-4

Detloff, M. R., Fisher, L. C., Mcgaughy, V., Longbrake, E. E., Popovich, P. G., and Basso, D. M. (2008). Remote activation of microglia and pro-inflammatory cytokines predict the onset and severity of below-level neuropathic pain after spinal cord injury in rats. *Exp. Neurol.* 212, 337–347. doi: 10.1016/j.expneurol.2008.04.009

Di Giovanni, S., Knoblach, S. M., Brandoli, C., Aden, S. A., Hoffman, E. P., and Faden, A. I. (2003). Gene profiling in spinal cord injury shows role of cell cycle in neuronal death. *Ann. Neurol.* 53, 454–468. doi: 10.1002/ana.10472

Doeppner, T. R., Doehring, M., Bretschneider, E., Zechariah, A., Kaltwasser, B., Muller, B., et al. (2013). MicroRNA-124 protects against focal cerebral ischemia via mechanisms involving Usp14-dependent REST degradation. *Acta Neuropathol.* 126, 251–265. doi: 10.1007/s00401-013-1142-5

Ferguson, A. R., Bolding, K. A., Huie, J. R., Hook, M. A., Santillano, D. R., Miranda, R. C., et al. (2008). Group I metabotropic glutamate receptors control metaplasticity of spinal cord learning through a protein kinase C-dependent mechanism. *J. Neurosci.* 28, 11939–11949. doi: 10.1523/JNEUROSCI.3098-08.2008

Frei, E., Klusman, I., Schnell, L., and Schwab, M. E. (2000). Reactions of oligodendrocytes to spinal cord injury: cell survival and myelin repair. *Exp. Neurol.* 163, 373–380. doi: 10.1006/exnr.2000.7379

Garraway, S. M., Petruska, J. C., and Mendell, L. M. (2003). BDNF sensitizes the response of lamina II neurons to high threshold primary afferent inputs. *Eur. J. Neurosci.* 18, 2467–2476. doi: 10.1046/j.1460-9568.2003.02982.x

Garraway, S. M., Turtle, J. D., Huie, J. R., Lee, K. H., Hook, M. A., Woller, S. A., et al. (2011). Intermittent noxious stimulation following spinal cord contusion injury impairs locomotor recovery and reduces spinal brain-derived neurotrophic factor-tropomyosin-receptor kinase signaling in adult rats. *Neuroscience* 199, 86–102. doi: 10.1016/j.neuroscience.2011.10.007

Gomez-Pinilla, F., Huie, J. R., Ying, Z., Ferguson, A. R., Crown, E. D., Baumbauer, K. M., et al. (2007). BDNF and learning: evidence that instrumental training promotes learning within the spinal cord by up-regulating BDNF expression. *Neuroscience* 148, 893–906. doi: 10.1016/j.neuroscience.2007.05.051

Grau, J. W., Washburn, S. N., Hook, M. A., Ferguson, A. R., Crown, E. D., Garcia, G., et al. (2004). Uncontrollable stimulation undermines recovery after spinal cord injury. *J. Neurotrauma* 21, 1795–1817. doi: 10.1089/neu.2004.21.1795

Gruner, J. A. (1992). A monitored contusion model of spinal cord injury in the rat. *J. Neurotrauma* 9, 123-126; discussion 126–128. doi: 10.1089/neu.1992.9.123

Hains, B. C., Klein, J. P., Saab, C. Y., Craner, M. J., Black, J. A., and Waxman, S. G. (2003). Upregulation of sodium channel Nav1.3 and functional involvement in neuronal hyperexcitability associated with central neuropathic pain after spinal cord injury. *J. Neurosci.* 23, 8881–8892.

Hains, B. C., and Waxman, S. G. (2006). Activated microglia contribute to the maintenance of chronic pain after spinal cord injury. *J. Neurosci.* 26, 4308–4317. doi: 10.1523/JNEUROSCI.0003-06.2006

Herrup, K., and Yang, Y. (2007). Cell cycle regulation in the postmitotic neuron: oxymoron or new biology? *Nat. Rev. Neurosci.* 8, 368–378. doi: 10.1038/nrn2124

Hook, M. A., Washburn, S. N., Moreno, G., Woller, S. A., Puga, D., Lee, K. H., et al. (2011). An IL-1 receptor antagonist blocks a morphine-induced attenuation of locomotor recovery after spinal cord injury. *Brain Behav. Immun.* 25, 349–359. doi: 10.1016/j.bbi.2010.10.018

Huie, J. R., Baumbauer, K. M., Lee, K. H., Bresnahan, J. C., Beattie, M. S., Ferguson, A. R., et al. (2012a). Glial tumor necrosis factor alpha (TNFalpha) generates metaplastic inhibition of spinal learning. *PLoS ONE* 7:e39751. doi: 10.1371/journal.pone.0039751

Huie, J. R., Garraway, S. M., Baumbauer, K. M., Hoy, K. C. Jr., Beas, B. S., et al. (2012b). Brain-derived neurotrophic factor promotes adaptive plasticity within the spinal cord and mediates the beneficial effects of controllable stimulation. *Neuroscience* 200, 74–90. doi: 10.1016/j.neuroscience.2011.10.028

Iyer, A., Zurolo, E., Prabowo, A., Fluiter, K., Spliet, W. G., Van Rijen, P. C., et al. (2012). MicroRNA-146a: a key regulator of astrocyte-mediated inflammatory response. *PLoS ONE* 7:e44789. doi: 10.1371/journal.pone.0044789

Kerr, B. J., Bradbury, E. J., Bennett, D. L., Trivedi, P. M., Dassan, P., French, J., et al. (1999). Brain-derived neurotrophic factor modulates nociceptive sensory inputs and NMDA-evoked responses in the rat spinal cord. *J. Neurosci.* 19, 5138–5148.

Kerr, B. J., and Patterson, P. H. (2005). Leukemia inhibitory factor promotes oligodendrocyte survival after spinal cord injury. *Glia* 51, 73–79. doi: 10.1002/glia.20177

Kusuda, R., Cadetti, F., Ravanelli, M. I., Sousa, T. A., Zanon, S., De Lucca, F. L., et al. (2011). Differential expression of microRNAs in mouse pain models. *Mol. Pain* 7, 17. doi: 10.1186/1744-8069-7-17

Kuzhandaivel, A., Nistri, A., Mazzone, G. L., and Mladinic, M. (2011). Molecular mechanisms underlying cell death in spinal networks in relation to locomotor activity after acute injury in vitro. *Front. Cell Neurosci.* 5:9. doi: 10.3389/fncel.2011.00009

Kynast, K. L., Russe, O. Q., Moser, C. V., Geisslinger, G., and Niederberger, E. (2013). Modulation of central nervous system-specific microRNA-124a alters the inflammatory response in the formalin test in mice. *Pain* 154, 368–376. doi: 10.1016/j.pain.2012.11.010

Lampert, A., Hains, B. C., and Waxman, S. G. (2006). Upregulation of persistent and ramp sodium current in dorsal horn neurons after spinal cord injury. *Exp. Brain Res.* 174, 660–666. doi: 10.1007/s00221-006-0511-x

Lee, S. T., Chu, K., Jung, K. H., Kim, J. H., Huh, J. Y., Yoon, H., et al. (2012). miR-206 regulates brain-derived neurotrophic factor in Alzheimer disease model. *Ann. Neurol.* 72, 269–277. doi: 10.1002/ana.23588

Lerch, J. K., Puga, D. A., Bloom, O., and Popovich, P. G. (2014). Glucocorticoids and macrophage migration inhibitory factor (MIF) are neuroendocrine modulators of inflammation and neuropathic pain after spinal cord injury. *Semin. Immunol.* doi: 10.1016/j.smim.2014.03.004 [Epub ahead of print].

Lewis, B. P., Shih, I. H., Jones-Rhoades, M. W., Bartel, D. P., and Burge, C. B. (2003). Prediction of mammalian microRNA targets. *Cell* 115, 787–798. doi: 10.1016/S0092-8674(03)01018-3

Li, Y., Shelat, H., and Geng, Y. J. (2012). IGF-1 prevents oxidative stress induced-apoptosis in induced pluripotent stem cells which is mediated by microRNA-1. *Biochem. Biophys. Res. Commun.* 426, 615–619. doi: 10.1016/j.bbrc.2012.08.139

Liu, D., Xu, G. Y., Pan, E., and Mcadoo, D. J. (1999). Neurotoxicity of glutamate at the concentration released upon spinal cord injury. *Neuroscience* 93, 1383–1389. doi: 10.1016/S0306-4522(99)00278-X

Liu, M., Roth, A., Yu, M., Morris, R., Bersani, F., Rivera, M. N., et al. (2013). The IGF2 intronic miR-483 selectively enhances transcription from IGF2 fetal promoters and enhances tumorigenesis. *Genes Dev.* 27, 2543–2548. doi: 10.1101/gad.224170.113

Liu, N. K., Wang, X. F., Lu, Q. B., and Xu, X. M. (2009). Altered microRNA expression following traumatic spinal cord injury. *Exp. Neurol.* 219, 424–429. doi: 10.1016/j.expneurol.2009.06.015

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25, 402–408. doi: 10.1006/meth.2001.1262

Masson, R. L. Jr., Sparkes, M. L., and Ritz, L. A. (1991). Descending projections to the rat sacrocaudal spinal cord. *J. Comp. Neurol.* 307, 120–130. doi: 10.1002/cne.903070111

McTigue, D. M., Horner, P. J., Stokes, B. T., and Gage, F. H. (1998). Neurotrophin-3 and brain-derived neurotrophic factor induce oligodendrocyte proliferation

and myelination of regenerating axons in the contused adult rat spinal cord. *J. Neurosci.* 18, 5354–5365.

Mekhail, M., Almazan, G., and Tabrizian, M. (2012). Oligodendrocyte-protection and remyelination post-spinal cord injuries: a review. *Prog. Neurobiol.* 96, 322–339. doi: 10.1016/j.pneurobio.2012.01.008

Montalban, E., Mattugini, N., Ciarapica, R., Provenzano, C., Savino, M., Scagnoli, F., et al. (2014). MiR-21 is an Ngf-modulated microRNA that supports Ngf signaling and regulates neuronal degeneration in PC12 cells. *Neuromolecular Med.* 16, 415–430. doi: 10.1007/s12017-014-8292-z

Nakanishi, K., Nakasa, T., Tanaka, N., Ishikawa, M., Yamada, K., Yamasaki, K., et al. (2010). Responses of microRNAs 124a and 223 following spinal cord injury in mice. *Spinal Cord* 48, 192–196. doi: 10.1038/sc.2009.89

Nyberg, F., and Sharma, H. S. (2002). Repeated topical application of growth hormone attenuates blood-spinal cord barrier permeability and edema formation following spinal cord injury: an experimental study in the rat using Evans blue, ([125])I-sodium and lanthanum tracers. *Amino Acids* 23, 231–239. doi: 10.1007/s00726-001-0134-2

Pappalardo-Carter, D. L., Balaraman, S., Sathyan, P., Carter, E. S., Chen, W. J., and Miranda, R. C. (2013). Suppression and epigenetic regulation of MiR-9 contributes to ethanol teratology: evidence from zebrafish and murine fetal neural stem cell models. *Alcohol. Clin. Exp. Res.* 37, 1657–1667. doi: 10.1111/acer.12139

Patterson, S. L., Abel, T., Deuel, T. A., Martin, K. C., Rose, J. C., and Kandel, E. R. (1996). Recombinant BDNF rescues deficits in basal synaptic transmission and hippocampal LTP in BDNF knockout mice. *Neuron* 16, 1137–1145. doi: 10.1016/S0896-6273(00)80140-3

Peruzzotti-Jametti, L., Donega, M., Giusto, E., Mallucci, G., Marchetti, B., and Pluchino, S. (2014). The role of the immune system in central nervous system plasticity after acute injury. *Neuroscience* doi: 10.1016/j.neuroscience.2014.04.036 [Epub ahead of print].

Place, R. F., Li, L. C., Pookot, D., Noonan, E. J., and Dahiya, R. (2008). MicroRNA-373 induces expression of genes with complementary promoter sequences. *Proc. Natl. Acad. Sci. U.S.A.* 105, 1608–1613. doi: 10.1073/pnas.0707594105

Ponomarev, E. D., Veremeyko, T., Barteneva, N., Krichevsky, A. M., and Weiner, H. L. (2011). MicroRNA-124 promotes microglia quiescence and suppresses EAE by deactivating macrophages via the C/EBP-alpha-PU.1 pathway. *Nat. Med.* 17, 64–70. doi: 10.1038/nm.2266

Schwab, M. E., and Strittmatter, S. M. (2014). Nogo limits neural plasticity and recovery from injury. *Curr. Opin. Neurobiol.* 27C, 53–60. doi: 10.1016/j.conb.2014.02.011

Selvamani, A., Sathyan, P., Miranda, R. C., and Sohrabji, F. (2012). An antagomir to microRNA Let7f promotes neuroprotection in an ischemic stroke model. *PLoS ONE* 7:e32662. doi: 10.1371/journal.pone.0032662

Sharma, H. S., Nyberg, F., Gordh, T., Alm, P., and Westman, J. (1997). Topical application of insulin like growth factor-1 reduces edema and upregulation of neuronal nitric oxide synthase following trauma to the rat spinal cord. *Acta Neurochir. Suppl.* 70, 130–133. doi: 10.1007/978-3-7091-6837-0_40

Silva, N. A., Sousa, N., Reis, R. L., and Salgado, A. J. (2014). From basics to clinical: a comprehensive review on spinal cord injury. *Prog. Neurobiol.* 114, 25–57. doi: 10.1016/j.pneurobio.2013.11.002

Strickland, E. R., Hook, M. A., Balaraman, S., Huie, J. R., Grau, J. W., and Miranda, R. C. (2011). MicroRNA dysregulation following spinal cord contusion: implications for neural plasticity and repair. *Neuroscience* 186, 146–160. doi: 10.1016/j.neuroscience.2011.03.063

Strickland, E. R., Woller, S. A., Hook, M. A., Grau, J. W., and Miranda, R. C. (2014). The association between spinal cord trauma-sensitive miRNAs and pain sensitivity, and their regulation by morphine. *Neurochem. Int.* doi: 10.1016/j.neuint.2014.05.005 [Epub ahead of print].

Vichaya, E. G., Baumbauer, K. M., Carcoba, L. M., Grau, J. W., and Meagher, M. W. (2009). Spinal glia modulate both adaptive and pathological processes. *Brain Behav. Immun.* 23, 969–976. doi: 10.1016/j.bbi.2009.05.001

Visvanathan, J., Lee, S., Lee, B., Lee, J. W., and Lee, S. K. (2007). The microRNA miR-124 antagonizes the anti-neural REST/SCP1 pathway during embryonic CNS development. *Genes Dev.* 21, 744–749. doi: 10.1101/gad.1519107

Wang, H., Zhu, H. Q., Wang, F., Zhou, Q., Gui, S. Y., and Wang, Y. (2013). MicroRNA-1 prevents high-fat diet-induced endothelial permeability in apoE knock-out mice. *Mol. Cell. Biochem.* 378, 153–159. doi: 10.1007/s11010-013-1606-x

Willemen, H. L., Huo, X. J., Mao-Ying, Q. L., Zijlstra, J., Heijnen, C. J., and Kavelaars, A. (2012). MicroRNA-124 as a novel treatment for persistent hyperalgesia. *J. Neuroinflammation* 9, 143. doi: 10.1186/1742-2094-9-143

Wu, J., Qian, J., Li, C., Kwok, L., Cheng, F., Liu, P., et al. (2010). miR-129 regulates cell proliferation by downregulating Cdk6 expression. *Cell Cycle* 9, 1809–1818. doi: 10.4161/cc.9.9.11535

Xu, G. Y., Hughes, M. G., Ye, Z., Hulsebosch, C. E., and Mcadoo, D. J. (2004). Concentrations of glutamate released following spinal cord injury kill oligodendrocytes in the spinal cord. *Exp. Neurol.* 187, 329–336. doi: 10.1016/j.expneurol.2004.01.029

Xu, X. M., Guenard, V., Kleitman, N., Aebischer, P., and Bunge, M. B. (1995). A combination of BDNF and NT-3 promotes supraspinal axonal regeneration into Schwann cell grafts in adult rat thoracic spinal cord. *Exp. Neurol.* 134, 261–272. doi: 10.1006/exnr.1995.1056

Yu, J. Y., Chung, K. H., Deo, M., Thompson, R. C., and Turner, D. L. (2008a). MicroRNA miR-124 regulates neurite outgrowth during neuronal differentiation. *Exp. Cell Res.* 314, 2618–2633. doi: 10.1016/j.yexcr.2008.06.002

Yu, X. Y., Song, Y. H., Geng, Y. J., Lin, Q. X., Shan, Z. X., Lin, S. G., et al. (2008b). Glucose induces apoptosis of cardiomyocytes via microRNA-1 and IGF-1. *Biochem. Biophys. Res. Commun.* 376, 548–552. doi: 10.1016/j.bbrc.2008.09.025

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Swimming against the tide: investigations of the C-bouton synapse

Adam S. Deardorff†, Shannon H. Romer†, Patrick M. Sonner and Robert E. W. Fyffe*

Boonshoft School of Medicine, Department of Neuroscience, Cell Biology and Physiology, Wright State University, Dayton, OH, USA

Edited by:
Hans Hultborn, University of Copenhagen, Denmark

Reviewed by:
C. J. Heckman, Northwestern University, USA
Claire Francesca Meehan, University of Copenhagen, Denmark

***Correspondence:**
Robert E. W. Fyffe, Boonshoft School of Medicine, Department of Neuroscience, Cell Biology and Physiology, Wright State University, 202A University Hall, 3640 Col. Glenn Highway, Dayton, OH 45435, USA
e-mail: robert.fyffe@wright.edu

†*Adam S. Deardorff and Shannon H. Romer have contributed equally to this work.*

C-boutons are important cholinergic modulatory loci for state-dependent alterations in motoneuron firing rate. m2 receptors are concentrated postsynaptic to C-boutons, and m2 receptor activation increases motoneuron excitability by reducing the action potential afterhyperpolarization. Here, using an intensive review of the current literature as well as data from our laboratory, we illustrate that C-bouton postsynaptic sites comprise a unique structural/functional domain containing appropriate cellular machinery (a "signaling ensemble") for cholinergic regulation of outward K^+ currents. Moreover, synaptic reorganization at these critical sites has been observed in a variety of pathologic states. Yet despite recent advances, there are still great challenges for understanding the role of C-bouton regulation and dysregulation in human health and disease. The development of new therapeutic interventions for devastating neurological conditions will rely on a complete understanding of the molecular mechanisms that underlie these complex synapses. Therefore, to close this review, we propose a comprehensive hypothetical mechanism for the cholinergic modification of α-MN excitability at C-bouton synapses, based on findings in several well-characterized neuronal systems.

Keywords: SK, Kv2.1, α-motoneuron, acetylcholine, C-boutons, afterhyperpolarization, subsurface cistern

INTRODUCTION

The neuromuscular system provides rapid and coordinated force generation, whereby the number and firing rate of recruited motor units are systematically adjusted to meet environmental demands (Monster and Chan, 1977; Henneman and Mendell, 1981; Clamann, 1993; Cope and Sokoloff, 1999). Indeed, the elegant simplicity with which animals navigate their environment relies on neural circuitry that is inherently modifiable, and the ability to perform a variety of motor tasks while responding quickly to unexpected perturbations and threats is essential for individual survival (Ladle et al., 2007; Miri et al., 2013). Control of α-MN repetitive firing properties is a therefore highly conserved and critical adaption of mammalian and non-mammalian species alike, and identifying the responsible spinal circuits has been of essential importance in our understanding of neuromuscular function and dysfunction (Miles and Sillar, 2011).

For more than 50 years, a particular class of synapse in the spinal cord ventral horn – the C-bouton – has generated sustained interest among α-MN anatomists and physiologists. Unambiguous identification of these conspicuously large cholinergic synaptic contacts and the characteristic postsynaptic SSC for which they are named has prompted numerous investigations into their distribution, source, function, and pathology. Yet despite the detailed morphologic and physiologic information generated by many neuroscientists, it is humbling to consider (a) the incrementally slow trajectory by which our understanding of this enigmatic synapse has grown and (b) that as yet there is no definitive and fully functional hypothesis regarding their distribution, their postsynaptic subcellular machinery, their contribution to motor control and behavior, and their regulation/dysregulation in health and disease.

Recently, we have learned the most elementary effect of C-boutons on α-MN *f-I* gain during static intracellular current injection occurs via dramatic reductions in the strength of the action potential AHP (Miles et al., 2007), which is mediated by postsynaptic small conductance Ca^{2+}-activated K^+ (SK) channels (Deardorff et al., 2013). However, the mystery of the C-bouton and its cholinergic effects on MN biophysical properties and integrative capabilities is by no means solved, as has been suggested (Frank, 2009). Using an isolated spinal cord preparation, Miles et al. (2007) demonstrate a putative role for C-boutons in ensuring appropriate levels of motor output during drug induced fictive locomotion. But complexity arises upon behavioral assessment of adult mice with selective genetic inactivation of C-bouton synaptic inputs, which during locomotion exhibit normal flexor–extensor alternation and normal EMG amplitude. Motor deficits in these mice primarily manifest during *high-output* tasks such as swimming (Zagoraiou et al., 2009). These data convincingly implicate C-boutons in the task-dependent regulation of α-MN excitability via reduction of outward K^+ currents, but questions remain regarding (a) the functional impact of C-bouton input during different behaviors, (b) the manner in which C-bouton activity is modulated to match motor demands, (c) the expression of abnormal force generation as well as spasticity, rigidity, or tremor as a

Abbreviations: AHP, afterhyperpolarization; ChAT, choline acetyltransferase; IaIN, Ia inhibitory interneuron; IR, immunoreactivity; m2 receptor, type 2 muscarinic acetylcholine receptor; MN, motoneuron; RyR, ryanodine receptors; S1R, sigma-1 receptors; SK, small conductance calcium-activated potassium channel; SSC, subsurface cistern; VAChT, vesicular acetylcholine transporter; VGluT, vesicular glutamate transporter.

consequence of C-bouton dysfunction, and (d) the mechanism of interaction between underlying acetylcholine receptors (AChRs) and K^+ channels.

To aid in the development of new *in vivo* and *in vitro* experimental strategies to answer these and related questions, this review details our current understanding of the cellular, synaptic, and genetic properties that underlie C-bouton function and proposes a hitherto unexplored mechanism for the cholinergic modification of α-MN excitability. It should be noted that the title of this review is intended to reflect and pay homage to the many dedicated and careful neuroscientists who have undertaken MN synaptological investigations over the years. This review will therefore also provide historical perspective on the foundational advances in our understanding of this complex and elusive, yet important, synapse. Neuroscientists have spent 50+ years at the C-bouton swimming against the tide. Significant progress has been slow and hard fought. And though we are a long way from shore, we must remember – as our murine colleagues have demonstrated – without C-boutons we cannot swim at all.

THE C-BOUTON SIGNALING ENSEMBLE: A CONTEMPORARY VIEW OF A CLASSIC SYNAPSE

We are riding the crest of a wave. With the turn of the century and the application of advanced morphologic analyses, cellular neurophysiology, and selective genetic perturbations, we have built a decidedly robust picture of C-bouton form and function. C-boutons are an essential piece of an integrated control system set to regulate α-MN activity through a complex anatomical substrate: a signaling ensemble (**Figures 1** and **2**) precisely organized for highly nuanced orchestration of somatic K^+ currents.

PRECISE ANATOMICAL LOCALIZATION AND ORGANIZATION OF SIGNALING COMPONENTS: AN ENSEMBLE OF APPOSED PROTEINS AND MOSAIC MEMBRANE DOMAINS

C-type synaptic sites comprise three closely apposed membranous domains (**Figure 2**), spanning a breadth of <25 nm, and across which the distribution of synaptic and signaling proteins are precisely regulated. Clear and consistent immunohistochemical data demonstrate membrane clusters of α-MN Kv2.1 channels, SK2/3 channels, and m2 receptors directly apposing C-bouton presynaptic terminals (Skinner et al., 1999; Hellstrom et al., 2003; Muennich and Fyffe, 2004; Wilson et al., 2004; Deardorff et al., 2013). When visualized under high resolution, these SK2/3 channel and m2 receptor clusters are composed of an intricate, non-uniform aggregation of smaller "threadlike" structures that are woven together and closely approximate/appose C-bouton pre-synaptic vesicle release sites, which are enriched with bassoon (A. S. Deardorff, S. H. Romer, R. E. W. Fyffe, unpublished; see **Figure 1**). Beneath the postsynaptic membrane, in α-MN SSCs, the gap junction protein connexin32 shows a similar threadlike distribution pattern (Yamamoto et al., 1990, 1991; Zampieri et al., 2014), indicating that connexin32, SK channels/m2 receptors, and transmitter release machinery are precisely aligned across the three membranous domains. Kv2.1 channels appear to "fill in" the remaining postsynaptic α-MN membrane surface not occupied by SK channels or m2 receptors. The demarcated postsynaptic area, therefore, is a highly structured and mosaic domain of interdigitating clusters of Kv2.1 channels and co-localized SK2/3 channels and m2 receptors. The orderly, stacked apposition of proteins on the cisternal, postsynaptic, and presynaptic membranes as well as the spatial interdigitation of distinct channel and receptor clusters demonstrates a coordinated and specific signaling organization across all membranous domains at C-bouton synaptic sites.

ADDITIONAL SIGNALING COMPONENTS

Additional studies have revealed, to varying levels of specificity, other signaling components that characterize the C-bouton ensemble. Certain elements, although identified within one or another membranous or cytoplasmic domain, are not well defined in regard to specific subdomain organization nor anatomic relation to other molecular components. In this category, C-bouton synaptic terminals express a range of exocytotic proteins consistent with those necessary for fast transmitter release (Hellstrom et al., 1999), are highly associated with presynaptic $P2X_7$ purinergic receptor immunoreactivity (∼90% of C-boutons; Deng and Fyffe, 2004), and may also express presynaptic nicotinic acetylcholine receptors (nAChRs; Khan et al., 2003). In addition, the α-MN SSC is highly enriched with S1Rs (Mavlyutov et al., 2010), and with closely associated neuregulin-1 (NG1) immunoreactivity (Gallart-Palau et al., 2014). Indole-*N*-methyl transferase (INMT), an enzyme that converts tryptamine into the S1R ligand dimethytryptamine (DMT), is also present in close proximity to S1Rs at C-bouton postsynaptic sites (Mavlyutov et al., 2012), but the extent to which S1Rs, themselves, are diffusely distributed within the entire cisternal membrane or co-localize/interdigitate with the well-characterized connexin32 immunoreactivity is not described.

The subcellular organization of Ca^{2+} sources necessary for SK channel activation also remains poorly characterized. However, α-MN SK2/3 channels require high voltage activated (HVA) N- and P/Q-type Ca^{2+} currents to generate the AHP (Viana et al., 1993; Umemiya and Berger, 1994; Bayliss et al., 1995; Li and Bennett, 2007), and SK channels typically couple to their Ca^{2+} source(s) by <200 nm (Fakler and Adelman, 2008; Jones and Stuart, 2013). Internally, SSCs may amplify or shape these Ca^{2+} signals via RyRs or connexin32, as they do in other cell types (see discussion Section "Subsurface Cisternae and the Generation of an Isolated Ca^{2+} Signal"). We, therefore, expect some proportion of HVA Ca^{2+} channels and RyRs to localize to the C-bouton postsynaptic membrane and/or to the associated SSC (**Figure 2**). In support, Wilson et al. (2004) provide evidence that P/Q-type Ca^{2+} channels are diffusely spread throughout the α-MN somatic membrane. By inference, some proportion must then appose C-boutons. The presence of N-type Ca^{2+} channels on α-MNs, however, has only been demonstrated physiologically (Carlin et al., 2000; Wilson et al., 2004).

THE CREST OF A WAVE

The unique aggregation of cytoplasmic and membrane bound pre- and postsynaptic proteins that constitute the C-bouton signaling ensemble provides mechanistic insight into the cholinergic modulation of α-MN firing rate and has advanced new research at a comparatively faster pace than that of many other α-MN synaptic

inputs. While uncertainties still confound our arrival at a "simple" molecular mechanism governing C-bouton synaptic function, experiments in other cell systems can help push us forward against the tide. Further exploration of this complex synapse is clearly necessitated. However, we must first review other salient features of the C-bouton system.

MOMENTS AND MILESTONES: ULTRASTRUCTURE

Pioneering EM investigations (Wyckoff and Young, 1956) provided accurate anatomical description and categorization of the structurally diverse presynaptic terminals contacting spinal α-MNs, and in general, most authors still conform to the descriptive abbreviations (S-, F-, C-, T-, and M-Boutons) introduced by Bodian (1966a,b) and Conradi (1969a). (An additional bouton type, the P bouton, makes presynaptic connections with specific excitatory boutons in contact with the MN surface and may form triadic arrangements; Conradi, 1969a; Fyffe and Light, 1984). Those boutons Conradi classified as "C-type" are defined by and named for a signature 10–15 nm thick postsynaptic SSC ("C-type" for cistern): a broad, flat disc of smooth endoplasmic reticulum juxtaposed a mere 5–8 nm below the postsynaptic membrane and spanning the length of the apposing presynaptic terminal (**Figure 3**; Conradi, 1969a). The SSC is continuous with several lamellae of rough endoplasmic reticulum oriented in parallel with the cell membrane and frequently observed alongside free ribosomal rosettes in the subcisternal cytoplasm

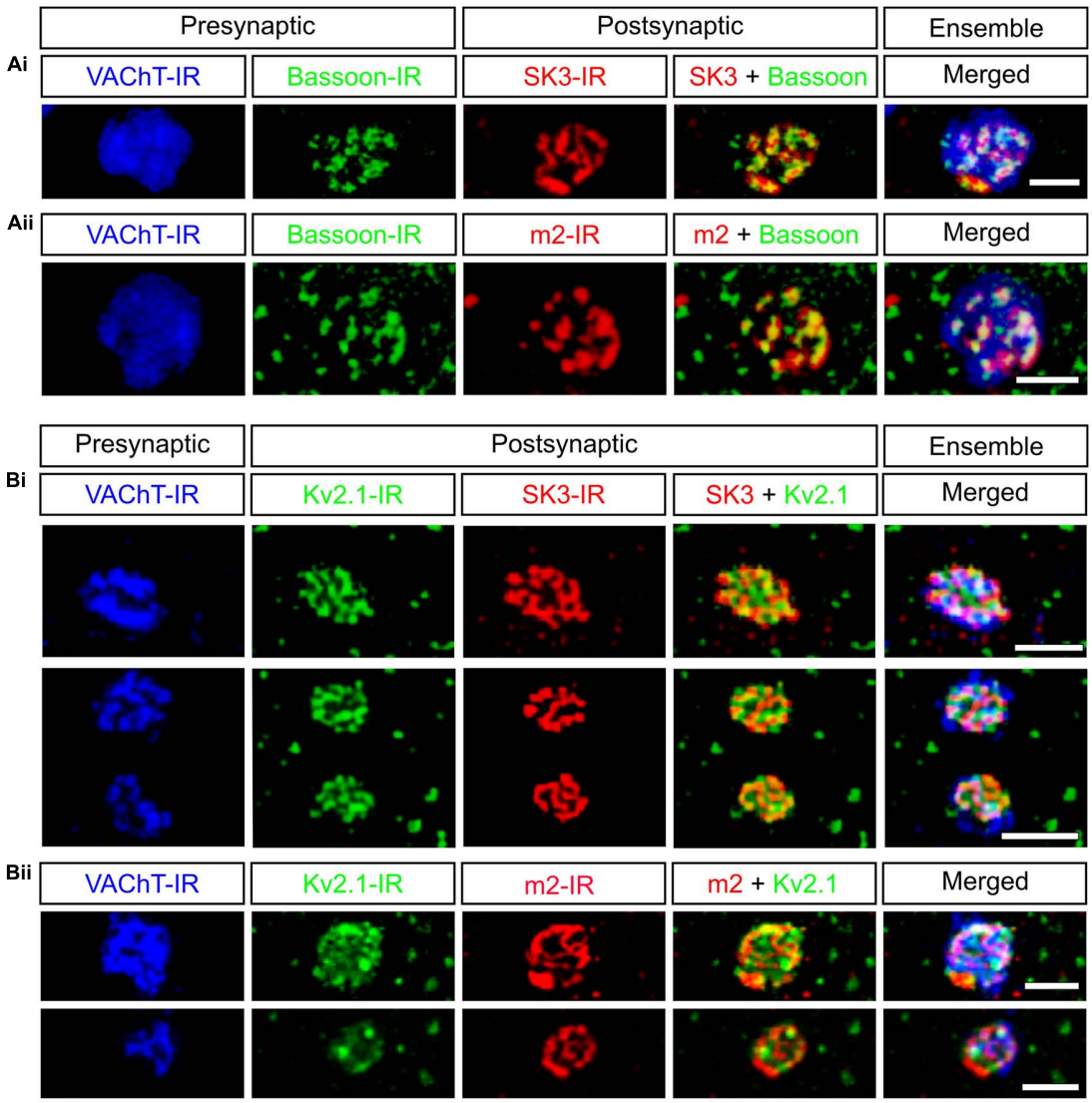

FIGURE 1 | C-bouton synaptic sites contain a complex signaling ensemble. Presynaptic bassoon-IR and postsynaptic SK3-IR and m2-IR share a striking subsynaptic fenestrated appearance within the C-bouton. All images are small confocal stacks (3 × 1 μm Z-stacks) of *en face* C-boutons, indicated with VAChT-IR (blue), on rat lumbar α-motoneurons. **(A)** Presynaptic active zone protein bassoon (green) is aligned with postsynaptic ion channels SK3 (**Ai**, red) and m2 receptors (**Aii**, red). **(B)** Kv2.1-IR (green) intercalates with SK3-IR (Bi, red) and m2-IR **(Bii)**, "filling in" the C-bouton postsynaptic membrane. Scale bars are 2.0 μm.

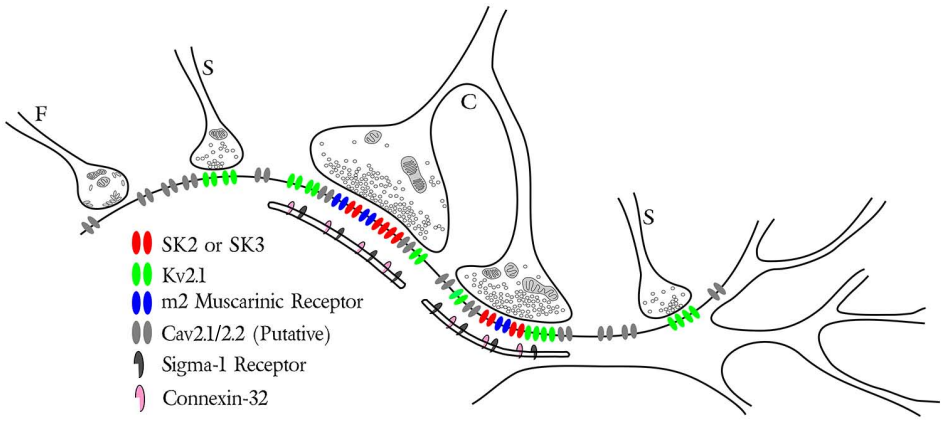

FIGURE 2 | Synaptic distribution of specific ion channels and receptors on soma and proximal dendrites of motoneurons. The schematic illustrates three types of motoneuron presynaptic boutons including the glycinergic/GABAergic F-type, glutamatergic S-type and cholinergic C-type with its associated postsynaptic subsurface cistern. Note the specific localization of m2 muscarinic receptors (blue) with SK channels (red) and Kv2.1 channels (green) postsynaptic to the C-bouton. Small Kv2.1 clusters are also found postsynaptic to some S-type synapses (see Muennich and Fyffe, 2004). The P/Q- and N-type Ca^{2+} channels Cav2.1/2.2 (light gray) are illustrated throughout the membrane, although the precise subcellular localization of this channel is currently unknown. Both connexin 32 (pink) and the sigma-1 receptor (dark gray) are specifically associated with the C-bouton subsurface cistern.

(**Figure 3**). Across a particularly narrow synaptic cleft (3–8 nm; see discussion Davidoff and Irintchev, 1986), the C-boutons themselves contain a dense cytoplasmic matrix of glycogen particles and neurofilaments tightly packed with 25–55 nm (diameter) clear spherical/pleomorphic vesicles, abundant mitochondria, and occasionally a small number of large dense core vesicles intermingled therein (**Figure 3**; Bodian, 1966a,b; Conradi, 1969a; McLaughlin, 1972b; Hamos and King, 1980). Notably, several authors (Rosenbluth, 1962; Bodian, 1966a,b; Charlton and Gray, 1966; Van Harreveld and Khattab, 1967) identified these unique and prominent boutons prior to Conradi's (1969a) classic and thorough description of their synaptic ultrastructure – which remains the gold standard for their identification.

C-boutons are among the largest of α-MN somatic and proximal dendritic synaptic inputs, ranging in size from 3 to 8 μm in the cat (Conradi, 1969a; McLaughlin, 1972b; Conradi et al., 1979a), 3–6 μm in the primate (Bodian, 1966a,b), 3–5 μm in the opossum (Hamos and King, 1980), 3–6 μm in the human (Pullen, 1992), and 1–8 μm in the rodent (Alvarez et al., 1999). But despite their conspicuous size, they lack quintessential active zone ultrastructure, i.e., pronounced paramembraneous densities and associated pools of readily releasable vesicles (Bodian, 1966a,b; Conradi, 1969a; McLaughlin, 1972b; Bernstein and Bernstein, 1976), prompting early speculation that vesicle release occurs across the entire synaptic interface (McLaughlin, 1972b). However, small presynaptic dense projections and local vesicle aggregations have been subsequently described (Hamos and King, 1980; Connaughton et al., 1986; Davidoff and Irintchev, 1986), and are particularly pronounced in non-osmicated tissue stained with E-PTA (Pullen, 1988a) or uranyl acetate and lead citrate (Schroder, 1979). These observations are commonly accepted evidence for *specific* synaptic vesicle release sites. Supporting this notion, C-boutons express discrete *punctae* of the active zone specific protein bassoon rather than diffuse expression throughout the presynaptic membrane (A. S. Deardorff, S. H. Romer, R. E. W. Fyffe, unpublished; see **Figure 1**). Moreover, bassoon immunoreactivity precisely overlies postsynaptic SK channels and m2 receptors even though traditional postsynaptic densities are not typically observed under EM. The physiologic advantage of this characteristically atypical and peculiarly subtle active zone architecture, however, is not yet fully understood, and may be further complicated by interspecies variability (see Pullen, 1988a).

C-boutons are ubiquitous and highly specific to somatic α-MNs and have been identified on α-MN somata and proximal dendrites in all mammalian species studied thus far (see Yamamoto et al., 1991 for references). Detailed analyses of γ-MNs (Lagerback, 1985; Lagerback et al., 1986; Destombes et al., 1992), autonomic MNs (Mawe et al., 1986; Leedy et al., 1988), spinal interneurons (Johnson and Sears, 1988), and Renshaw cells (Lagerback and Ronnevi, 1982; Alvarez and Fyffe, 2007) confirm these cells lack C-type synaptic inputs. C-boutons, when properly identified (see discussion Section "Moments and Milestones: Transmitter Content"), are thus a useful anatomical criterion to distinguish somatic α-MNs in the brain and spinal cord (Conradi, 1969a; Pullen, 1988b; Deng and Fyffe, 2004; Muennich and Fyffe, 2004; Deardorff et al., 2013). Although there have been no extensive three-dimensional analyses of the total number of C-boutons per α-MN, our, and other, observations suggest on the order of 30–70 such contacts per cell (McLaughlin, 1972b; Hamos and King, 1980; Brannstrom, 1993; Brannstrom and Kellerth, 1998), and in general, there are a greater number of C-bouton synaptic contacts on large α-MNs innervating fast twitch muscle fibers, with this difference not simply due to the larger available somatic/dendritic surface area (Conradi et al., 1979a,b; Kellerth et al., 1979, 1983; Hellstrom et al., 2003). It should be noted, the features of C-boutons present on somatic α-MNs in ocular motor nuclei vary from those in the spinal cord and other brainstem motor nuclei. Specifically, C-boutons have been ultrastructurally identified (Tredici et al., 1976)

and α-MN SSCs express connexin32 (Yamamoto et al., 1991), but no large VAChT-IR synaptic contacts (Hellstrom et al., 2003) nor m2 receptors are present (Vilaro et al., 1992; Hellstrom et al., 2003).

MOMENTS AND MILESTONES: TRANSMITTER CONTENT

Correlative light-electron microscopic analysis of ChAT-IR confirmed C-boutons are cholinergic (Houser et al., 1983; Connaughton et al., 1986; Li et al., 1995), a suggestion first made decades prior with ultrastructural acetylcholinesterase (AChE) histochemistry (Lewis and Shute, 1966), which alone is not sufficient for cholinergic classification (Fibiger, 1982; Satoh et al., 1983; Sakamoto et al., 1985; Davidoff and Irintchev, 1986; Nagy et al., 1993). In support, VAChT is highly associated with small clear synaptic vesicles in the C-bouton presynaptic terminal (Gilmor et al., 1996) and there is a strong association throughout the brainstem and spinal cord between large ChAT-IR synaptic boutons on α-MNs and SSCs immunolabeled for connexin32 (Nagy et al., 1993). Immunoreactivity for the cholinergic markers ChAT or VAChT, combined with anatomical criteria such as bouton size and location, therefore makes C-boutons easily identifiable in adult/neonatal histologic sections (**Figure 3**; Barber et al., 1984; Phelps et al., 1984; Nagy et al., 1993; Hellstrom et al., 2003; Wilson et al., 2004; Zagoraiou et al., 2009; Alvarez et al., 2011; Deardorff et al., 2013). However, this approach should be applied with caution, as a small subset of cholinergic S-type terminals arising from recurrent α-MN axon collaterals and contacting α-MN somata may approximate C-boutons in size (Cullheim et al., 1977; Lagerback et al., 1981). Definitive confirmation of C-bouton phenotype requires ultrastructural verification of the C-bouton specific "cisternal signature" or alternatively – when systematically surveying an adequate sample of α-MNs under EM is unrealistic – light level co-localization of cholinergic makers with C-bouton specific pre- and/or postsynaptic proteins (see Section

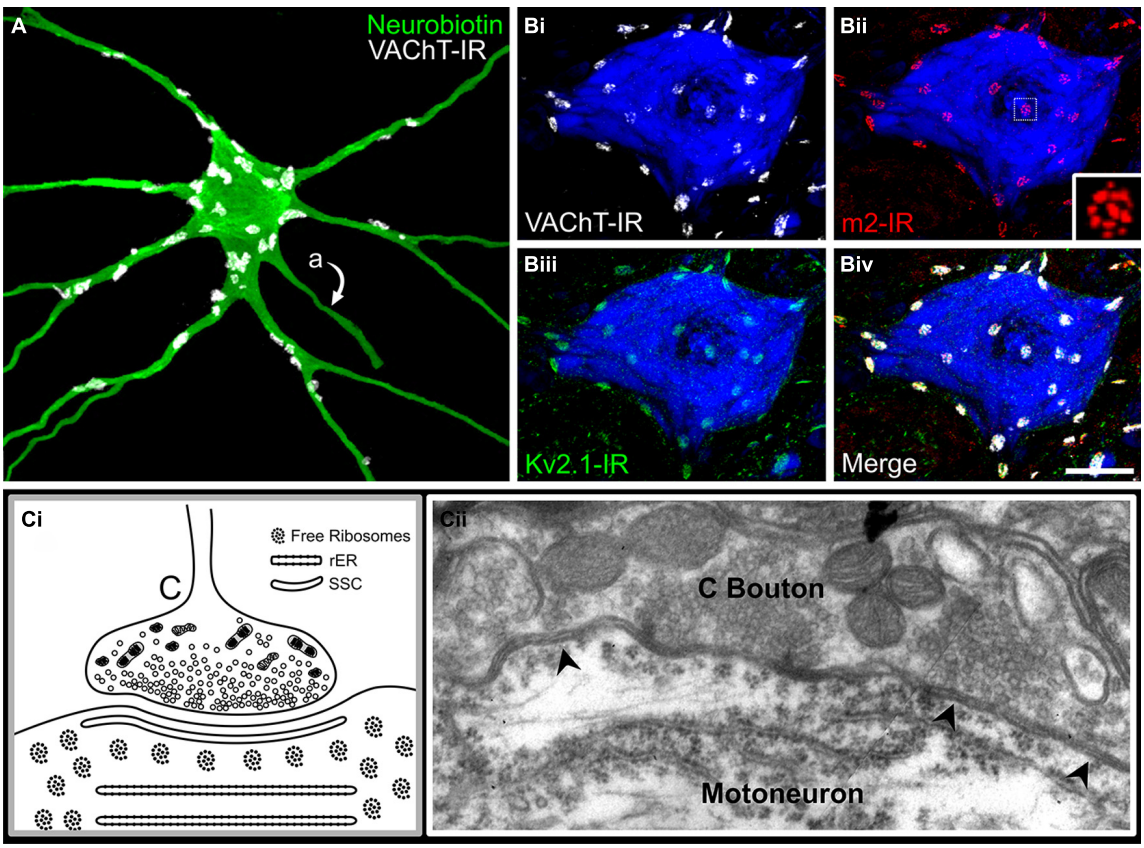

FIGURE 3 | The C-bouton synapse on mammalian α-motoneurons. (A) C-bouton synapses on intracellularly labeled and reconstructed adult rat lumbar α-MN are revealed by VAChT-IR (white). Large C-boutons densely innervate the soma and proximal dendrites of α-MNs but are absent from more distal locations. Also note that C-boutons are not located on motoneuron axons (indicated by "a"). **(B)** C-boutons, indicated by VAChT-IR (**Bi,iv**, white), are presynaptic to the muscarinic m2 receptor (**Bii,iv**, red) and large Kv2.1 clusters (**Biii,iv**, green). Note that m2 receptor immunoreactivity on the α-MN soma and proximal dendrites localize exclusively to C-bouton postsynaptic sites. **(Bii)** Inset shows subsynaptic fenestrated distribution of m2-IR. Images are confocal stacks of 12 × 1 μm Z-stacks with nissl stain (blue) to label adult rat neuronal somata. Scale bar is 20 μm. **(C)** Diagrammatic representation and electron micrograph of C-bouton ultrastructure in an adult rat. **(Ci)** Diagram illustrates densely packed, clear spherical or pleomorphic vesicles and abundant mitochondria. Closely apposed to the postsynaptic membrane is a 10–15 nm wide subsurface cistern (SSC) that is continuous with several lamellae of underlying rough endoplasmic reticulum (rER). Free ribosomal rosettes are typically visible in the subsynaptic region. **(Cii)** Electron micrograph of C-bouton synapse on an α-MN soma. Arrowheads indicate a SSC extending the entire appositional length of the bouton. Note key features present in electron micrograph illustrated in diagram **(Ci)**.

"The C-bouton Signaling Ensemble: A Contemporary View of a Classic Synapse").

MOMENTS AND MILESTONES: DISSECTING THE C-BOUTON CIRCUITRY

Unlike so many α-MN synaptic inputs, for which the neurons of origin are identifiable anatomically and physiologically (Jankowska and Lindstrom, 1972; Jankowska and Roberts, 1972a,b; Brown et al., 1981; Brown, 1983; Fyffe, 1991a,b; Burke and Glenn, 1996; Bui et al., 2003), the neuronal source of C-boutons has been elusive. Early *in vivo* lesion studies demonstrated that C-boutons do not degenerate following dorsal root section (Conradi, 1969b; McLaughlin, 1972a; Bodian, 1975); spinal cord hemisection/transection (McLaughlin, 1972c; Bodian, 1975; Pullen and Sears, 1978, 1983), or cortical ablation (Bodian, 1975). Neither are they labeled by injection of retrograde tracers into dorsal roots (Ralston and Ralston, 1979), nor intracellular staining of Ia afferents (Brown and Fyffe, 1978; Conradi et al., 1983; Fyffe and Light, 1984), Ib afferents (Brown and Fyffe, 1979), group II afferents (Fyffe, 1979), hair follicle afferents (Maxwell et al., 1982), or axons innervating cutaneous mechanoreceptors (Brown et al., 1978, 1980, 1981; Bannatyne et al., 1984; Maxwell et al., 1984). Similarly, intracellular labeling of α-MNs showed C-boutons do not arise from α-MN axon collaterals (Lagerback et al., 1981), which is corroborated by differential protein expression in C-type synapses versus cholinergic terminals in the Renshaw cell area (see Section "The C-bouton Signaling Ensemble: A Contemporary View of a Classic Synapse;" Hellstrom et al., 1999; Deng and Fyffe, 2004).

Though these data collectively indicate the intraspinal derivation of C-boutons, no investigator to date has intracellularly labeled a cholinergic spinal interneuron and traced its axon to an α-MN C-type synaptic contact *in vivo* or *in vitro*; the definitive test for synaptic connectivity. Advanced molecular labeling techniques, however, have very convincingly demonstrated that C-boutons arise from cholinergic V0-embryonic ($V0_C$) interneurons identifiable transcriptionally and phenotypically by the expression of the V0-specific homeobox protein Dbx1, the paired-like homeodomain transcription factor Pitx2, and the cholinergic proteins ChAT or VAChT (Miles et al., 2007; Zagoraiou et al., 2009). (For complete information on V0 cell ontogeny, we refer the reader to studies by Moran-Rivard et al. (2001), Pierani et al. (2001), and Lanuza et al. (2004) as well as the review by Arber (2012)). $V0_C$ interneurons correspond to a known population of cholinergic partition cells (Barber et al., 1984; Phelps et al., 1984; Arvidsson et al., 1997) located lateral to the central canal in Rexed's lamina X and medial lamina VII (Miles et al., 2007; Zagoraiou et al., 2009). They can be subdivided into ipsilateral and bilateral projecting subpopulations and span several segments rostral and caudal to their innervated motor pools (Stepien et al., 2010). Cholinergic partition cells, C-type synaptic boutons, and the "signaling ensemble" appear early in postnatal development, and are well established by approximately 1 month of age (Phelps et al., 1984; Wetts and Vaughn, 2001; Wilson et al., 2004).

The specific placement of $V0_C$ interneurons within segmental spinal circuitry is not fully characterized [see preliminary circuit diagrams in Zagoraiou et al. (2009) and Witts et al. (2014)]. Preliminary analysis of $V0_C$ connectivity demonstrates $V0_C$ interneurons receive synaptic input from several sources, including descending serotonergic pathways, local and/or descending VGluT2 projections, inhibitory interneurons (e.g., V2b cells), lamina II/III nociceptive interneurons, and non-proprioceptive primary mechanosensors (Zagoraiou et al., 2009; Witts et al., 2014; Zampieri et al., 2014; Zhang et al., 2014). Each $V0_C$ cell sends divergent axonal projections to several α-MNs of the same or functionally equivalent motor pools and avoids α-MNs innervating antagonist muscles (Stepien et al., 2010). Numerous *en passant* synaptic varicosities arising from a single $V0_C$ axon contact the soma and proximal dendrites of a one or more α-MNs, which in turn receive convergent input from several $V0_C$ cells (Stepien et al., 2010). Although the precise levels of convergence/divergence are unknown, this pattern of connectivity establishes a large number of release sites from each presynaptic axon onto the α-MN, likely reflecting a high probability of transmitter release and contributing to a high safety factor for strong cholinergic neuromodulation (e.g., Walmsley et al., 1998).

Recent work shows $V0_C$ interneurons also project numerous *small* synaptic contacts onto V1-derived IaINs (Siembab et al., 2010). These synapses are morphologically dissimilar to C-boutons (Siembab et al., 2010), and their postsynaptic effects are currently unknown. Still, it is intriguing to consider that $V0_C$ interneurons project to the only two neuronal types (α-MNS and IaINs) in the ventral horn known to receive both recurrent inhibition and group Ia excitatory drive. Whether $V0_C$ interneurons, like Renshaw cells, send parallel projections to α-MNs and the "corresponding" IaINs (i.e., those with the same Ia connections; Hultborn et al., 1971a,b,c) has yet to be elucidated. Nevertheless, these data provide further insight into segmental motor circuitry and prompt new questions into both circuit function and synaptic specificity of the $V0_C$ neuronal class.

MOMENTS AND MILESTONES: AHP, SK, AND MOTOR UNIT TYPE

Early *in vivo* use of the SK channel blocker, apamin, established that SK channels are uniquely responsible for generating α-MN AHP currents (Zhang and Krnjevic, 1987). *In vitro* investigation subsequently confirmed these findings (Viana et al., 1993; Lape and Nistri, 2000), and showed that α-MN SK currents are reduced following m2 receptor activation at C-bouton synaptic sites (Lape and Nistri, 2000; Miles et al., 2007). Consistent with these electrophysiological data, our lab has recently shown that not only are SK channels highly enriched in the C-bouton postsynaptic membrane (Deardorff et al., 2013), but individual α-MNs express a variable complement of SK2 and SK3 channel isoforms consistent with observed co-variability in α-MN size and AHP duration (Deardorff et al., 2013). In the rodent, all α-MNs express SK2, but SK3 expression is markedly heterogeneous and cell-type-specific (**Figure 4**) varying in intensity from negligible (<2× background) to modest (2 to 3× background) to strong (>3× background) between individual α-MNs in a single tissue section. SK3 channels, which have a longer deactivation time constant than SK2 (Xia et al., 1998), are *only* expressed (with SK2) at C-bouton postsynaptic sites in smaller α-MNs with longer duration/larger

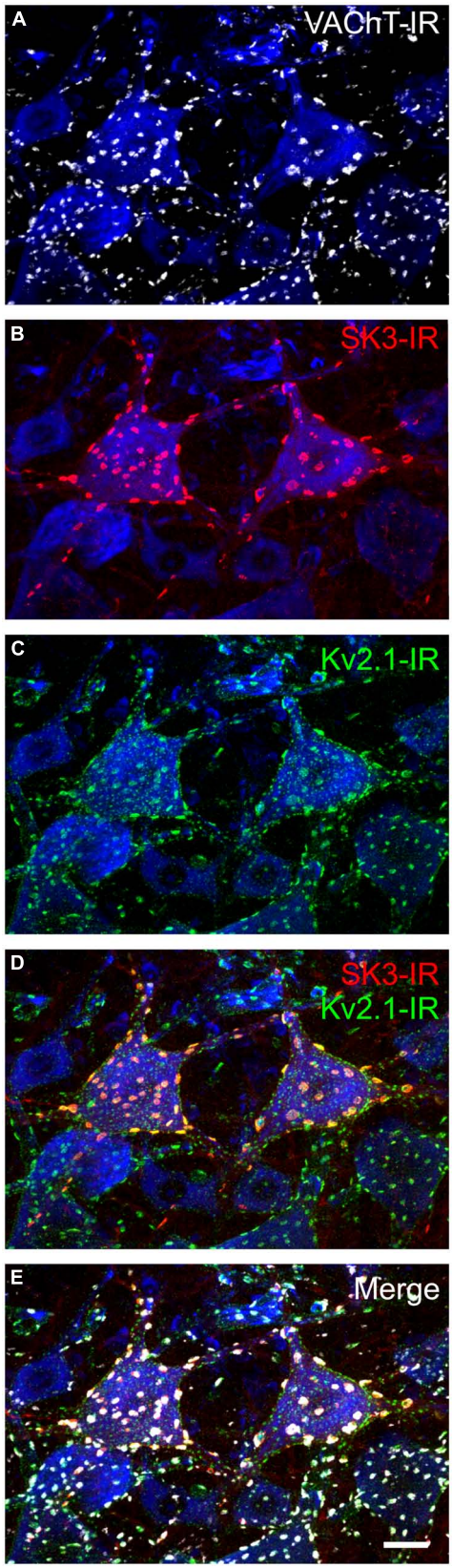

FIGURE 4 | Continued

FIGURE 4 | Continued
The potassium ion channel SK3 is part of the C-bouton signaling ensemble in a subset of α-motoneurons. Images are confocal stacks of 26 × 1 μm Z-stacks with nissl stain (blue) to label rat lumbar neuronal somata. Scale bar is 20 μm. **(A)** VAChT-IR (white) C-boutons form synapses onto all rat lumbar α-MNs on the soma and proximal dendrites. **(B)** SK3-IR (red) located within surface membrane of a subset of α-MNs in large distinct clusters. In rodents, SK3 channels, having slower intrinsic activation and deactivation kinetics than SK2 channels (Xia et al., 1998), are preferentially expressed in small, presumably S-type, α-MNs with long duration and large amplitude mAHP currents (Deardorff et al., 2013). **(C)** Large and small Kv2.1-IR (green) clusters are located within the surface membrane of all α-MNs. **(D,E)** The large SK3-IR and Kv2.1-IR clusters colocalize within the surface membrane of α-MNs and are apposed to VAChT-IR C-boutons.

amplitude AHPs (**Figure 5**). Conversely, larger α-MNs with significantly shorter duration/smaller amplitude AHPs express only SK2 (with little or no SK3-IR; **Figure 5**).

SK3-expressing α-MNs share other physiological properties predictive of S-type MNs (i.e., slower conduction velocity, lower rheobase, and higher input resistance; Deardorff et al., 2013). SK3-IR within the signaling ensemble can therefore provide "brush stroke" differentiation of rodent α-MNs along their physiological spectrum, and is a useful tool for histologic analysis of α-MN subtypes in development and disease (Brownstone and Magown, 2013). Altogether these data strongly indicate that the relative proportion of SK2/SK3 isoforms and the channel cluster size and density regulates AHP duration and amplitude, and the variability of these proportions accounts, in part, for the fact that AHP properties are continuous variables across a population of α-MNs (Deardorff et al., 2013). SK channel expression may, therefore, explain the "speed match" between AHP duration of a given α-MN and the contractile speed of its innervated muscle fibers (Bakels and Kernell, 1993; Gardiner, 1993). However, critical additional factors include the source and amplitude of the necessary Ca^{2+} signal, the coupling of these signals to the SK channels and, potentially, the presence/absence of I_h currents (Gustafsson and Pinter, 1985). Nevertheless, differential SK channel expression at the C-bouton undoubtedly contributes to α-MN input–output gain across the spectrum of α-MN subtypes by regulating AHP properties.

SWIMMING FORWARD: A MECHANISM FOR CHOLINERGIC MODULATION

We return now to the crest of our wave. The constancy of form and the intricacy of protein expression imply a fundamental logic to C-bouton organization and engagement during motor activity. Here, we assert the signaling ensemble is built around an organizing principle (i.e., the SSC) that allows for the generation of isolated Ca^{2+} signals at multiple sites on the soma. From this starting point, our intent here is to swim forward toward the synthesis of a comprehensive mechanistic hypothesis for the cholinergic modulation of α-MN firing rate. We base our rationale in the now recognized functional requirement for C-boutons in "swimming" (Zagoraiou et al., 2009), in the observation that cholinergic C-bouton function is *not required* for regular locomotion (Zagoraiou et al., 2009), and in the probable interactions of the key components of the

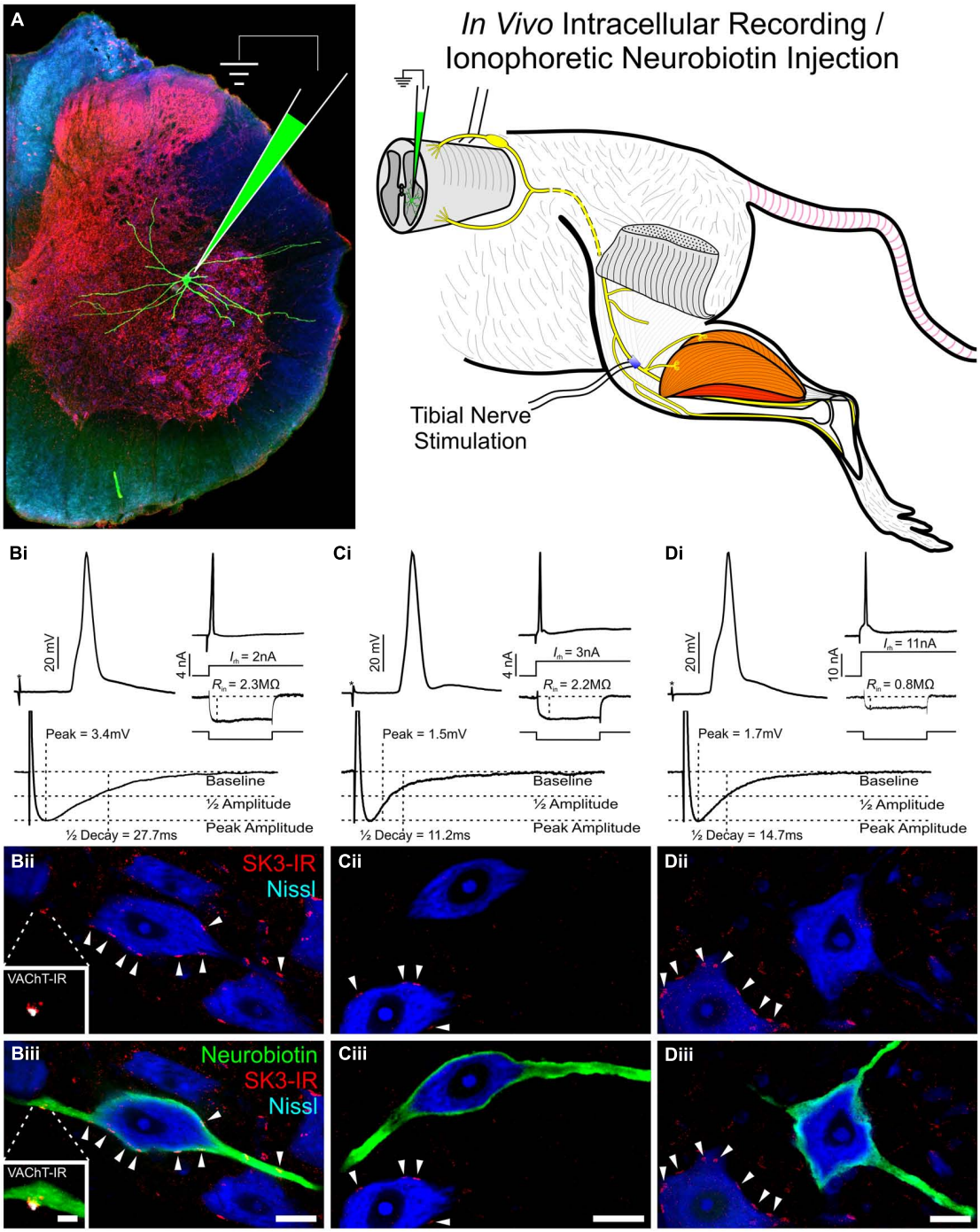

FIGURE 5 | Subset of rat lumbar α-motoneurons with SK3-IR have significantly longer AHP 1/2 decay time and increased amplitude. Data shown is review of previous study reported by Deardorff et al. (2013). **(A)** Diagrammatic representation of experimental paradigms. In an adult *in vivo* rat preparation, tibial α-MNs, identified by antidromic activation of the tibial nerve, were penetrated with a sharp recording electrode. Neuronal electrical properties were recorded and neurons were filled with neurobiotin (green) for *post hoc* identification. Spinal cord tissue was harvested and processed for SK3-IR. **(B–D)** Neuronal electrical properties are of α-MNs depicted in micrographs below. Asterisk (*) denotes stimulus artifact. Micrographs are single optical confocal sections through the soma of intracellularly labeled α-MNs (green) processed for SK3-IR (red) and the general neuronal stain nissl (Blue). Scale bars are 20 μm. **(B)** SK3-IR (+) (**Bii** and **Biii** arrowheads) α-MNs have long duration and large amplitude AHP, low rheobase, and high input resistance. Micrograph insets show VAChT-IR (White) C-bouton in apposition to an SK3-IR (+) cluster. Inset scale bar is 5 μm. **(C,D)** SK3-IR (−) α-MNs have short duration and small amplitude AHPs. However, even among these SK3-IR (−) cells, rheobase and input resistance show high variance along the continuum of α-MN properties. Please note the nearby SK3-IR (+) cells (**C,Dii,iii** arrowheads).

C-bouton signaling ensemble (**Figures 1** and **2**), most of which are known to generate, regulate, or be regulated by local intracellular Ca^{2+}.

Although C-boutons may boost recruitment gain, as proposed elsewhere (Zagoraiou et al., 2009; Brownstone and Magown, 2013), we propose that the cholinergic modulation produced by C-boutons is highly task-dependent and will be maximal only during the moderate to strong physiological drive necessary for high-output motor tasks like swimming (Zagoraiou et al., 2009; **Figure 6**). The mechanism we suggest accounts for the minimal appreciable requirements and effects observed during conditions of low and/or transient drive, which are appropriate for spinal reflexes and/or low-output tasks such as walking (Zagoraiou et al., 2009; **Figures 6Ai,Bi**). We extend this notion further to conditions of extremely powerful physiological (or pathological) drive, during which time any effects of C-bouton activity on firing rate are negated by the molecular dynamics and kinetics of the respective m2 receptors and SK/Kv channels (**Figures 6Aiv,Biv**). That is, while the cumulative, combined effects of these isolated Ca^{2+} signals on specific AHP and delayed rectifier K^+ currents are likely to be quite significant throughout the α-MN activity spectrum, the functional impact of the C-bouton circuitry is only observed when imposed upon a restricted window of moderate to strong excitatory drive. We believe our synthesis, which is primarily based on interpretation of disparate datasets, will promote testable hypotheses. Elements of this synthetic approach are considered in the following sections.

SUBSURFACE CISTERNAE AND THE GENERATION OF AN ISOLATED Ca^{2+} SIGNAL

It is widely accepted that neuronal SSCs function as an intracellular Ca^{2+} store with multiple roles in Ca^{2+} homeostasis and mobilization (see Yamamoto et al., 1991 and Fuchs et al., 2014 for references). Indeed, Henkart et al. (1976) proposed that SSCs "are designed to release Ca^{2+} into the cytoplasm with whatever further effects this might produce." SSCs serve also as a physical diffusion barrier that spatially and functionally restricts this Ca^{2+} signal from those originating in other cellular compartments and, during increased cellular activity, act as a Ca^{2+} sink to rapidly absorb and shuttle free Ca^{2+} from the cisternal microdomain (Yamamoto et al., 1990, 1991; Fuchs et al., 2014). Ca^{2+} release by RyR-rich SSCs serve, in part, to activate nearby SK channels in cochlear hair cells, which share some synaptic similarities with C-boutons (Evans et al., 2000; Lioudyno et al., 2004; Grant et al., 2006), and in sympathetic ganglion cells (Akita and Kuba, 2000). RyR release of Ca^{2+} may also result in an increase in nearby Kv2.1 channel conductances, via Ca^{2+}-dependent dephosphorylation pathways, as it does in hippocampal and cortical pyramidal cells (Du et al., 1998; Antonucci et al., 2001; Misonou et al., 2005). Moreover, vesicles observed budding from the cytoplasmic surface of SSCs in cochlear hair cells and α-MNs are thought to be involved in removal of excess free Ca^{2+} from the subsynaptic cytoplasm (Yamamoto et al., 1991; Fuchs et al., 2014). In light of these factors, the SSC itself is highly indicative that the functional regulation of the C-bouton signaling ensemble (which includes SK and Kv2.1 channels) occurs through precise control of an isolated Ca^{2+} microdomain, the mechanistic underpinnings of which are considered below.

INVOLVEMENT OF THE SIGNALING ENSEMBLE WITH THE ISOLATED Ca^{2+} SIGNAL

The unique aggregation of cellular elements at C-bouton synaptic sites and their coordinated regulation *by* and/or *of* the isolated Ca^{2+} signal enables exquisite control over α-MN K^+ currents. Consider first the generation of the α-MN AHP. Membrane bound N- and P/Q-type Ca^{2+} currents necessary for α-MN SK channel activation (see "Additional Signaling Components") generate this Ca^{2+} signal, which is isolated and shaped by the SSC. The AHP currents influence repetitive discharge properties of α-MNs, in part, via reductions in the variability in the interspike interval, the slope of the *f-I* relation, and the maximal rate of primary-range firing (Kernell, 2006; Brownstone and Magown, 2013).

A primary effect of m2 receptor activation by C-bouton synapses is a reduction of the AHP (Lape and Nistri, 2000; Miles et al., 2007). Though their signaling pathway(s) in α-MNs are undefined, m2 receptors typically exert their effects by inhibiting N-type Ca^{2+} channels, as observed in sympathetic ganglion (Hille, 1994; Herlitze et al., 1996; Shapiro et al., 1999), cortical pyramidal (Stewart et al., 1999), neostriatal (Howe and Surmeier, 1995), and basal forebrain neurons (Allen and Brown, 1993). Ca^{2+} influx through these channels is required for activation of SK channels and dictates the number of SK channels that open. N-type channel blockade by m2 receptors is usually mediated by $G_{i/o}$ protein coupled βγ subunits, which cause a depolarizing shift in the voltage dependence of channel activation (Hille, 1994; Herlitze et al., 1996; Ikeda, 1996; Jeong and Ikeda, 1999; Shapiro et al., 1999) and is negated by strong or repeated membrane depolarization (Hille, 1994).

The m2/cholinergic effect exerted by active C-boutons is quite simple and intuitive at this level: preventing N-type Ca^{2+} influx (which is largely triggered by synaptically evoked action potentials) from activating SK channels during moderate to strong physiologic drive of the MNs (**Figures 6Aii,iii,Bii,iii**). This would be consistent with *observed* reduction of the AHP and enhanced α-MN excitability when m2 receptors are, presumably, activated during swimming or other tasks requiring high motor output (e.g., **Figures 6Aii,Aiii**; Miles et al., 2007; Zagoraiou et al., 2009). This "upstream" mechanism of AHP modulation will have a minimal appreciable effect on individual AHPs and α-MN firing rate during low levels of physiologic drive causing transient or "subprimary" range firing (Manuel et al., 2009; Turkin et al., 2010), due to the *physiological* triggering of SK channel activation by a short duration, suprathreshold stimulus (i.e., an action potential) occurring at intervals that may be longer than the duration of the AHP itself (**Figures 6Ai,Bi**). This may account for observations that C-bouton function is not required for regular locomotion (Zagoraiou et al., 2009). Moreover during powerful and/or pathologic excitatory drive the m2-mediated diminution of N-type channel activity is negated (Hille, 1994), resulting in a break of the m2 generated effect and an increase in AHP size (**Figures 6Aiv,Biv**).

At high levels of excitatory drive we must also consider the results of modulation of other components of the signaling ensemble. Although the m2 mediated effect on AHP is significant in a particular physiological range, the *whole* microdomain has an important role in setting α-MN firing rate. With this in mind,

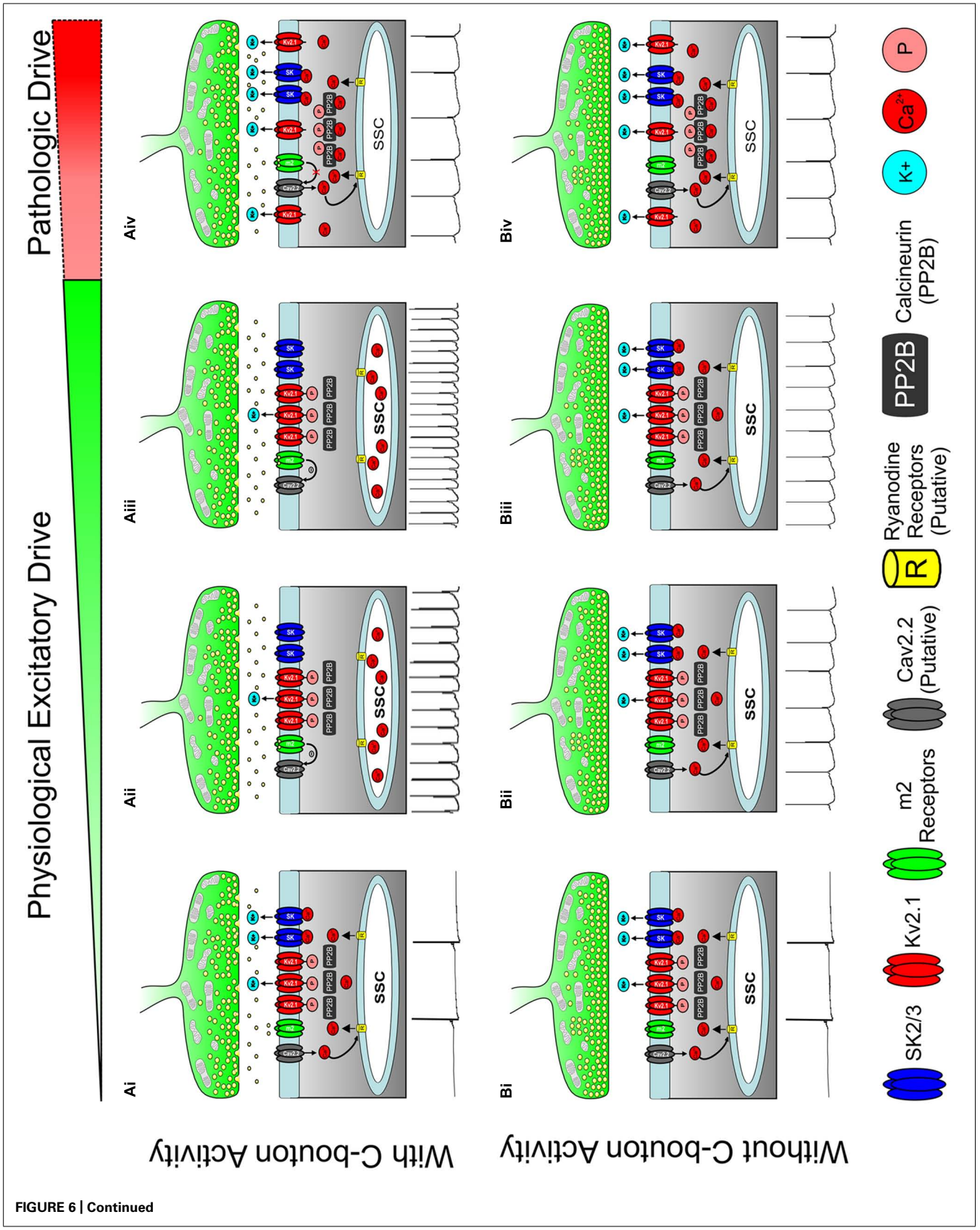

FIGURE 6 | Continued

FIGURE 6 | Continued
Hypothesis for state dependent regulation of motoneuron activity through the C-Bouton signaling ensemble. (A) C-boutons increase motoneuron firing frequency along a widow of the α-MN activity spectrum. **(Ai)** With low or transient physiological drive, m2 activation is not likely to mediate an effect on AHP duration or firing rate. **(Aii,iii)** As excitatory drive increases, persistent m2 receptor activation inhibits local Ca_V channels through a G_i/G_o coupled pathway, preventing both the SK channel activation and Kv2.1 dephosphorylation. Thus, outward K^+ current is reduced and neuronal firing rate is increased (relative to **Bii** and **Biii**) as illustrated with spike train below. **(Aiv)** m2-mediated effects on Ca_V channels are negated by prolonged or repeated membrane depolarization (Hille, 1994) as may occur during extremely strong or pathologic excitatory drive. Here, Ca^{2+} influx through N-type calcium channels activates SK channels to generate AHP and to dephosphorylate Kv2.1 to increase outward K^+ current and reduce firing frequency, as illustrated with spike train below. **(Bi–iii)** As excitatory drive increases without C-bouton activity, the N-type Ca^{2+} influx activates SK channels to generate AHP. Thus, the outward K^+ current maintains a lower firing frequency than in corresponding images in A. Spike trains illustrated below. **(Biv)** As in **(Aiv)**, during prolonged or pathologic excitatory drive, N-type Ca^{2+} influx results in both SK channel activation and Kv2.1 dephosphorylation, thereby increasing outward K^+ current and homeostatically decreasing firing rate, illustrated with spike train below. All spike trains depicted in this figure are added for illustrative purposes only and do not represent electrophysiological recordings or computer simulations.

the Ca^{2+} dependent generation of the AHP and its regulation by m2 receptor activation is one part of a coordinated series of molecular events that occur at the C-bouton, but is reliant on the complex interplay of other components in the signaling ensemble. For example, as excitatory drive increases how does the combinatorial contribution of SK and/or Kv2.1 change in the presence or absence of cholinergic input?

In the highly clustered configuration (typically) observed in hippocampal and cortical pyramidal cells, and α-MNs, Kv2.1 channels are phosphorylated and have a high activation and deactivation threshold and slow kinetics (Murakoshi et al., 1997; Misonou et al., 2004, 2005; Surmeier and Foehring, 2004; Mohapatra and Trimmer, 2006; Misonou, 2010). Interestingly, some investigators have postulated that clustered Kv2.1 channels serve primarily non-conducting functions (O'Connell et al., 2010; Fox et al., 2013); for the purposes of this discussion we will consider a more traditional role for the channels in αMNs. Importantly, upon prolonged/pathologic excitatory drive, Ca^{2+}/calcineurin dependent dephosphorylation pathways (**Figures 6Aiv,Biv**) rapidly decluster Kv2.1 while simultaneously lowering its activation and deactivation threshold and accelerating its kinetics (Surmeier and Foehring, 2004; Park et al., 2006; Mohapatra et al., 2009). In α-MNs, prolonged excitatory drive causes rapid Kv2.1 channel declustering (Romer et al., 2014) by a Ca^{2+}/calcineurin dependent mechanism (S. H. Romer, A. S. Deardorff, R. E. W. Fyffe, unpublished), though corresponding alterations in channel kinetics are uncharacterized.

Data from other cell types shows clustered Kv2 channels maintain steady state firing by regulating membrane potential during the interspike interval (Johnston et al., 2008; Guan et al., 2013; Liu and Bean, 2014), while declustered/dephosphorylated Kv2 channels serve to homeostatically lower firing rate (Surmeier and Foehring, 2004; Park et al., 2006; Mohapatra et al., 2009). In this way, Kv2 channels may increase or decrease cell excitability depending on the kinetics of channel activation (Liu and Bean, 2014). Brownstone et al. (2011) propose C-bouton activity during fictive locomotion (Miles et al., 2007; Zagoraiou et al., 2009) may contribute to steady state firing rates via the regulation of Kv2.1 phosphorylation and clustering. This is consistent with our hypothesis that m2-mediated inhibition of HVA-Ca^{2+} current prevents the activation of Ca^{2+}/calcineurin dependent dephosphorylation pathways and thus maintains Kv2.1 clustering. However, if prolonged/pathologic excitatory drive causes large changes in intracellular Ca^{2+} sufficient to allow diffusion of Ca^{2+} from neighboring compartments, there would be rapid Kv2.1 channel declustering (Romer et al., 2014) by a Ca^{2+}/calcineurin dependent mechanism (S. H. Romer, A. S. Deardorff, R. E. W. Fyffe, unpublished), negating the influence of C-boutons.

Several other components of this complex signaling ensemble likely serve to fine tune the efficacy of neuromodulation. Presynaptic nAChRs and $P2X_7$ receptors may provide an additional regulatory mechanism for synaptic transmission, particularly if ATP is co-released with ACh as it is at other central and peripheral cholinergic synapses (Burnstock et al., 1997), and cisternal S1Rs are known to reduce the sensitivity of m2 receptors to ACh (Walker and Bourguignon, 1990; Kim et al., 2010). Altogether, we suggest the C-bouton signaling ensemble is a highly integrated system, organized around an anatomically segregated Ca^{2+} microdomain, for precise and nuanced regulation of cell firing. Moreover, it has a built-in fail-safe mechanism against excitotoxicity, in that this strategically organized ensemble can both be driven by, or override, the synaptic circuitry of the C-bouton.

AN ALTERNATIVE MECHANISM

Others have suggested, based on muscarine's minimal effect on global α-MN Ca^{2+} currents, that m2 receptor activation results in the direct blockade of α-MN SK channels (Miles et al., 2007; Witts et al., 2014). In support of their view, the direct phosphorylation of SK channels by protein kinase A (PKA) and casein kinase 2 (CK2) can, respectively, cause channel internalization (Kohler et al., 1996; Ren et al., 2006; Fakler and Adelman, 2008; Faber, 2009) and reduced Ca^{2+} sensitivity (Bildl et al., 2004; Allen et al., 2007). Moreover, neurotransmitter-initiated signaling cascades have been shown to modulate SK channel gating through CK2- or protein kinase C (PKC)-mediated phosphorylation (Maingret et al., 2008; Buchanan et al., 2010; Giessel and Sabatini, 2010). Although m2 receptors typically inhibit protein kinase activity, they can activate phosphorylation pathways in smooth muscle (Zhou et al., 2003). Therefore it is possible the direct phosphorylation of SK channels by protein kinases could provide an alternate mechanism through which m2 receptors reduce the AHP in α-MNs.

However, evidence that N- and P/Q-type Ca^{2+} channels are diffusely distributed throughout the α-MN somatic membrane (Wilson et al., 2004), and that α-MN SSCs function as Ca^{2+} diffusion barriers indicates that m2 receptor activation need only inhibit those α-MN Ca_V channels located within or very near to the C-bouton postsynaptic membrane to exert an effect on the AHP. In this case, m2 influence over the signaling ensemble would be masked in studies of global Ca^{2+} currents. The activation of

CK2- or PKC-mediated phosphorylation would also be a novel finding for neuronal m2 receptors, necessitating future studies characterizing this undescribed signaling pathway. Moreover, such a mechanism would act as a binary switch, turning on and off AHP when necessary and not requiring an elaborate signaling ensemble nor the SSC. Our hypothesis, however, of a signaling ensemble organized around fine control of a Ca^{2+} micro-signaling domain is capable of highly nuanced and graded modulation of outward K^+ current.

C-BOUTONS IN HUMAN HEALTH AND DISEASE

Dynamic reorganization of C-boutons and components of the postsynaptic signaling ensemble has been noted in a variety of pathologic conditions and in conditions of altered excitability (Saxena et al., 2013; Romer et al., 2014; Witts et al., 2014). The bulk of the data has thus far been obtained in animal models, and there is no consensus on whether C-bouton plasticity in these conditions is compensatory or pathologic. In part, the uncertainty results from the diversity of disease/injury models that affect C-boutons and the complexity of the signaling ensemble.

Analysis of effects on C-bouton structure in models of amyotrophic lateral sclerosis (ALS), spinal cord injury, and peripheral nerve injury demonstrate diverse and sometimes conflicting reports. In ALS, there has been interest in potential neuroprotective roles for C-boutons and this view is bolstered by studies that show an early increase in C-bouton size (Pullen and Athanasiou, 2009; Herron and Miles, 2012; Saxena et al., 2013); however, diminished C-bouton and V0c interneuronal ChAT/VAChT content (Nagao et al., 1998; Casas et al., 2013) and S1R expression (Casas et al., 2013; see Witts et al., 2014) have also been observed in similar murine models of the disease. The structural changes in animal models may also reflect a propensity for C-bouton reorganization to occur first in larger, less excitable, and more vulnerable α-MNs (Saxena et al., 2013), and the changes may be more pronounced in males (Herron and Miles, 2012). There is minimal data from autopsied human spinal cord from ALS patients, mostly from late stages of the disease, showing continued presence of C-boutons on degeneration-resistant sphinteric α-MNs (Pullen, 1992). Additionally, the duration of the AHP in human MNs is possibly related to disease progression (i.e., an initial shortening followed by prolongation; Piotrkiewicz and Hausmanowa-Petrusewicz, 2011).

C-bouton organization is affected by both spinal cord and peripheral nerve injury, which generally appear to cause transient or persistent loss of and/or disconnection of C-boutons from α-MNs and changes in expression and localization of SK, HCN, and Kv2.1 channels (Kerns and Hinsman, 1973; Sumner, 1975; Alvarez et al., 2011; Romer et al., 2012, 2014). These specific changes may account for some, but not all, of the physiological changes that have been observed (Kuno et al., 1974a,b; Cope et al., 1986; Bichler et al., 2007a,b; Bullinger et al., 2011; Prather et al., 2011), including altered post-spike AHP duration and repetitive firing properties (Kuno et al., 1974a; Gustafsson and Pinter, 1984).

The significance of C-bouton plasticity remains uncertain. After injury, the specific loss or disconnection could lead to postsynaptic receptors (m2) becoming constitutively active, analogous to observations made of the serotoninergic system (Fouad et al., 2010; Kong et al., 2010, 2011; Murray et al., 2010, 2011; Hultborn et al., 2013), but this has not been explored. Given the high vulnerability of large, F-type α-MNS in ALS, it would be interesting to determine if the graded expression of SK channel isoforms will promote new testable hypotheses regarding disease pathogenesis and C-bouton mediated compensatory adjustments (Brownstone and Magown, 2013; Deardorff et al., 2013).

CONCLUSION

Multiple neuromodulatory systems and a myriad of ion channels are available for the task dependent regulation of MN excitability. The serotonergic system, for example, originates in the brainstem raphe nucleus, provides extensive synaptic input onto α-MN dendrites (Alvarez et al., 1998) and is strongly linked to both behavioral and pathologic alterations of persistent inward Ca^{2+} currents (Li and Bennett, 2003, 2007; Heckmann et al., 2005; Brownstone, 2006; Heckman et al., 2008; Norton et al., 2008; Powers et al., 2008). While numerous studies have focused on *inward* current modulation, the state dependent regulation of α-MN *outward* current has only recently been investigated (see Manuel et al., 2012). New evidence has shown that a cholinergic modulatory system originating from spinal interneurons ($V0_C$ interneurons), and contributing dense synaptic coverage to α-MN somata, modulates the strength of motor output via reductions in α-MN outward K^+ current (Miles et al., 2007; Zagoraiou et al., 2009). It is interesting to consider that while serotonin increases MN excitability by *amplifying inward* current, acetylcholine does so by *reducing outward* current. The dynamic interplay of these two different, but rather synergistic, systems endows the CNS with remarkable control over MN output, and the interaction between the AHP and L-type Ca^{2+} currents responsible for PIC may be a critical factor in regulating α-MN firing properties (Manuel et al., 2014).

Here, we illustrate large, cholinergic presynaptic terminals, termed C-boutons (Conradi, 1969a), are important modulatory loci for state-dependent alterations in MN repetitive firing, largely mediating their effects through a unique and highly specialized signaling ensemble organized for the state-dependent regulation of outward K^+ currents. To effectively manipulate signal transduction at C-bouton synaptic sites may be critical in the development of new therapeutic interventions for a variety of devastating neurological conditions. However, advances in patient care will first require a complete understanding of both the transduction mechanisms, as well as which cases (if any) C-bouton synaptic reorganization and/or alterations in α-MN AHP (and other intrinsic α-MN properties) contribute to disease pathology or, alternatively, maintain α-MN viability.

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REFERENCES

Akita, T., and Kuba, K. (2000). Functional triads consisting of ryanodine receptors, Ca($^{2+}$) channels, and Ca($^{2+}$)-activated K(+) channels in bullfrog sympathetic neurons. Plastic modulation of action potential. *J. Gen. Physiol.* 116, 697–720. doi: 10.1085/jgp.116.5.697

Allen, D., Fakler, B., Maylie, J., and Adelman, J. P. (2007). Organization and regulation of small conductance Ca^{2+}-activated K+ channel multiprotein complexes. *J. Neurosci.* 27, 2369–2376. doi: 10.1523/JNEUROSCI.3565-06.2007

Allen, T. G., and Brown, D. A. (1993). M2 muscarinic receptor-mediated inhibition of the Ca^{2+} current in rat magnocellular cholinergic basal forebrain neurones. *J. Physiol.* 466, 173–189.

Alvarez, F. J., Dewey, D. E., McMillin, P., and Fyffe, R. E. (1999). Distribution of cholinergic contacts on Renshaw cells in the rat spinal cord: a light microscopic study. *J. Physiol.* 515(Pt 3), 787–797. doi: 10.1111/j.1469-7793.1999.787ab.x

Alvarez, F. J., and Fyffe, R. E. (2007). The continuing case for the Renshaw cell. *J. Physiol.* 584, 31–45. doi: 10.1113/jphysiol.2007.136200

Alvarez, F. J., Pearson, J. C., Harrington, D., Dewey, D., Torbeck, L., and Fyffe, R. E. (1998). Distribution of 5-hydroxytryptamine-immunoreactive boutons on alpha-motoneurons in the lumbar spinal cord of adult cats. *J. Comp. Neurol.* 393, 69–83. doi: 10.1002/(SICI)1096-9861(19980330)393:1<69::AID-CNE7>3.0.CO;2-O

Alvarez, F. J., Titus-Mitchell, H. E., Bullinger, K. L., Kraszpulski, M., Nardelli, P., and Cope, T. C. (2011). Permanent central synaptic disconnection of proprioceptors after nerve injury and regeneration. I. Loss of VGLUT1/IA synapses on motoneurons. *J. Neurophysiol.* 106, 2450–2470. doi: 10.1152/jn.01095.2010

Antonucci, D. E., Lim, S. T., Vassanelli, S., and Trimmer, J. S. (2001). Dynamic localization and clustering of dendritic Kv2.1 voltage-dependent potassium channels in developing hippocampal neurons. *Neuroscience* 108, 69–81. doi: 10.1016/S0306-4522(01)00476-6

Arber, S. (2012). Motor circuits in action: specification, connectivity, and function. *Neuron* 74, 975–989. doi: 10.1016/j.neuron.2012.05.011

Arvidsson, U., Riedl, M., Elde, R., and Meister, B. (1997). Vesicular acetylcholine transporter (VAChT) protein: a novel and unique marker for cholinergic neurons in the central and peripheral nervous systems. *J. Comp. Neurol.* 378, 454–467. doi: 10.1002/(SICI)1096-9861(19970224)378:4<454::AID-CNE2>3.0.CO;2-1

Bakels, R., and Kernell, D. (1993). Matching between motoneurone and muscle unit properties in rat medial gastrocnemius. *J. Physiol.* 463, 307–324.

Bannatyne, B. A., Maxwell, D. J., Fyffe, R. E., and Brown, A. G. (1984). Fine structure of primary afferent axon terminals of slowly adapting cutaneous receptors in the cat. *Q. J. Exp. Physiol.* 69, 547–557.

Barber, R. P., Phelps, P. E., Houser, C. R., Crawford, G. D., Salvaterra, P. M., and Vaughn, J. E. (1984). The morphology and distribution of neurons containing choline acetyltransferase in the adult rat spinal cord: an immunocytochemical study. *J. Comp. Neurol.* 229, 329–346. doi: 10.1002/cne.902290305

Bayliss, D. A., Umemiya, M., and Berger, A. J. (1995). Inhibition of N- and P-type calcium currents and the after-hyperpolarization in rat motoneurones by serotonin. *J. Physiol.* 485(Pt 3), 635–647.

Bernstein, J. J., and Bernstein, M. E. (1976). Ventral horn synaptology in the rat. *J. Neurocytol.* 5, 109–123. doi: 10.1007/BF01176185

Bichler, E. K., Carrasco, D. I., Rich, M. M., Cope, T. C., and Pinter, M. J. (2007a). Rat motoneuron properties recover following reinnervation in the absence of muscle activity and evoked acetylcholine release. *J. Physiol.* 585, 47–56. doi: 10.1113/jphysiol.2007.135541

Bichler, E. K., Nakanishi, S. T., Wang, Q. B., Pinter, M. J., Rich, M. M., and Cope, T. C. (2007b). Enhanced transmission at a spinal synapse triggered in vivo by an injury signal independent of altered synaptic activity. *J. Neurosci.* 27, 12851–12859. doi: 10.1523/JNEUROSCI.1997-07.2007

Bildl, W., Strassmaier, T., Thurm, H., Andersen, J., Eble, S., Oliver, D., et al. (2004). Protein kinase CK2 is coassembled with small conductance $Ca^{(2+)}$-activated K+ channels and regulates channel gating. *Neuron* 43, 847–858. doi: 10.1016/j.neuron.2004.08.033

Bodian, D. (1966a). Electron microscopy: two major synaptic types on spinal motoneurons. *Science* 151, 1093–1094. doi: 10.1126/science.151.3714.1093

Bodian, D. (1966b). Synaptic types on spinal motoneurons: electron microscopic study. *Bull. Johns Hopkins Hosp.* 119, 16–46.

Bodian, D. (1975). Origin of specific synaptic types in the motoneuron neuropil of the monkey. *J. Comp. Neurol.* 159, 225–243. doi: 10.1002/cne.901590205

Brannstrom, T. (1993). Quantitative synaptology of functionally different types of cat medial gastrocnemius alpha-motoneurons. *J. Comp. Neurol.* 330, 439–454. doi: 10.1002/cne.903300311

Brannstrom, T., and Kellerth, J. O. (1998). Changes in synaptology of adult cat spinal alpha-motoneurons after axotomy. *Exp. Brain Res.* 118, 1–13. doi: 10.1007/s002210050249

Brown, A. G. (1983). *Organization of the Spinal Cord.* New York: Springer Verlag.

Brown, A. G., and Fyffe, R. E. (1978). The morphology of group Ia afferent fibre collaterals in the spinal cord of the cat. *J. Physiol.* 274, 111–127.

Brown, A. G., and Fyffe, R. E. (1979). The morphology of group Ib afferent fibre collaterals in the spinal cord of the cat. *J. Physiol.* 296, 215–226.

Brown, A. G., Fyffe, R. E., and Noble, R. (1980). Projections from Pacinian corpuscles and rapidly adapting mechanoreceptors of glabrous skin to the cat's spinal cord. *J. Physiol.* 307, 385–400.

Brown, A. G., Fyffe, R. E., Rose, P. K., and Snow, P. J. (1981). Spinal cord collaterals from axons of type II slowly adapting units in the cat. *J. Physiol.* 316, 469–480.

Brown, A. G., Rose, P. K., and Snow, P. J. (1978). Morphology and organization of axon collaterals from afferent fibres of slowly adapting type I units in cat spinal cord. *J. Physiol.* 277, 15–27.

Brownstone, R. M. (2006). Beginning at the end: repetitive firing properties in the final common pathway. *Prog. Neurobiol.* 78, 156–172. doi: 10.1016/j.pneurobio.2006.04.002

Brownstone, R. M., Krawitz, S., and Jordan, L. M. (2011). Reversal of the late phase of spike frequency adaptation in cat spinal motoneurons during fictive locomotion. *J. Neurophysiol.* 105, 1045–1050. doi: 10.1152/jn.00411.2010

Brownstone, R. M., and Magown, P. (2013). Matchmaking: SK channels, C-boutons and motor units. *J. Physiol.* 591, 747–748. doi: 10.1113/jphysiol.2012.249573

Buchanan, K. A., Petrovic, M. M., Chamberlain, S. E., Marrion, N. V., and Mellor, J. R. (2010). Facilitation of long-term potentiation by muscarinic M(1) receptors is mediated by inhibition of SK channels. *Neuron* 68, 948–963. doi: 10.1016/j.neuron.2010.11.018

Bui, T. V., Cushing, S., Dewey, D., Fyffe, R. E., and Rose, P. K. (2003). Comparison of the morphological and electrotonic properties of Renshaw cells, Ia inhibitory interneurons, and motoneurons in the cat. *J. Neurophysiol.* 90, 2900–2918. doi: 10.1152/jn.00533.2003

Bullinger, K. L., Nardelli, P., Pinter, M. J., Alvarez, F. J., and Cope, T. C. (2011). Permanent central synaptic disconnection of proprioceptors after nerve injury and regeneration. II. Loss of functional connectivity with motoneurons. *J. Neurophysiol.* 106, 2471–2485. doi: 10.1152/jn.01097.2010

Burke, R. E., and Glenn, L. L. (1996). Horseradish peroxidase study of the spatial and electrotonic distribution of group Ia synapses on type-identified ankle extensor motoneurons in the cat. *J. Comp. Neurol.* 372, 465–485. doi: 10.1002/(SICI)1096-9861(19960826)372:3<465::AID-CNE9>3.0.CO;2-0

Burnstock, G., Campbell, G., Satchell, D., and Smythe, A. (1997). Evidence that adenosine triphosphate or a related nucleotide is the transmitter substance released by non-adrenergic inhibitory nerves in the gut. 1970. *Br. J. Pharmacol.* 120, 337–357; discussion 334–336. doi: 10.1111/j.1476-5381.1997.tb06815.x

Carlin, K. P., Jiang, Z., and Brownstone, R. M. (2000). Characterization of calcium currents in functionally mature mouse spinal motoneurons. *Eur. J. Neurosci.* 12, 1624–1634. doi: 10.1046/j.1460-9568.2000.00050.x

Casas, C., Herrando-Grabulosa, M., Manzano, R., Mancuso, R., Osta, R., and Navarro, X. (2013). Early presymptomatic cholinergic dysfunction in a murine model of amyotrophic lateral sclerosis. *Brain Behav.* 3, 145–158. doi: 10.1002/brb3.104

Charlton, B. T., and Gray, E. G. (1966). Comparative electron microscopy of synapses in the vertebrate spinal cord. *J. Cell Sci.* 1, 67–80.

Clamann, H. P. (1993). Motor unit recruitment and the gradation of muscle force. *Phys. Ther.* 73, 830–843.

Connaughton, M., Priestley, J. V., Sofroniew, M. V., Eckenstein, F., and Cuello, A. C. (1986). Inputs to motoneurones in the hypoglossal nucleus of the rat: light and electron microscopic immunocytochemistry for choline acetyltransferase, substance P and enkephalins using monoclonal antibodies. *Neuroscience* 17, 205–224. doi: 10.1016/0306-4522(86)90237-X

Conradi, S. (1969a). Ultrastructure and distribution of neuronal and glial elements on the motoneuron surface in the lumbosacral spinal cord of the adult cat. *Acta Physiol. Scand. Suppl.* 332, 5–48.

Conradi, S. (1969b). Ultrastructure of dorsal root boutons on lumbosacral motoneurons of the adult cat, as revealed by dorsal root section. *Acta Physiol. Scand. Suppl.* 332, 85–115.

Conradi, S., Cullheim, S., Gollvik, L., and Kellerth, J. O. (1983). Electron microscopic observations on the synaptic contacts of group Ia muscle spindle afferents in the cat lumbosacral spinal cord. *Brain Res.* 265, 31–39. doi: 10.1016/0006-8993(83)91330-6

Conradi, S., Kellerth, J. O., and Berthold, C. H. (1979a). Electron microscopic studies of serially sectioned cat spinal alpha-motoneurons. II. A method for the description of architecture and synaptology of the cell body and proximal dendritic segments. *J. Comp. Neurol.* 184, 741–754. doi: 10.1002/cne.901840407

Conradi, S., Kellerth, J. O., Berthold, C. H., and Hammarberg, C. (1979b). Electron microscopic studies of serially sectioned cat spinal alpha-motoneurons. IV. Motoneurons innervating slow-twitch (type S) units of the soleus muscle. *J. Comp. Neurol.* 184, 769–782. doi: 10.1002/cne.901840409

Cope, T. C., Bodine, S. C., Fournier, M., and Edgerton, V. R. (1986). Soleus motor units in chronic spinal transected cats: physiological and morphological alterations. *J. Neurophysiol.* 55, 1202–1220.

Cope, T. C., and Sokoloff, A. J. (1999). Orderly recruitment tested across muscle boundaries. *Prog. Brain Res.* 123, 177–190. doi: 10.1016/S0079-6123(08)62855-1

Cullheim, S., Kellerth, J. O., and Conradi, S. (1977). Evidence for direct synaptic interconnections between cat spinal alpha-motoneurons via the recurrent axon collaterals: a morphological study using intracellular injection of horseradish peroxidase. *Brain Res.* 132, 1–10. doi: 10.1016/0006-8993(77)90702-8

Davidoff, M. S., and Irintchev, A. P. (1986). Acetylcholinesterase activity and type C synapses in the hypoglossal, facial and spinal-cord motor nuclei of rats. An electron-microscope study. *Histochemistry* 84, 515–524. doi: 10.1007/BF00482985

Deardorff, A. S., Romer, S. H., Deng, Z., Bullinger, K. L., Nardelli, P., Cope, T. C., et al. (2013). Expression of postsynaptic Ca^{2+}-activated K+ (SK) channels at C-bouton synapses in mammalian lumbar-motoneurons. *J. Physiol.* 591, 875–897. doi: 10.1113/jphysiol.2012.240879

Deng, Z., and Fyffe, R. E. (2004). Expression of P2X7 receptor immunoreactivity in distinct subsets of synaptic terminals in the ventral horn of rat lumbar spinal cord. *Brain Res.* 1020, 53–61. doi: 10.1016/j.brainres.2004.06.014

Destombes, J., Horcholle-Bossavit, G., Thiesson, D., and Jami, L. (1992). Alpha and gamma motoneurons in the peroneal nuclei of the cat spinal cord: an ultrastructural study. *J. Comp. Neurol.* 317, 79–90. doi: 10.1002/cne.903170106

Du, J., Tao-Cheng, J. H., Zerfas, P., and Mcbain, C. J. (1998). The K+ channel, Kv2.1, is apposed to astrocytic processes and is associated with inhibitory postsynaptic membranes in hippocampal and cortical principal neurons and inhibitory interneurons. *Neuroscience* 84, 37–48. doi: 10.1016/S0306-4522(97)00519-8

Evans, M. G., Lagostena, L., Darbon, P., and Mammano, F. (2000). Cholinergic control of membrane conductance and intracellular free Ca^{2+} in outer hair cells of the guinea pig cochlea. *Cell Calcium* 28, 195–203. doi: 10.1054/ceca.2000.0145

Faber, E. S. (2009). Functions and modulation of neuronal SK channels. *Cell Biochem. Biophys.* 55, 127–139. doi: 10.1007/s12013-009-9062-7

Fakler, B., and Adelman, J. P. (2008). Control of K(Ca) channels by calcium nano/microdomains. *Neuron* 59, 873–881. doi: 10.1016/j.neuron.2008.09.001

Fibiger, H. C. (1982). The organization and some projections of cholinergic neurons of the mammalian forebrain. *Brain Res.* 257, 327–388. doi: 10.1016/0165-0173(82)90011-X

Fouad, K., Rank, M. M., Vavrek, R., Murray, K. C., Sanelli, L., and Bennett, D. J. (2010). Locomotion after spinal cord injury depends on constitutive activity in serotonin receptors. *J. Neurophysiol.* 104, 2975–2984. doi: 10.1152/jn.00499.2010

Fox, P. D., Loftus, R. J., and Tamkun, M. M. (2013). Regulation of Kv2.1 K(+) conductance by cell surface channel density. *J. Neurosci.* 33, 1259–1270. doi: 10.1523/JNEUROSCI.3008-12.2013

Frank, E. (2009). A new class of spinal interneurons: the origin and function of C boutons is solved. *Neuron* 64, 593–595. doi: 10.1016/j.neuron.2009.11.030

Fuchs, P. A., Lehar, M., and Hiel, H. (2014). Ultrastructure of cisternal synapses on outer hair cells of the mouse cochlea. *J. Comp. Neurol.* 522, 717–729. doi: 10.1002/cne.23478

Fyffe, R. E. (1979). The morphology of group II muscle afferent fibre collaterals [proceedings]. *J. Physiol.* 296, 39P–40P.

Fyffe, R. E. (1991a). Glycine-like immunoreactivity in synaptic boutons of identified inhibitory interneurons in the mammalian spinal cord. *Brain Res.* 547, 175–179. doi: 10.1016/0006-8993(91)90590-R

Fyffe, R. E. (1991b). Spatial distribution of recurrent inhibitory synapses on spinal motoneurons in the cat. *J. Neurophysiol.* 65, 1134–1149.

Fyffe, R. E., and Light, A. R. (1984). The ultrastructure of group Ia afferent fiber synapses in the lumbosacral spinal cord of the cat. *Brain Res.* 300, 201–209. doi: 10.1016/0006-8993(84)90831-X

Gallart-Palau, X., Tarabal, O., Casanovas, A., Sabado, J., Correa, F. J., Hereu, M., et al. (2014). Neuregulin-1 is concentrated in the postsynaptic subsurface cistern of C-bouton inputs to alpha-motoneurons and altered during motoneuron diseases. *FASEB J.* 28, 3618–3632. doi: 10.1096/fj.13-248583

Gardiner, P. F. (1993). Physiological properties of motoneurons innervating different muscle unit types in rat gastrocnemius. *J. Neurophysiol.* 69, 1160–1170.

Giessel, A. J., and Sabatini, B. L. (2010). M1 muscarinic receptors boost synaptic potentials and calcium influx in dendritic spines by inhibiting post-synaptic SK channels. *Neuron* 68, 936–947. doi: 10.1016/j.neuron.2010.09.004

Gilmor, M. L., Nash, N. R., Roghani, A., Edwards, R. H., Yi, H., Hersch, S. M., et al. (1996). Expression of the putative vesicular acetylcholine transporter in rat brain and localization in cholinergic synaptic vesicles. *J. Neurosci.* 16, 2179–2190.

Grant, L., Slapnick, S., Kennedy, H., and Hackney, C. (2006). Ryanodine receptor localisation in the mammalian cochlea: an ultrastructural study. *Hear. Res.* 219, 101–109. doi: 10.1016/j.heares.2006.06.002

Guan, D., Armstrong, W. E., and Foehring, R. C. (2013). Kv2 channels regulate firing rate in pyramidal neurons from rat sensorimotor cortex. *J. Physiol.* 591, 4807–4825. doi: 10.1113/jphysiol.2013.257253

Gustafsson, B., and Pinter, M. J. (1984). Effects of axotomy on the distribution of passive electrical properties of cat motoneurones. *J. Physiol.* 356, 433–442.

Gustafsson, B., and Pinter, M. J. (1985). Factors determining the variation of the afterhyperpolarization duration in cat lumbar alpha-motoneurones. *Brain Res.* 326, 392–395. doi: 10.1016/0006-8993(85)90053-8

Hamos, J. E., and King, J. S. (1980). The synaptic organization of the motor nucleus of the trigeminal nerve in the opossum. *J. Comp. Neurol.* 194, 441–463. doi: 10.1002/cne.901940210

Heckman, C. J., Hyngstrom, A. S., and Johnson, M. D. (2008). Active properties of motoneurone dendrites: diffuse descending neuromodulation, focused local inhibition. *J. Physiol.* 586, 1225–1231. doi: 10.1113/jphysiol.2007.145078

Heckmann, C. J., Gorassini, M. A., and Bennett, D. J. (2005). Persistent inward currents in motoneuron dendrites: implications for motor output. *Muscle Nerve* 31, 135–156. doi: 10.1002/mus.20261

Hellstrom, J., Arvidsson, U., Elde, R., Cullheim, S., and Meister, B. (1999). Differential expression of nerve terminal protein isoforms in VAChT-containing varicosities of the spinal cord ventral horn. *J. Comp. Neurol.* 411, 578–590. doi: 10.1002/(SICI)1096-9861(19990906)411:4<578::AID-CNE4>3.0.CO;2-L

Hellstrom, J., Oliveira, A. L., Meister, B., and Cullheim, S. (2003). Large cholinergic nerve terminals on subsets of motoneurons and their relation to muscarinic receptor type 2. *J. Comp. Neurol.* 460, 476–486. doi: 10.1002/cne.10648

Henkart, M., Landis, D. M., and Reese, T. S. (1976). Similarity of junctions between plasma membranes and endoplasmic reticulum in muscle and neurons. *J. Cell Biol.* 70, 338–347. doi: 10.1083/jcb.70.2.338

Henneman, E., and Mendell, L. M. (1981). "Functional organization of the motoneuron pool and its inputs," in *Handbook of Physiology*, ed. V. E. Brooks (Bethesda, MD: American Physiological Society), 423–507.

Herlitze, S., Garcia, D. E., Mackie, K., Hille, B., Scheuer, T., and Catterall, W. A. (1996). Modulation of Ca^{2+} channels by G-protein beta gamma subunits. *Nature* 380, 258–262. doi: 10.1038/380258a0

Herron, L. R., and Miles, G. B. (2012). Gender-specific perturbations in modulatory inputs to motoneurons in a mouse model of amyotrophic lateral sclerosis. *Neuroscience* 226, 313–323. doi: 10.1016/j.neuroscience.2012.09.031

Hille, B. (1994). Modulation of ion-channel function by G-protein-coupled receptors. *Trends Neurosci.* 17, 531–536. doi: 10.1016/0166-2236(94)90157-0

Houser, C. R., Crawford, G. D., Barber, R. P., Salvaterra, P. M., and Vaughn, J. E. (1983). Organization and morphological characteristics of cholinergic neurons: an immunocytochemical study with a monoclonal antibody to choline acetyltransferase. *Brain Res.* 266, 97–119. doi: 10.1016/0006-8993(83)91312-4

Howe, A. R., and Surmeier, D. J. (1995). Muscarinic receptors modulate N-, P-, and L-type Ca^{2+} currents in rat striatal neurons through parallel pathways. *J. Neurosci.* 15, 458–469.

Hultborn, H., Jankowska, E., and Lindstrom, S. (1971a). Recurrent inhibition from motor axon collaterals of transmission in the Ia inhibitory pathway to motoneurones. *J. Physiol.* 215, 591–612.

Hultborn, H., Jankowska, E., and Lindstrom, S. (1971b). Recurrent inhibition of interneurones monosynaptically activated from group Ia afferents. *J. Physiol.* 215, 613–636.

Hultborn, H., Jankowska, E., Lindstrom, S., and Roberts, W. (1971c). Neuronal pathway of the recurrent facilitation of motoneurones. *J. Physiol.* 218, 495–514.

Hultborn, H., Zhang, M., and Meehan, C. F. (2013). Control and role of plateau potential properties in the spinal cord. *Curr. Pharm. Des.* 19, 4357–4370. doi: 10.2174/1381612811319240004

Ikeda, S. R. (1996). Voltage-dependent modulation of N-type calcium channels by G-protein beta gamma subunits. *Nature* 380, 255–258. doi: 10.1038/380255a0

Jankowska, E., and Lindstrom, S. (1972). Morphology of interneurones mediating Ia reciprocal inhibition of motoneurones in the spinal cord of the cat. *J. Physiol.* 226, 805–823.

Jankowska, E., and Roberts, W. J. (1972a). An electrophysiological demonstration of the axonal projections of single spinal interneurones in the cat. *J. Physiol.* 222, 597–622.

Jankowska, E., and Roberts, W. J. (1972b). Synaptic actions of single interneurones mediating reciprocal Ia inhibition of motoneurones. *J. Physiol.* 222, 623–642.

Jeong, S. W., and Ikeda, S. R. (1999). Sequestration of G-protein beta gamma subunits by different G-protein alpha subunits blocks voltage-dependent modulation of Ca^{2+} channels in rat sympathetic neurons. *J. Neurosci.* 19, 4755–4761.

Johnson, I. P., and Sears, T. A. (1988). Ultrastructure of interneurons within motor nuclei of the thoracic region of the spinal cord of the adult cat. *J. Anat.* 161, 171–185.

Johnston, J., Griffin, S. J., Baker, C., Skrzypiec, A., Chernova, T., and Forsythe, I. D. (2008). Initial segment Kv2.2 channels mediate a slow delayed rectifier and maintain high frequency action potential firing in medial nucleus of the trapezoid body neurons. *J. Physiol.* 586, 3493–3509. doi: 10.1113/jphysiol.2008.153734

Jones, S. L., and Stuart, G. J. (2013). Different calcium sources control somatic versus dendritic SK channel activation during action potentials. *J. Neurosci.* 33, 19396–19405. doi: 10.1523/JNEUROSCI.2073-13.2013

Kellerth, J. O., Berthold, C. H., and Conradi, S. (1979). Electron microscopic studies of serially sectioned cat spinal alpha-motoneurons. III. Motoneurons innervating fast-twitch (type FR) units of the gastrocnemius muscle. *J. Comp. Neurol.* 184, 755–767. doi: 10.1002/cne.901840408

Kellerth, J. O., Conradi, S., and Berthold, C. H. (1983). Electron microscopic studies of serially sectioned cat spinal alpha-motoneurons: V. Motoneurons innverating fast-twitch (type FF) units of the gastrocnemius muscle. *J. Comp. Neurol.* 214, 451–458. doi: 10.1002/cne.902140409

Kernell, D. (2006). *The Motoneurone and Its Muscle Fibres.* Oxford: Oxford University Press. doi: 10.1093/acprof:oso/9780198526551.001.0001

Kerns, J. M., and Hinsman, E. J. (1973). Neuroglial response to sciatic neurectomy. II. Electron microscopy. *J. Comp. Neurol.* 151, 255–280. doi: 10.1002/cne.901510304

Khan, I., Osaka, H., Stanislaus, S., Calvo, R. M., Deerinck, T., Yaksh, T. L., et al. (2003). Nicotinic acetylcholine receptor distribution in relation to spinal neurotransmission pathways. *J. Comp. Neurol.* 467, 44–59. doi: 10.1002/cne.10913

Kim, F. J., Kovalyshyn, I., Burgman, M., Neilan, C., Chien, C. C., and Pasternak, G. W. (2010). Sigma 1 receptor modulation of G-protein-coupled receptor signaling: potentiation of opioid transduction independent from receptor binding. *Mol. Pharmacol.* 77, 695–703. doi: 10.1124/mol.109.057083

Kohler, M., Hirschberg, B., Bond, C. T., Kinzie, J. M., Marrion, N. V., Maylie, J., et al. (1996). Small-conductance, calcium-activated potassium channels from mammalian brain. *Science* 273, 1709–1714. doi: 10.1126/science.273.5282.1709

Kong, X. Y., Wienecke, J., Chen, M., Hultborn, H., and Zhang, M. (2011). The time course of serotonin 2A receptor expression after spinal transection of rats: an immunohistochemical study. *Neuroscience* 177, 114–126. doi: 10.1016/j.neuroscience.2010.12.062

Kong, X. Y., Wienecke, J., Hultborn, H., and Zhang, M. (2010). Robust upregulation of serotonin 2A receptors after chronic spinal transection of rats: an immunohistochemical study. *Brain Res.* 1320, 60–68. doi: 10.1016/j.brainres.2010.01.030

Kuno, M., Miyata, Y., and Munoz-Martinez, E. J. (1974a). Differential reaction of fast and slow alpha-motoneurones to axotomy. *J. Physiol.* 240, 725–739.

Kuno, M., Miyata, Y., and Munoz-Martinez, E. J. (1974b). Properties of fast and slow alpha motoneurones following motor reinnervation. *J. Physiol.* 242, 273–288.

Ladle, D. R., Pecho-Vrieseling, E., and Arber, S. (2007). Assembly of motor circuits in the spinal cord: driven to function by genetic and experience-dependent mechanisms. *Neuron* 56, 270–283. doi: 10.1016/j.neuron.2007.09.026

Lagerback, P. A. (1985). An ultrastructural study of cat lumbosacral gamma-motoneurons after retrograde labelling with horseradish peroxidase. *J. Comp. Neurol.* 240, 256–264. doi: 10.1002/cne.902400304

Lagerback, P. A., Cullheim, S., and Ulfhake, B. (1986). Electron microscopic observations on the synaptology of cat sciatic gamma-motoneurons after intracellular staining with horseradish peroxidase. *Neurosci. Lett.* 70, 23–27. doi: 10.1016/0304-3940(86)90431-3

Lagerback, P. A., and Ronnevi, L. O. (1982). An ultrastructural study of serially sectioned Renshaw cells. II. Synaptic types. *Brain Res.* 246, 181–192. doi: 10.1016/0006-8993(82)91166-0

Lagerback, P. A., Ronnevi, L. O., Cullheim, S., and Kellerth, J. O. (1981). An ultrastructural study of the synaptic contacts of alpha-motoneurone axon collaterals. I. Contacts in lamina IX and with identified alpha-motoneurone dendrites in lamina VII. *Brain Res.* 207, 247–266. doi: 10.1016/0006-8993(81)90363-2

Lanuza, G. M., Gosgnach, S., Pierani, A., Jessell, T. M., and Goulding, M. (2004). Genetic identification of spinal interneurons that coordinate left–right locomotor activity necessary for walking movements. *Neuron* 42, 375–386. doi: 10.1016/S0896-6273(04)00249-1

Lape, R., and Nistri, A. (2000). Current and voltage clamp studies of the spike medium afterhyperpolarization of hypoglossal motoneurons in a rat brain stem slice preparation. *J. Neurophysiol.* 83, 2987–2995.

Leedy, M. G., Bresnahan, J. C., Mawe, G. M., and Beattie, M. S. (1988). Differences in synaptic inputs to preganglionic neurons in the dorsal and lateral band subdivisions of the cat sacral parasympathetic nucleus. *J. Comp. Neurol.* 268, 84–90. doi: 10.1002/cne.902680109

Lewis, P. R., and Shute, C. C. (1966). The distribution of cholinesterase in cholinergic neurons demonstrated with the electron microscope. *J. Cell Sci.* 1, 381–390.

Li, W., Ochalski, P. A., Brimijoin, S., Jordan, L. M., and Nagy, J. I. (1995). C-terminals on motoneurons: electron microscope localization of cholinergic markers in adult rats and antibody-induced depletion in neonates. *Neuroscience* 65, 879–891. doi: 10.1016/0306-4522(94)00511-3

Li, X., and Bennett, D. J. (2007). Apamin-sensitive calcium-activated potassium currents (SK) are activated by persistent calcium currents in rat motoneurons. *J. Neurophysiol.* 97, 3314–3330. doi: 10.1152/jn.01068.2006

Li, Y., and Bennett, D. J. (2003). Persistent sodium and calcium currents cause plateau potentials in motoneurons of chronic spinal rats. *J. Neurophysiol.* 90, 857–869. doi: 10.1152/jn.00236.2003

Lioudyno, M., Hiel, H., Kong, J. H., Katz, E., Waldman, E., Parameshwaran-Iyer, S., et al. (2004). A "synaptoplasmic cistern" mediates rapid inhibition of cochlear hair cells. *J. Neurosci.* 24, 11160–11164. doi: 10.1523/JNEUROSCI.3674-04.2004

Liu, P. W., and Bean, B. P. (2014). Kv2 Channel regulation of action potential repolarization and firing patterns in superior cervical ganglion neurons and hippocampal CA1 pyramidal neurons. *J. Neurosci.* 34, 4991–5002. doi: 10.1523/JNEUROSCI.1925-13.2014

Maingret, F., Coste, B., Hao, J., Giamarchi, A., Allen, D., Crest, M., et al. (2008). Neurotransmitter modulation of small-conductance Ca^{2+}-activated K+ channels by regulation of Ca^{2+} gating. *Neuron* 59, 439–449. doi: 10.1016/j.neuron.2008.05.026

Manuel, M., Iglesias, C., Donnet, M., Leroy, F., Heckman, C. J., and Zytnicki, D. (2009). Fast kinetics, high-frequency oscillations, and subprimary firing range in adult mouse spinal motoneurons. *J. Neurosci.* 29, 11246–11256. doi: 10.1523/JNEUROSCI.3260-09.2009

Manuel, M., Li, Y., Elbasiouny, S. M., Murray, K., Griener, A., Heckman, C. J., et al. (2012). NMDA induces persistent inward and outward currents that cause rhythmic bursting in adult rodent motoneurons. *J. Neurophysiol.* 108, 2991–2998. doi: 10.1152/jn.00518.2012

Manuel, M., Zytnicki, D., and Meunier, C. (2014). The dendritic location of the L-type current and its deactivation by the somatic AHP current both contribute to firing bistability in motoneurons. *Front. Comput. Neurosci.* 8:4. doi: 10.3389/fncom.2014.00004

Mavlyutov, T. A., Epstein, M. L., Andersen, K. A., Ziskind-Conhaim, L., and Ruoho, A. E. (2010). The sigma-1 receptor is enriched in postsynaptic sites of C-terminals in mouse motoneurons. An anatomical and behavioral study. *Neuroscience* 167, 247–255. doi: 10.1016/j.neuroscience.2010.02.022

Mavlyutov, T. A., Epstein, M. L., Liu, P., Verbny, Y. I., Ziskind-Conhaim, L., and Ruoho, A. E. (2012). Development of the sigma-1 receptor in C-terminals of motoneurons and colocalization with the N,N′-dimethyltryptamine forming enzyme, indole-N-methyl transferase. *Neuroscience* 206, 60–68. doi: 10.1016/j.neuroscience.2011.12.040

Mawe, G. M., Bresnahan, J. C., and Beattie, M. S. (1986). A light and electron microscopic analysis of the sacral parasympathetic nucleus after labelling primary afferent and efferent elements with HRP. *J. Comp. Neurol.* 250, 33–57. doi: 10.1002/cne.902500104

Maxwell, D. J., Bannatyne, B. A., Fyffe, R. E., and Brown, A. G. (1982). Ultrastructure of hair follicle afferent fibre terminations in the spinal cord of the cat. *J. Neurocytol.* 11, 571–582. doi: 10.1007/BF01262425

Maxwell, D. J., Bannatyne, B. A., Fyffe, R. E., and Brown, A. G. (1984). Fine structure of primary afferent axon terminals projecting from rapidly adapting mechanoreceptors of the toe and foot pads of the cat. *Q. J. Exp. Physiol.* 69, 381–392.

McLaughlin, B. J. (1972a). Dorsal root projections to the motor nuclei in the cat spinal cord. *J. Comp. Neurol.* 144, 461–474. doi: 10.1002/cne.901440405

McLaughlin, B. J. (1972b). The fine structure of neurons and synapses in the motor nuclei of the cat spinal cord. *J. Comp. Neurol.* 144, 429–460. doi: 10.1002/cne.901440404

McLaughlin, B. J. (1972c). Propriospinal and supraspinal projections to the motor nuclei in the cat spinal cord. *J. Comp. Neurol.* 144, 475–500. doi: 10.1002/cne.901440406

Miles, G. B., Hartley, R., Todd, A. J., and Brownstone, R. M. (2007). Spinal cholinergic interneurons regulate the excitability of motoneurons during locomotion. *Proc. Natl. Acad. Sci. U.S.A.* 104, 2448–2453. doi: 10.1073/pnas.0611134104

Miles, G. B., and Sillar, K. T. (2011). Neuromodulation of vertebrate locomotor control networks. *Physiology (Bethesda)* 26, 393–411. doi: 10.1152/physiol.00013.2011

Miri, A., Azim, E., and Jessell, T. M. (2013). Edging toward entelechy in motor control. *Neuron* 80, 827–834. doi: 10.1016/j.neuron.2013.10.049

Misonou, H. (2010). Homeostatic regulation of neuronal excitability by K(+) channels in normal and diseased brains. *Neuroscientist* 16, 51–64. doi: 10.1177/1073858409341085

Misonou, H., Mohapatra, D. P., Menegola, M., and Trimmer, J. S. (2005). Calcium- and metabolic state-dependent modulation of the voltage-dependent Kv2.1 channel regulates neuronal excitability in response to ischemia. *J. Neurosci.* 25, 11184–11193. doi: 10.1523/JNEUROSCI.3370-05.2005

Misonou, H., Mohapatra, D. P., Park, E. W., Leung, V., Zhen, D., Misonou, K., et al. (2004). Regulation of ion channel localization and phosphorylation by neuronal activity. *Nat. Neurosci.* 7, 711–718. doi: 10.1038/nn1260

Mohapatra, D. P., Misonou, H., Pan, S. J., Held, J. E., Surmeier, D. J., and Trimmer, J. S. (2009). Regulation of intrinsic excitability in hippocampal neurons by activity-dependent modulation of the KV2.1 potassium channel. *Channels (Austin)* 3, 46–56. doi: 10.4161/chan.3.1.7655

Mohapatra, D. P., and Trimmer, J. S. (2006). The Kv2.1 C terminus can autonomously transfer Kv2.1-like phosphorylation-dependent localization, voltage-dependent gating, and muscarinic modulation to diverse Kv channels. *J. Neurosci.* 26, 685–695. doi: 10.1523/JNEUROSCI.4620-05.2006

Monster, A. W., and Chan, H. (1977). Isometric force production by motor units of extensor digitorum communis muscle in man. *J. Neurophysiol.* 40, 1432–1443.

Moran-Rivard, L., Kagawa, T., Saueressig, H., Gross, M. K., Burrill, J., and Goulding, M. (2001). Evx1 is a postmitotic determinant of v0 interneuron identity in the spinal cord. *Neuron* 29, 385–399. doi: 10.1016/S0896-6273(01)00213-6

Muennich, E. A., and Fyffe, R. E. (2004). Focal aggregation of voltage-gated, Kv2.1 subunit-containing, potassium channels at synaptic sites in rat spinal motoneurones. *J. Physiol.* 554, 673–685. doi: 10.1113/jphysiol.2003.056192

Murakoshi, H., Shi, G., Scannevin, R. H., and Trimmer, J. S. (1997). Phosphorylation of the Kv2.1 K+ channel alters voltage-dependent activation. *Mol. Pharmacol.* 52, 821–828. doi: 10.1124/mol.52.5.821

Murray, K. C., Nakae, A., Stephens, M. J., Rank, M., D'amico, J., Harvey, P. J., et al. (2010). Recovery of motoneuron and locomotor function after spinal cord injury depends on constitutive activity in 5-HT2C receptors. *Nat. Med.* 16, 694–700. doi: 10.1038/nm.2160

Murray, K. C., Stephens, M. J., Ballou, E. W., Heckman, C. J., and Bennett, D. J. (2011). Motoneuron excitability and muscle spasms are regulated by 5-HT2B and 5-HT2C receptor activity. *J. Neurophysiol.* 105, 731–748. doi: 10.1152/jn.00774.2010

Nagao, M., Misawa, H., Kato, S., and Hirai, S. (1998). Loss of cholinergic synapses on the spinal motor neurons of amyotrophic lateral sclerosis. *J. Neuropathol. Exp. Neurol.* 57, 329–333. doi: 10.1097/00005072-199804000-00004

Nagy, J. I., Yamamoto, T., and Jordan, L. M. (1993). Evidence for the cholinergic nature of C-terminals associated with subsurface cisterns in alpha-motoneurons of rat. *Synapse* 15, 17–32. doi: 10.1002/syn.890150103

Norton, J. A., Bennett, D. J., Knash, M. E., Murray, K. C., and Gorassini, M. A. (2008). Changes in sensory-evoked synaptic activation of motoneurons after spinal cord injury in man. *Brain* 131, 1478–1491. doi: 10.1093/brain/awn050

O'Connell, K. M., Loftus, R., and Tamkun, M. M. (2010). Localization-dependent activity of the Kv2.1 delayed-rectifier K+ channel. *Proc. Natl. Acad. Sci. U.S.A.* 107, 12351–12356. doi: 10.1073/pnas.1003028107

Park, K. S., Mohapatra, D. P., Misonou, H., and Trimmer, J. S. (2006). Graded regulation of the Kv2.1 potassium channel by variable phosphorylation. *Science* 313, 976–979. doi: 10.1126/science.1124254

Phelps, P. E., Barber, R. P., Houser, C. R., Crawford, G. D., Salvaterra, P. M., and Vaughn, J. E. (1984). Postnatal development of neurons containing choline acetyltransferase in rat spinal cord: an immunocytochemical study. *J. Comp. Neurol.* 229, 347–361. doi: 10.1002/cne.902290306

Pierani, A., Moran-Rivard, L., Sunshine, M. J., Littman, D. R., Goulding, M., and Jessell, T. M. (2001). Control of interneuron fate in the developing spinal cord by the progenitor homeodomain protein Dbx1. *Neuron* 29, 367–384. doi: 10.1016/S0896-6273(01)00212-4

Piotrkiewicz, M., and Hausmanowa-Petrusewicz, I. (2011). Motoneuron after-hyperpolarisation duration in amyotrophic lateral sclerosis. *J. Physiol.* 589, 2745–2754. doi: 10.1113/jphysiol.2011.204891

Powers, R. K., Nardelli, P., and Cope, T. C. (2008). Estimation of the contribution of intrinsic currents to motoneuron firing based on paired motoneuron discharge records in the decerebrate cat. *J. Neurophysiol.* 100, 292–303. doi: 10.1152/jn.90296.2008

Prather, J. F., Nardelli, P., Nakanishi, S. T., Ross, K. T., Nichols, T. R., Pinter, M. J., et al. (2011). Recovery of proprioceptive feedback from nerve crush. *J. Physiol.* 589, 4935–4947. doi: 10.1113/jphysiol.2011.210518

Pullen, A. H. (1988a). Feline 'C'-type terminals possess synaptic sites associated with a hypolemmal cistern and Nissl body. *Neurosci. Lett.* 84, 143–148. doi: 10.1016/0304-3940(88)90398-9

Pullen, A. H. (1988b). Quantitative synaptology of feline motoneurones to external anal sphincter muscle. *J. Comp. Neurol.* 269, 414–424. doi: 10.1002/cne.902690308

Pullen, A. H. (1992). Presynaptic terminal loss from alpha-motoneurones following the retrograde axonal transport of diphtheria toxin. *Acta Neuropathol.* 83, 488–498. doi: 10.1007/BF00310025

Pullen, A. H., and Athanasiou, D. (2009). Increase in presynaptic territory of C-terminals on lumbar motoneurons of G93A SOD1 mice during disease progression. *Eur. J. Neurosci.* 29, 551–561. doi: 10.1111/j.1460-9568.2008.06602.x

Pullen, A. H., and Sears, T. A. (1978). Modification of "C" synapses following partial central deafferentation of thoracic motoneurones. *Brain Res.* 145, 141–146. doi: 10.1016/0006-8993(78)90802-8

Pullen, A. H., and Sears, T. A. (1983). Trophism between C-type axon terminals and thoracic motoneurones in the cat. *J. Physiol.* 337, 373–388.

Ralston, H. J., and Ralston, D. D. (1979). Identification of dorsal root synaptic terminals on monkey ventral horn cells by electron microscopic autoradiography. *J. Neurocytol.* 8, 151–166. doi: 10.1007/BF01175558

Ren, Y., Barnwell, L. F., Alexander, J. C., Lubin, F. D., Adelman, J. P., Pfaffinger, P. J., et al. (2006). Regulation of surface localization of the small conductance Ca^{2+}-activated potassium channel, Sk2, through direct phosphorylation by cAMP-dependent protein kinase. *J. Biol. Chem.* 281, 11769–11779. doi: 10.1074/jbc.M513125200

Romer, S., Deardorff, A., Ahmed, S., Schwieterman, R., and Fyffe, R. (2012). "Alterations in the ion channels SK3, HCN-1 and Kv2.1 in rodent lumbar alpha-motoneurons following peripheral axotomy," in *Society for Neuroscience: 42nd Annual Meeting*, New Orleans, LA.

Romer, S. H., Dominguez, K. M., Gelpi, M. W., Deardorff, A. S., Tracy, R. C., and Fyffe, R. E. (2014). Redistribution of Kv2.1 ion channels on spinal motoneurons following peripheral nerve injury. *Brain Res.* 1547, 1–15. doi: 10.1016/j.brainres.2013.12.012

Rosenbluth, J. (1962). Subsurface cisterns and their relationship to the neuronal plasma membrane. *J. Cell Biol.* 13, 405–421. doi: 10.1083/jcb.13.3.405

Sakamoto, H., Ohsato, K., and Atsumi, S. (1985). Acetylcholinesterase activity at the synapses of presynaptic boutons with presumed alpha-motoneurons in chicken ventral horn. Light- and electron-microscopic studies. *Histochemistry* 83, 291–298. doi: 10.1007/BF00684373

Satoh, K., Armstrong, D. M., and Fibiger, H. C. (1983). A comparison of the distribution of central cholinergic neurons as demonstrated by acetylcholinesterase pharmacohistochemistry and choline acetyltransferase immunohistochemistry. *Brain Res. Bull.* 11, 693–720. doi: 10.1016/0361-9230(83)90013-8

Saxena, S., Roselli, F., Singh, K., Leptien, K., Julien, J. P., Gros-Louis, F., et al. (2013). Neuroprotection through excitability and mTOR required in ALS motoneurons to delay disease and extend survival. *Neuron* 80, 80–96. doi: 10.1016/j.neuron.2013.07.027

Schroder, H. D. (1979). Paramembranous densities of 'C' terminal-motoneuron synapses in the spinal cord of the rat. *J. Neurocytol.* 8, 47–52. doi: 10.1007/BF01206457

Shapiro, M. S., Loose, M. D., Hamilton, S. E., Nathanson, N. M., Gomeza, J., Wess, J., et al. (1999). Assignment of muscarinic receptor subtypes mediating G-protein modulation of $Ca(^{2+})$ channels by using knockout mice. *Proc. Natl. Acad. Sci. U.S.A.* 96, 10899–10904. doi: 10.1073/pnas.96.19.10899

Siembab, V. C., Smith, C. A., Zagoraiou, L., Berrocal, M. C., Mentis, G. Z., and Alvarez, F. J. (2010). Target selection of proprioceptive and motor axon synapses on neonatal V1-derived Ia inhibitory interneurons and Renshaw cells. *J. Comp. Neurol.* 518, 4675–4701. doi: 10.1002/cne.22441

Skinner, H. J., Girling, K. J., Whitehurst, A., and Nathanson, M. H. (1999). Influence of metoclopramide on plasma cholinesterase and duration of action of mivacurium. *Br. J. Anaesth.* 82, 542–545. doi: 10.1093/bja/82.4.542

Stepien, A. E., Tripodi, M., and Arber, S. (2010). Monosynaptic rabies virus reveals premotor network organization and synaptic specificity of cholinergic partition cells. *Neuron* 68, 456–472. doi: 10.1016/j.neuron.2010.10.019

Stewart, A. E., Yan, Z., Surmeier, D. J., and Foehring, R. C. (1999). Muscarine modulates Ca^{2+} channel currents in rat sensorimotor pyramidal cells via two distinct pathways. *J. Neurophysiol.* 81, 72–84.

Sumner, B. E. (1975). A quantitative study of subsurface cisterns and their relationships in normal and axotomized hypoglossal neurones. *Exp. Brain Res.* 22, 175–183. doi: 10.1007/BF00237687

Surmeier, D. J., and Foehring, R. (2004). A mechanism for homeostatic plasticity. *Nat. Neurosci.* 7, 691–692. doi: 10.1038/nn0704-691

Tredici, G., Pizzini, G., and Milanesi, S. (1976). The ultrastructure of the nucleus of the oculomotor nerve (somatic efferent portion) of the cat. *Anat. Embryol. (Berl.)* 149, 323–346. doi: 10.1007/BF00315448

Turkin, V. V., O'Neill, D., Jung, R., Iarkov, A., and Hamm, T. M. (2010). Characteristics and organization of discharge properties in rat hindlimb motoneurons. *J. Neurophysiol.* 104, 1549–1565. doi: 10.1152/jn.00379.2010

Umemiya, M., and Berger, A. J. (1994). Properties and function of low- and high-voltage-activated Ca^{2+} channels in hypoglossal motoneurons. *J. Neurosci.* 14, 5652–5660.

Van Harreveld, A., and Khattab, F. I. (1967). Electron microscopy of asphyxiated spinal cords of cats. *J. Neuropathol. Exp. Neurol.* 26, 521–536. doi: 10.1097/00005072-196710000-00001

Viana, F., Bayliss, D. A., and Berger, A. J. (1993). Multiple potassium conductances and their role in action potential repolarization and repetitive firing behavior of neonatal rat hypoglossal motoneurons. *J. Neurophysiol.* 69, 2150–2163.

Vilaro, M. T., Wiederhold, K. H., Palacios, J. M., and Mengod, G. (1992). Muscarinic M2 receptor mRNA expression and receptor binding in cholinergic and non-cholinergic cells in the rat brain: a correlative study using in situ hybridization histochemistry and receptor autoradiography. *Neuroscience* 47, 367–393. doi: 10.1016/0306-4522(92)90253-X

Walker, G., and Bourguignon, L. Y. (1990). Membrane-associated 41-kDa GTP-binding protein in collagen-induced platelet activation. *FASEB J.* 4, 2925–2933.

Walmsley, B., Alvarez, F. J., and Fyffe, R. E. (1998). Diversity of structure and function at mammalian central synapses. *Trends Neurosci.* 21, 81–88. doi: 10.1016/S0166-2236(97)01170-3

Wetts, R., and Vaughn, J. E. (2001). Development of cholinergic terminals around rat spinal motor neurons and their potential relationship to developmental cell death. *J. Comp. Neurol.* 435, 171–183. doi: 10.1002/cne.1200

Wilson, J. M., Rempel, J., and Brownstone, R. M. (2004). Postnatal development of cholinergic synapses on mouse spinal motoneurons. *J. Comp. Neurol.* 474, 13–23. doi: 10.1002/cne.20089

Witts, E. C., Zagoraiou, L., and Miles, G. B. (2014). Anatomy and function of cholinergic C bouton inputs to motor neurons. *J. Anat.* 224, 52–60. doi: 10.1111/joa.12063

Wyckoff, R. W., and Young, J. Z. (1956). The motorneuron surface. *Proc. R. Soc. Lond. B Biol. Sci.* 144, 440–450. doi: 10.1098/rspb.1956.0002

Xia, X. M., Fakler, B., Rivard, A., Wayman, G., Johnson-Pais, T., Keen, J. E., et al. (1998). Mechanism of calcium gating in small-conductance calcium-activated potassium channels. *Nature* 395, 503–507. doi: 10.1038/26758

Yamamoto, T., Hertzberg, E. L., and Nagy, J. I. (1990). Epitopes of gap junctional proteins localized to neuronal subsurface cisterns. *Brain Res.* 527, 135–139. doi: 10.1016/0006-8993(90)91071-N

Yamamoto, T., Hertzberg, E. L., and Nagy, J. I. (1991). Subsurface cisterns in alpha-motoneurons of the rat and cat: immunohistochemical detection with antibodies against connexin32. *Synapse* 8, 119–136. doi: 10.1002/syn.890080206

Zagoraiou, L., Akay, T., Martin, J. F., Brownstone, R. M., Jessell, T. M., and Miles, G. B. (2009). A cluster of cholinergic premotor interneurons modulates mouse locomotor activity. *Neuron* 64, 645–662. doi: 10.1016/j.neuron.2009.10.017

Zampieri, N., Jessell, T. M., and Murray, A. J. (2014). Mapping sensory circuits by anterograde transsynaptic transfer of recombinant rabies virus. *Neuron* 81, 766–778. doi: 10.1016/j.neuron.2013.12.033

Zhang, J., Lanuza, G. M., Britz, O., Wang, Z., Siembab, V. C., Zhang, Y., et al. (2014). V1 and v2b interneurons secure the alternating flexor–extensor motor activity mice require for limbed locomotion. *Neuron* 82, 138–150. doi: 10.1016/j.neuron.2014.02.013

Zhang, L., and Krnjevic, K. (1987). Apamin depresses selectively the after-hyperpolarization of cat spinal motoneurons. *Neurosci. Lett.* 74, 58–62. doi: 10.1016/0304-3940(87)90051-6

Zhou, H., Das, S., and Murthy, K. S. (2003). Erk1/2- and p38 MAP kinase-dependent phosphorylation and activation of cPLA2 by m3 and m2 receptors. *Am. J. Physiol. Gastrointest. Liver Physiol.* 284, G472–G480.

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Cholinergic mechanisms in spinal locomotion—potential target for rehabilitation approaches

Larry M. Jordan[1]*, J. R. McVagh[1], B. R. Noga[2], A. M. Cabaj[3,4], H. Majczyński[3], Urszula Sławińska[3], J. Provencher[5], H. Leblond[5] and Serge Rossignol[5]*

[1] Department of Physiology and Pathophysiology, Spinal Cord Research Centre, University of Manitoba, Winnipeg, MB, Canada
[2] Department of Neurological Surgery, The Miami Project to Cure Paralysis, University of Miami, Miami, FL, USA
[3] Department of Neurophysiology, Nencki Institute of Experimental Biology PAS, Warsaw, Poland
[4] Department of Nerve-Muscle Engineering, Institute of Biocybernetics and Biomedical Engineering PAS, Warsaw, Poland
[5] Groupe de Recherche sur le Système Nerveux Central and Department of Neuroscience, Faculty of Medicine, Université de Montréal, Montreal, QC, Canada

Edited by:
Shawn Hochman, Emory University, USA

Reviewed by:
Sandrine S. Bertrand, Université Bordeaux 2, France
Andrea Nistri, Scuola Internazionale Superiore di Studi Avanzati, Italy
David Magnuson, University of Louisville, USA

***Correspondence:**
Larry M. Jordan, Department of Physiology, Spinal Cord Research Centre, The University of Manitoba, 745 Bannatyne ave., BMSB 425, Winnipeg, MB R3E 0W3, Canada
e-mail: larry@scrc.umanitoba.ca;
Serge Rossignol, Department of Neuroscience, Faculty of Medicine, Université de Montréal, Desmarais Pavillion, 2960 Chemin de la Tour, Montreal, QC H3T 1J4, Canada
e-mail: serge.rossignol@umontreal.ca

Previous experiments implicate cholinergic brainstem and spinal systems in the control of locomotion. Our results demonstrate that the endogenous cholinergic propriospinal system, acting *via* M_2 and M_3 muscarinic receptors, is capable of consistently producing well-coordinated locomotor activity in the *in vitro* neonatal preparation, placing it in a position to contribute to normal locomotion and to provide a basis for recovery of locomotor capability in the absence of descending pathways. Tests of these suggestions, however, reveal that the spinal cholinergic system plays little if any role in the induction of locomotion, because MLR-evoked locomotion in decerebrate cats is not prevented by cholinergic antagonists. Furthermore, it is not required for the development of stepping movements after spinal cord injury, because cholinergic agonists do not facilitate the appearance of locomotion after spinal cord injury, unlike the dramatic locomotion-promoting effects of clonidine, a noradrenergic α-2 agonist. Furthermore, cholinergic antagonists actually improve locomotor activity after spinal cord injury, suggesting that plastic changes in the spinal cholinergic system interfere with locomotion rather than facilitating it. Changes that have been observed in the cholinergic innervation of motoneurons after spinal cord injury do not decrease motoneuron excitability, as expected. Instead, the development of a "hyper-cholinergic" state after spinal cord injury appears to enhance motoneuron output and suppress locomotion. A cholinergic suppression of afferent input from the limb after spinal cord injury is also evident from our data, and this may contribute to the ability of cholinergic antagonists to improve locomotion. Not only is a role for the spinal cholinergic system in suppressing locomotion after SCI suggested by our results, but an obligatory contribution of a brainstem cholinergic relay to reticulospinal locomotor command systems is not confirmed by our experiments.

Keywords: spinal rhythm generation, cholinergic mechanisms, *in vitro* neonatal rat, decerebrate cat, chronic spinal cat, chronic spinal rat

INTRODUCTION

Acetylcholine (ACh) is thought to be a transmitter in the brainstem system for initiation of locomotion (Garcia-Rill, 1986; Jordan, 1998; Dubuc et al., 2008; Ryczko and Dubuc, 2013), and is important at the spinal level because cholinergic propriospinal cells may be involved in control of the Central Pattern Generator (CPG) for locomotion (McCance and Phillis, 1968; Huang et al., 2000; Jordan and Schmidt, 2002; Zagoraiou et al., 2009; Miles and Sillar, 2011; Tillakaratne et al., 2014). In this study we address three controversial issues: the importance of the brainstem cholinergic system in the induction of locomotion in adult animals, the capacity for the spinal cholinergic propriospinal system to provide coordinated locomotor output, and the importance of the spinal cholinergic propriospinal system in the recovery of locomotor capability in the absence of descending locomotor control.

Abbreviations: 4-DAMP, 4-diphenylacetoxy-N-methylpiperidine methiodide; 5-HT, 5-hydroxytryptamine, serotonin; ACh, acetylcholine; AChE, acetylcholine esterase; AChR, acetylcholine receptor; aCSF, artificial cerebral spinal fluid; AF, adductor femoris muscle; AHP, afterhyperpolarization; AIC, afferent input control; BB, biceps brachii muscle; b.w., body weight; CIN, commissural interneurons; CNF, nucleus cuneiformis; CNS, central nervous system; CPG, central pattern generator; EDRO, edrophonium; EMG, electromyogram; ENG, electroneurogram; GL, gastrocnemius lateralis muscle; GM, gastrocnemius medialis muscle; IaINs, Ia inhibitory interneurons; I.D., internal diameter; IP, iliopsoas muscle; i.p., intraperitoneal; i.t., intrathecal; L_2, L_3, L_4, L_5, lumbar spinal cord segments; LRC, locomotor rhythm control; M_1, M_2, M_3, M_4, muscarinic receptors; METHOC, methoctramine; MLR, Mesencephalic Locomotor Region; MNs, motoneurons; MT-3, muscarinic toxin-3; NEO, neostigmine; NMDA, *N*-Methyl-D-aspartate; O.D., outside diameter; PPN, pedunculopontine nucleus; PT, posterior tibial nerve; Pitx2, transcription factor; s.c., subcutaneously; SCI, spinal cord injury; SD, standard deviation; Sol, soleus muscle; SP, superficial peroneal nerve; Srt, sartorius muscle; St, semitendinosus muscle; T9, T10, T13, thoracic spinal cord segments; TA, tibialis anterior muscle; TB, triceps brachii muscle; VAChT, vesicular acetylcholine transporter; VL, vastus lateralis muscle; WAG rats, Wistar Albino Glaxo rats.

A role for brainstem cholinergic neurons in the production of locomotion resulting from stimulation of the mesencephalic locomotor region (MLR) in a number of species is now widely accepted (Sholomenko et al., 1991; Dubuc et al., 2008; Smetana et al., 2010; Ryczko and Dubuc, 2013), but the requirement for cholinergic involvement in mammals remains controversial (McCance et al., 1968a,b; Jordan, 1998; Takakusaki et al., 2003). The MLR was originally described (Shik et al., 1966) as coextensive with the nucleus cuneiformis (CNF), but subsequent evidence has been obtained to implicate ACh, acting at muscarinic receptors, in the production of locomotor behavior in mammals (Garcia-Rill and Skinner, 1987; Garcia-Rill et al., 1987), and it has been suggested that the major output of the MLR to the reticular formation is a cholinergic projection from the pedunculopontine nucleus (PPN) (Garcia-Rill, 1986). More recent work by Takakusaki et al. (2003, 2008) compared the effects of CNF and PPN stimulation and confirmed the CNF as effective for inducing locomotion, but the PPN stimuli induces muscle tone suppression. Garcia-Rill et al. (2011), while confirming that the PPN is involved in the control of muscle tone, attempted to attribute the effectiveness of CNF stimulation for production of locomotion to the presence of cholinergic neurons within the effective sites in the CNF. If this is the case, then cholinergic antagonists should impair MLR-evoked locomotion whether the stimulus is localized to the CNF or the PPN. We elected to determine if cholinergic antagonists could alter MLR-evoked locomotion in decerebrate cats. At the same time, we tested the notion that cholinergic propriospinal neurons contribute to the normal control of locomotion in adult animals.

The mammalian spinal cord contains several types of cholinergic neurons, including motoneurons, preganglionic autonomic neurons, partition cells (lamina VII), at least two populations of central canal neurons (lamina X) and small dorsal horn cells scattered in lamina III-V (Barber et al., 1984; Houser et al., 1984; Phelps et al., 1984; Borges and Iversen, 1986). These cells are likely contributors to the effects of ACh in the spinal cord to the control of afferent input, including analgesia (Eisenach, 1999; Wess et al., 2003; Umana et al., 2013). Cholinergic cells in laminae VII and X are implicated in the control of locomotion (Huang et al., 2000; Tillakaratne et al., 2014). Cholinergic excitation of neurons involved in the control of locomotion has been demonstrated (Dai et al., 2009; Dai and Jordan, 2010), and activity of cholinergic propriospinal cells during locomotion has been documented (Webster and Jones, 1988; Carr et al., 1994, 1995; Huang et al., 2000). Activity of premotoneuron cholinergic Pitx2+ V0c neurons during locomotor episodes *in vitro* has been demonstrated (Zagoraiou et al., 2009).

There is considerable evidence that ACh can induce rhythmic activity in isolated spinal cord preparations, but usually the rhythm is not locomotor-like: although there is a left-right alternation, there is a synchronous activity in flexor and extensor motoneurons on each side. However, there are examples in the literature of well-coordinated locomotor-like activity produced by cholinesterase inhibitors, which increase release of ACh from intrinsic cholinergic neurons (Smith and Feldman, 1987; Smith et al., 1988a; Cowley and Schmidt, 1994; Kiehn et al., 1996; Anglister et al., 2008). In this study we showed that enhancing the efficacy of the propriospinal cholinergic system in neonatal animals is sufficient for production of consistent well-coordinated locomotor activity *in vitro*, and we determine the muscarinic receptors involved in this effect. These findings formed the basis for further investigation of the importance of spinal cholinergic neurons in the production of locomotion in adult animals and in animals with SCI.

Considerable attention has recently been drawn to the potential for the intraspinal cholinergic propriospinal system to influence functional recovery after loss of the descending command pathways that normally trigger locomotion. Recovery of hindlimb stepping has been related to sprouting of cholinergic fibers after SCI (Jakeman et al., 1998). It has been demonstrated that spinal motoneurons receive cholinergic C-terminals that excite motoneurons by reducing the AHP, and the cholinergic neurons that are the source of these terminals have been identified (McCance and Phillis, 1968; Zagoraiou et al., 2009; Miles and Sillar, 2011; Witts et al., 2014). Cholinergic commissural cells have also been implicated in the control of locomotion and suggested as a basis for cholinergic influences after SCI (Smith et al., 1988a,b; Martin et al., 1989; Huang et al., 2000). Changes in cholinergic propriospinal cells and inputs to motoneurons after SCI have been documented (Kapitza et al., 2012; Skup et al., 2012; Witts et al., 2014), and reduced numbers of cholinergic C-terminals onto motoneurons has been suggested as a basis for rapid exhaustion of the central drive required for the performance of locomotor movements in patients with SCI (Kapitza et al., 2012). Changes in the cholinergic control of sensory inputs would also be expected if atrophy or other alterations in cholinergic neurons involved in this process occur after SCI. Given this background of plasticity in the cholinergic propriospinal system, and its putative involvement in locomotor activity after injury, we tested whether this system is recruited during locomotor recovery in adult animals with SCI, and determined the consequences of plasticity in this system after SCI.

METHODS

Experimental procedures (neonatal rats and decerebrate cats) were approved by the University of Manitoba Animal Care Committee and conform to the standards of the Canadian Council of Animal Care. The experiments on spinal rats were carried out with the approval of the First Ethics Committee for Animal Experimentation in Poland, according to the European Union and the Polish Law on Animal Protection.

IN VITRO NEONATAL RAT PREPARATIONS

Dissection

Experiments were performed on 102 Sprague-Dawley rats aged 1–4 days. Following induction of anesthesia with Halothane®, animals were quickly decerebrated and eviscerated then transferred to a 50 ml chamber lined with Sylgard® containing artificial cerebral spinal fluid (aCSF): 128 mM NaCl, 3 mM KCl, 1 μM $MgSO_4$, 21 mM $NaHCO_3$, 1.5 mM $CaCl_2.2HO_3$, 0.5 mM $NaH_2PO_4.2H_2O$, and 30 mM glucose, oxygenated with 95% 0_2/5% CO_2. The entire spinal cord was exposed by removing the vertebral bodies, then the isolated spinal cord was placed in

the bath with the hindlimbs attached ($n = 65$). In some experiments ($n = 37$) the hindlimbs were removed leaving only the isolated spinal cord, to determine whether the feedback from the attached moving limb influenced the results. In these cases, a 10 ml chamber was used. Dorsal and ventral roots were cut from cervical C1 to thoracic T12 and the surrounding tissue removed. The dorsal/ventral roots in the lumbosacral region remained intact in the hindlimb-attached preparations. When present, the pelvis was stabilized with insect pins inserted into the underlying Sylgard® to prevent movement from occurring during application of neurochemicals. Bath temperature was maintained between 5° and 19°C during surgery, while recordings were obtained at room temperature (20°–25°C). The results obtained using hindlimb—attached and isolated spinal cord preparations were indistinguishable, and the data were pooled.

Recordings

Electroneurograms (ENGs) were obtained using plastic suction electrodes filled with aCSF attached to lumbar roots L2 and L5 bilaterally, whether the hindlimbs were attached or not. Each ventral root recording was band pass-filtered (30 Hz to 3 kHz) and stored on a PC for subsequent analysis using software developed within our group. Details can be found at www.scrc.umanitoba.ca/doc/. Briefly, raw waveform recordings from the ventral roots were filtered at 1 Hz then rectified. Sections of the rectified waveforms containing sustained locomotor activity were analyzed in order to determine step cycle duration (onset of burst of activity in one root to the onset of the next burst in the same root) and frequency.

Circular statistics, as described previously (Kriellaars et al., 1994; Kjaerulff and Kiehn, 1996; Cowley et al., 2005; Liu and Jordan, 2005), were used to establish relationships between ventral root recordings. Briefly, we determined the relationships among locomotor-like bursts of activity recorded from pairs of ventral roots in order to compare the mean phase values of homolateral ventral roots to evaluate flexor (L2) vs. extensor (L5) coordination, as well as bilateral ventral roots, to examine right/left coordination. The angle (θ) of the mean vector represents the relationship between the reference onset and another waveform. The vector points to the direction of the maximum concentration of data points. The *r*-value, the correlation coefficient, represents the concentration of data around that vector. It is widely accepted that L2 ventral root activity consists largely of flexor motoneuron activity and L5 ventral root activity corresponds to extensor motoneuron activity (Kjaerulff and Kiehn, 1996). Cycles were selected for analysis when a sustained pattern of rhythmic activity was established.

Induction of rhythmic activity

Pharmacological substances were added by micropipette directly to the bath solution. The aCSF was stirred by the air mixture bubbling in the chamber. The AChE inhibitor edrophonium (EDRO), mean dose 50 μM, (25–100 μM, 10 mM stock solution) was added directly to the chamber to induce rhythmic activity. A second AChE inhibitor, neostigmine (NEO) in concentration 25 μM, was also used to confirm the effects of EDRO. Antagonists were added to the bath only when a sustained pattern of ventral root activity compatible with hindlimb stepping was produced.

Antagonists

Both nicotinic and muscarinic antagonists were examined in 46 preparations with well-coordinated locomotion induced by EDRO in order to determine the cholinergic receptors involved. The antagonists chosen were tubocurarine, a nicotinic antagonist (1–15 μM, $n = 8$), and the muscarinic antagonists atropine (M_{1234} antagonist, 100 nM, $n = 5$), telenzepine (M_1 antagonist, 500 nM–20 μM, $n = 12$), methoctramine (METHOC; M_2 antagonist, 1–50 μM, $n = 14$), 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) (M_3 antagonist, 3 nM–1 μM, $n = 12$), and muscarinic toxin-3 (MT-3) (M_4 antagonist, 5–300 nM, $n = 3$). Statistical analysis of the effects of antagonists was carried out using Student's *t*-Test for Paired Samples. All drugs were obtained from Sigma.

DECEREBRATE CAT PREPARATIONS

Fifteen adult cats were initially anesthetized with a mixture of nitrous oxide and Halothane® and then intubated. The left common carotid was cannulated and blood pressure monitored. A cannula was inserted into the femoral vein for the delivery of fluids and drugs. Animals were given intravenous injections of dexamethasone (4 mg, Hexadrol phosphate, Organon) to reduce brainstem swelling. The head of each animal was fixed in a stereotaxic frame with all four limbs free to step on the treadmill. The hindquarters were suspended by a sling under the belly of the cat. The animals were decerebrated at the precollicular-postmammillary level and the anesthesia discontinued. Following a recovery period of at least 1 h, stepping on the moving treadmill was induced by electrical stimulation (square wave pulse 0.5 ms duration, 10–30 Hz, 50–200 μA) applied in the MLR (Shik et al., 1966) with monopolar steel electrodes. The electrical threshold for the initiation of hindlimb locomotion was determined by slowly increasing the strength of stimulation until locomotion ensued. Changes in the threshold for electrically induced locomotion were reported relative to the normal variability in threshold measurements observed with repeated control trials.

Locomotion of the hindlimbs was normally monitored using bilateral intramuscular electrodes for electromyographic (EMG) recordings similar to those described by English (1984) or English and Weeks (1989). Teflon-coated stainless-steel wires with exposed wire tip of 1–2 mm were implanted in various combinations of the following muscles: GL, TA, St and VL muscles. In three experiments, bilateral forelimb locomotion was also monitored with similar electrodes implanted within BB and TB muscles. The EMGs were amplified and captured using custom software.

Locomotion at four different treadmill speeds (0.3, 0.4, 0.6, 0.8 m/s) was assessed prior to and after separate intravenous doses of the muscarinic antagonist atropine (1.2–2.4 mg/kg) and/or the classical ganglionic nicotinic antagonist mecamylamine hydrochloride (3–9 mg/kg). Mecamylamine was given alone in 8 cats. The remaining 7 animals received both mecamylamine and atropine (atropine administered first in 4 animals; mecamylamine administered first in the remaining 3 animals).

Mecamylamine was administered by small injections (1–3 doses of 1.0 ml) of 1.0–4.5 mg/kg/ml (injection time of <1 min). The injections of mecamylamine were given over a period of up to 50 min (Noga et al., 1987) to limit the fall in blood pressure which may be seen after its administration. Atropine was also infused gradually (over a 2–3 min period) to limit the transient drop in blood pressure which could also occur with its administration. The choice of drugs and the range of doses used was based upon the following information: (a) Renshaw cell phasic activity associated with fictive locomotion is blocked by equal or lower doses of mecamylamine (Noga et al., 1987); (b) Renshaw cell activation produced by stretch of the Achilles tendon is blocked by equal or lower doses of atropine (Ryall and Haas, 1975); (c) responses of cells recorded from a variety of locations within the brain to either iontophoretic application of acetylcholine or to electrical stimulation of afferent pathways is blocked by intravenous application of atropine at quantities similar to or less than that given in the present study (Crawford and Curtis, 1966; McCance et al., 1968b; Lake, 1973). It is clear from radiolabeled tracer studies that atropine readily passes through the blood-brain barrier (Proakis and Harris, 1978).

The following parameters were measured: step cycle length, EMG burst duration, and the number of EMG bursts per muscle per step cycle. A linear envelope signal was first obtained by passing each rectified EMG signal through an 8 or 16 Hz low pass filter. All integrated EMG channels were digitized simultaneously at 200 Hz per channel. Intralimb coordination was determined by examining the temporal relationship between EMG activity of the flexor and extensor muscles from the same limb. Interlimb coordination was qualitatively assessed (for presence of walk, trot, or gallop) and only differences following the administration of the antagonists compared to the pre-drug controls for each treadmill speed are reported. The onset for each step cycle was determined from the EMG channel with the sharpest burst onsets and most consistent activity throughout the experiment. All step cycles in each channel were then normalized, the EMG activity averaged and the standard deviations computed. The area of the EMG signal (mean and standard deviation) between the onset and offset of each burst (relative to the baseline) was calculated as a quantitative measure of muscle activity during each burst in the step cycle. The average step cycle duration and burst duration (as a percentage of the step cycle) was computed for each trial.

SPINAL CAT PREPARATIONS

Chronic experiments were performed in 2 spinalized cats for a period of 90 days with several injections of drugs per week. Acute experiments ($n = 9$) were performed in unanesthetized decerebrate cats a few hours after a complete spinalization ($n = 4$) or around 1 week after spinalization ($n = 5$). All procedures were conducted according to the Guide for the Care and Use of Experimental Animals (Canada), using protocols approved by the Ethics Committee of Université de Montréal.

Implantation of chronic EMG electrodes

All surgical procedures were performed in aseptic conditions and under general anesthesia. The cats received buprenorphine (0.01 mg/kg s.c.) 1 h before surgery. They were pre-medicated with ketamine (10 mg/kg), acepromazine maleate (AtravetTM, 0.1 mg/kg), and glycopyrrolate (0.01 mg/kg) injected intramuscularly and anesthetized by isoflurane 1–3% in 95% O_2 maintained through an endotracheal tube. Lactate Ringer solution was administrated during the surgery through an intravenous catheter in the cephalic vein. The body temperature and heart rate were monitored during the whole surgery. The animal was secured in a stereotaxic frame. The EMG electrode implantation was as in Belanger et al. (1996) and Bouyer et al. (2001). The implanted muscles were the following: Srt, St, VL, GL, and TA. The intrathecal implantation was as in Chau et al. (1998). A fentanyl (2.5 mg, 25 μg/h) transdermal patch was fixed to the skin for 10 days after surgery. The postoperative care was as in previous publications (Belanger et al., 1996; Chau et al., 1998).

Recording and analysis procedures

The EMG signal was differentially amplified (bandwidth 100 Hz to 3 kHz) and digitized at 2 kHz. The EMGs were synchronized to the video images of the hindlimbs using a digital time code, recorded on the computer and on the video. Reflective markers were placed on the skin overlying the iliac crest, the femoral head, the knee joint, the lateral malleolus, the metatarsophalangeal joint and the tip of the fourth toe. Video images of the locomotor movement (left side) were captured by a digital camera and recorded on a videocassette recorder at 30 frames/s.

The bursts of activity of all EMGs were rectified and integrated to quantify their amplitude. Their onset and offset were identified automatically by homemade software and corrected manually by the experimenter in order to determine temporal parameters of each cycle. The video images were digitized and the x-y coordinates of different joint markers were obtained at the frequency of 60 fields/s by de-interlacing each video frame. These coordinates were used to calculate angular joint movements and could be displayed as continuous angular displacements or stick diagrams of one step cycle (see for instance **Figure 6**). The step length was calculated as the distance between two consecutive contacts of the same foot. The time interval between the onset and the offset of each muscle was measured and synchronized to the onset of the St EMG activity.

Spinalization and drug administration

Spinal cord transection was performed in aseptic conditions and under general anesthesia following a protocol established in Belanger et al. (1996) and Bouyer et al. (2001). Drug administration was made either *via* a cannula inserted at T13 with the tip at L4 (Chau et al., 1998) or in a bath over L3–L4 (Marcoux and Rossignol, 2000). For intrathecal injections, concentrations of stock solutions of drugs (atropine, 4-DAMP, dihydro-β-erythroidine, EDRO) were adjusted so that the desired total dose could be injected in a single 100 μl bolus unless otherwise indicated. The procedure was first to empty the cannula which had a dead space of 100 μl and then introduce the 100 μl of the drug and fill in the dead space again with saline.

Cutaneous reflex testing

In acute and 1 week spinal cats, cutaneous reflexes were tested by stimulating cutaneous nerves through cuff electrodes inserted

around the Superficial Peroneal (SP) and Posterior Tibial (PT) nerves on both sides and recording EMG responses through percutaneously inserted electrodes in St, TA, GL, and Srt muscles. Reflex responses were evaluated starting 5–10 min up to 2 h after the injection of drugs. The stimulating cuffs were stable and allowed tests of threshold several times during the experiment.

SPINAL RAT PREPARATION

Spinal cord transection

The experiments were performed on 3-month-old WAG female rats in which, under Isofluorane anesthesia (2% and Butomidor, 0.05 mg/kg b.w.) and sterile conditions the spinal cord was completely transected at a low thoracic level (T9/10). To prevent the possibility of axonal regrowth through the cavity of the lesion, 2–3 mm of spinal cord tissue was aspirated using a glass pipette as described previously (Sławińska et al., 2012). Then the muscles and fascia overlying the paravertebral muscles were closed in layers using sterile sutures, and the skin was closed with stainless-steel surgical clips. After surgery, the animals received a non-steroidal anti-inflammatory and analgesic treatment (Tolfedine 4 mg/kg). During the following 7–10 days, the animals received antibiotics (Gentamicin 2 mg/kg and Baytril 5 mg/kg) and the bladder was emptied manually twice a day until the voiding reflex was re-established.

Intrathecal cannula implantation

An intrathecal cannula was implanted such that the tip was targeted to the level of L3/L4 root entrance to the spinal cord. The procedure for intrathecal cannula implantation was similar to that used previously (Majczyński et al., 2005, 2006), except in this case the cannula was inserted from the caudal end of the vertebral canal. The implantation was performed in aseptic conditions under equithesin anesthesia (i.p. 0.35 ml/100 g b.w.). A silastic cannula (0.012 in I.D. × 0.025 in O.D.) was inserted into the subarachnoid space through a small opening made at the L3 vertebral level and was pushed rostrally. The cannula was fixed by sewing it to the L4 spinous process and stabilized by suturing the overlying back muscles in place. The other end of the cannula was guided under the skin to reach the skull and connected to a custom-made adaptor cemented to the bone. After surgery, the animals received a non-steroidal anti-inflammatory and analgesic treatment and antibiotics. Two to five days after surgery the patency and correct placement of the cannula was verified by injection of 15 μl of 2% lidocaine followed by 15 μl of saline (0.9%). Drugs were injected as a bolus of 20 μl of drug diluted with sterile saline and followed by a bolus of about 15 μl of sterile saline to wash the drug from the cannula. Drug injections were separated by an interval of at least 72 h. A similar volume of sterile 0.9% saline was used as a control injection.

Implantation of EMG recording electrodes

Ten weeks after spinal cord transection, bipolar EMG recording electrodes were implanted in the Sol and TA muscles of both hindlimbs as previously described (Sławińska et al., 2000, 2012, 2013). The electrodes were made of Teflon-coated stainless-steel wire (0.24 mm in diameter; AS633, Cooner Wire, Chastworth). The tips of the electrodes with 1–1.5 mm of the insulation removed were pulled through a cutaneous incision at the back of animal, and each of the hook electrodes was inserted into the appropriate muscle, where it was secured by a suture. The distance between the tips of electrodes was approximately 1–2 mm. The ground electrode was placed under the skin on the back of the animal at some distance from the hindlimb muscles. The connector with the wires fixed to it and covered with dental cement and silicone was secured to the back of the animal.

Locomotor function investigation

The hindlimb locomotor movements of spinal rats were investigated on a treadmill where the animals were kept with their forelimbs on a platform above the treadmill and their hindlimbs were touching the moving belt. Stimulation of the tail was used to elicit locomotor-like hindlimb movements and then pharmacological agents (carbachol, and 10–15 min later atropine) were applied intrathecally (both 20 μl, 1 mM). Simultaneously with EMG recordings a digital video camera was used to record the hindlimb movements. The animals were tested before and at various times after the drug application. EMG recordings were used to determine the quality of any locomotor activity induced by the tail pinching as described before (Sławińska et al., 2012, 2014).

RESULTS

IN VITRO EXPERIMENTS IN NEONATAL RATS

The intrinsic cholinergic propriospinal system is a potential substrate for locomotor recovery after SCI (Jordan and Schmidt, 2002), because other transmitter systems capable of inducing locomotion (e.g., noradrenergic, serotonergic, and dopaminergic) arise from brain cells forming descending pathways (Rossignol et al., 2002), leaving only intrinsic spinal circuitry to account for any recovery in cases of complete SCI such as used in the animal models described here. Thus, there is an obvious need to understand in detail the patterns of rhythmic activity which the endogenous cholinergic propriospinal system can produce, and to determine the receptor(s) involved. Further, although atropine blocks the rhythmic motor activity and excitation of motoneurons produced by cholinergic agonists, there is currently no information about the muscarinic receptor subtypes responsible for the cholinergic induction of rhythmic activity in the neonatal rat. Here we show that enhancement of the effects of the endogenous cholinergic propriospinal system with an acetylcholine esterase (AChE) inhibitor can give rise to well-coordinated locomotion, including alternation between ipsilateral flexor and extensor motoneuron groups, in contrast to the synchronous flexor/extensor activity commonly induced by application of exogenous cholinergic agonists (Cowley and Schmidt, 1994). We also provide evidence that M_2 and M_3 muscarinic receptors are involved in the cholinergic control of locomotor activity.

After baseline recordings of 30 s duration, EDRO (25–100 μM) was applied to the bath. Episodes of rhythmic activity (**Figures 1A,B**), with superimposed slower rhythms, were produced in 88% (64/73) of experiments after a period of 5–370 s (mean ± *SD*; 69.9 ± 75.5 s). In the remaining nine

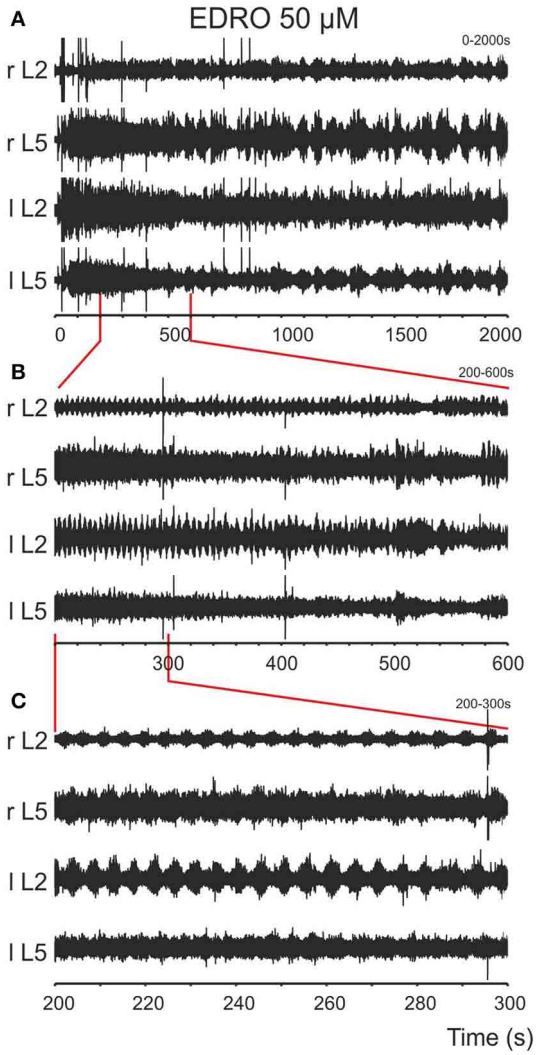

FIGURE 1 | Episodes of rhythmic activity are induced after bath application of EDRO. (A) EDRO applied to the bath 30 s after the onset of the recording induced episodes of rhythmic activity with waxing and waning amplitude of the signal in all channels for several minutes. **(B)** Expanded sample from the same recording showing episodes of rhythmic activity (the area indicated by vertical lines) separated by periods of decreased activity. **(C)** Expanded sample of the recordings in **(B)** (the area indicated by vertical lines) showing well-coordinated locomotion characterized by alternating flexor/extensor (L2/L5) and right/left ENG activity (r/l).

experiments, the activity was either tonic or was observed in one or two ventral roots only. In 44 of the 64 experiments in which EDRO produced rhythmic activity (69%), the activity consisted of sustained patterns of ventral root activity characterized by right/left and ipsilateral flexor/extensor alternation typical of locomotion (**Figures 1C**, **2**). The mean number of episodes of locomotion was 6.5 per 30 min recording period. The mean duration of each episode of activity was 110.4 ± 56.1 s (range 50–184 s). The mean rhythm frequency was 0.21 ± 0.05 Hz (0.13–0.33 Hz), and the mean step duration was 4.90 ± 1.31 s (range 1.8–7.8 s).

Besides locomotion, episodes of ipsilateral flexor/extensor and right/left synchrony, episodes of ipsilateral flexor/extensor synchrony but alternating right/left sides, episodes of right/left synchrony and ipsilateral flexor/extensor alternation, or a combination of these patterns were observed, often within the same experiment. No relation was observed between the concentration of EDRO applied and the type of pattern produced in these experiments.

A second AChE inhibitor, NEO (25 µM), was also effective in inducing locomotor-like activity in the 2 experiments in which it was applied (data not shown). The mean rhythm frequency (0.22 ± 0.017 Hz), duration (4.51 ± 1.02 s) and number of episodes, 4 per 30 min recording, were similar to that produced by EDRO. However, the mean duration of each episode was 267.5 ± 488.4 s (range 20–1000 s), a more than two-fold increase over the EDRO induced locomotion. These results confirm that decreasing the breakdown of ACh in the spinal cord is sufficient to trigger locomotor activity.

In order to determine if the rhythmic activity produced by EDRO was locomotor-like, 19 of the best 44 experiments were chosen for analysis of coordination among the ventral root recordings. Raw ENG waveforms (**Figure 2A**) were rectified and filtered for detailed analysis to determine the relationships between flexor/extensor and right/left activity (**Figure 2B**). Polar plots (**Figure 2C**) were produced from the rectified and filtered waveforms from this example (red vectors), showing the flexor and extensor ENGs (r L2 vs. r L5) were out of phase ($\theta = 205°$). The right and left flexor ventral root ENGs (r L2 vs. l L2) were also out of phase ($\theta = 201°$), indicating right/left alternation. Successive steps extracted from the rectified and filtered waveforms in **Figure 2B** were aligned with the onset of r L2 activity and overlaid (**Figure 2D**) to demonstrate the consistent alternation between flexor (L2) and extensor (L5) and left (l) and right (r) waveforms. The mean rhythm frequency in this experiment was 0.21 Hz and the mean duration of each step cycle was 4.88 ± 0.64 s.

Figure 2C also shows the mean angles (green vectors) in degrees and the mean *r*-values of right flexor/extensor alternation and right/left alternation for the 19 experiments (black vectors). The overall mean angle for flexor/extensor alternation (r L2 vs. r L5) was 170.0 ± 25.4° (124.9°–206.5°), $r = 0.84 \pm 0.1$ (0.66–0.98). The overall mean angle and the mean *r*-value right/left alternation (r L2 vs. l L2) was 186.1 ± 21.94° (153.6°–235.1°), $r = 0.87 \pm 0.08$ (0.67–0.96). These data indicate that well-coordinated locomotion occurred consistently with EDRO enhancement of the endogenous cholinergic system, without any requirement for cross-wavelet/coherence analysis to reveal "hidden" patterns of activity, as suggested by Lev-Tov and co-workers (Anglister et al., 2008).

THE EFFECTS OF ACh RECEPTOR (AChR) ANTAGONISTS

Nicotinic antagonists

In order to determine which AChR subtype is required for EDRO induced locomotion, we conducted experiments with both nicotinic and muscarinic antagonists. The non-specific nicotinic antagonist tubocurarine was used and had no effect in 8 experiments at concentrations of 1–20 µM, up to 70 times the K_i

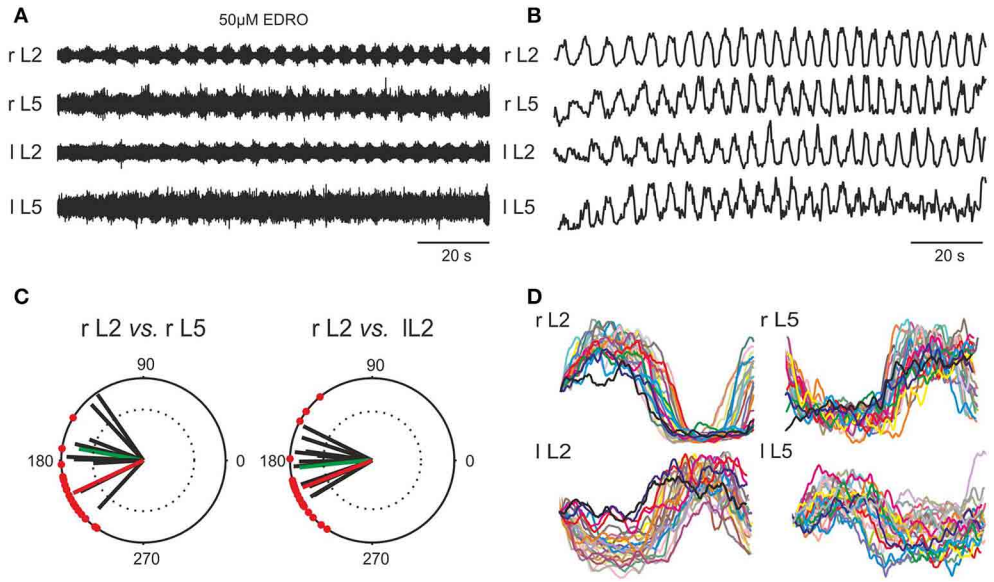

FIGURE 2 | Induction of locomotion *in vitro* by 50 μM EDRO. (A) ENG recordings showing sustained alternating flexor/extensor and left/right activity. **(B)** Rectified and filtered waveforms of the traces in **(A)**. **(C)** Polar plots derived from pooled data from 19 experiments showing the circular distribution of mean phase values and mean angles of the right flexor ventral root ENG (r L2) vs. the right extensor ventral root ENG (r L5), and right (r L2) vs. left (l L2) flexor ENGs (green vectors). The superimposed r vectors show the concentration of phase values around the mean angle. Mean rhythm frequency (Hz) of all filtered and rectified r L2 waveforms: 0.213 ± 0.052 Hz (0.13–0.33 Hz). Mean step duration of all rectified and filtered r L2 waveforms: 4.908 ± 1.312 s (4.85–7.87 s). The phase value for ipsilateral flexor/extensor activity ($\theta = 205°$) as well as the bilateral flexor (r L2 vs. l L2) phase value ($\theta = 201°$) for the example shown in **(A,B,D)** is shown in **(C)** with the red vector. **(D)** Overlay of step cycles (triggered on the onset of l L2 activity) of each rectified and filtered waveform shown in **(B)**.

of tubocurarine (280 nM) (Khan et al., 1994). It can therefore be concluded that EDRO-induced locomotion does not require nicotinic receptors.

Muscarinic antagonists

To determine if muscarinic receptors have a role in EDRO-induced locomotion, we examined the effects of the non-specific muscarinic antagonist atropine. Atropine has high affinity for all M receptor subtypes. In each of six experiments atropine decreased the amplitude of ENG bursts and blocked locomotion at concentrations from 105 nM to 1 μM. This result indicates that muscarinic receptors are required for the locomotion produced by EDRO, confirming previous observations (Smith et al., 1988a). In addition, however, we found that in 4 of 6 preparations prior to blocking locomotor activity completely, atropine produced an initial increase in the frequency (not burst amplitude) of locomotion that persisted until locomotion ceased (up to 16 min after the addition of atropine). The mean frequency prior to atropine was 0.207 ± 0.06 Hz, while after atropine treatment the frequency increased to a mean of 0.28 ± 0.06 Hz. This is a highly significant change ($p < 0.003$). This effect is shown in **Figure 3A**. In many cases the duration of the episode was prolonged, further suggesting a facilitatory action of atropine. This was not systematically investigated, however. To determine which muscarinic receptor subtypes might account for the effects of atropine, we examined the effects of antagonists to M_1(telenzepine), M_2 (methoctramine), M_3 (4-DAMP) and M_4 (MT-3) receptors. The M_5 receptor was not examined because M_5 receptors have not been observed in the ventral horn of the neonatal rat spinal cord (Wilson et al., 2004).

M_1 receptor antagonist

The M_1 receptor antagonist telenzepine was used in 12 experiments, starting with concentrations of 500 nM and increasing in concentration to 20 μM. Telenzepine failed to block locomotion at concentrations of 500 nM to 5 μM, (500–5000 fold greater than K_i). Telenzepine blocked EDRO induced locomotion only when the concentration reached 20 μM, (20,000 fold higher than K_i). In all cases where telenzepine blocked EDRO induced locomotion, the amplitudes of the ENG bursts were decreased but the frequency of stepping was not consistently altered (**Figure 3B1**). This implies that the action of telenzepine was not on CPG elements responsible for producing the locomotor rhythm, but on cells involved in controlling the output of motoneurons, or on the motoneurons themselves. The high doses required for telenzepine to effectively block locomotion imply that the effect was not due to a specific action at the M_1 receptor, but more likely because it also has affinity for the M_2 receptor, found on cholinergic propriospinal cells and on motoneurons, or because it has affinity at higher doses for the M_3 receptor. As noted below, the potent M_3 receptor antagonist 4-DAMP blocks EDRO-induced locomotion at doses in the nanomolar range.

M_2 receptor antagonist

We tested the M_2 receptor antagonist methoctramine (METHOC) in 14 experiments with concentrations from 1

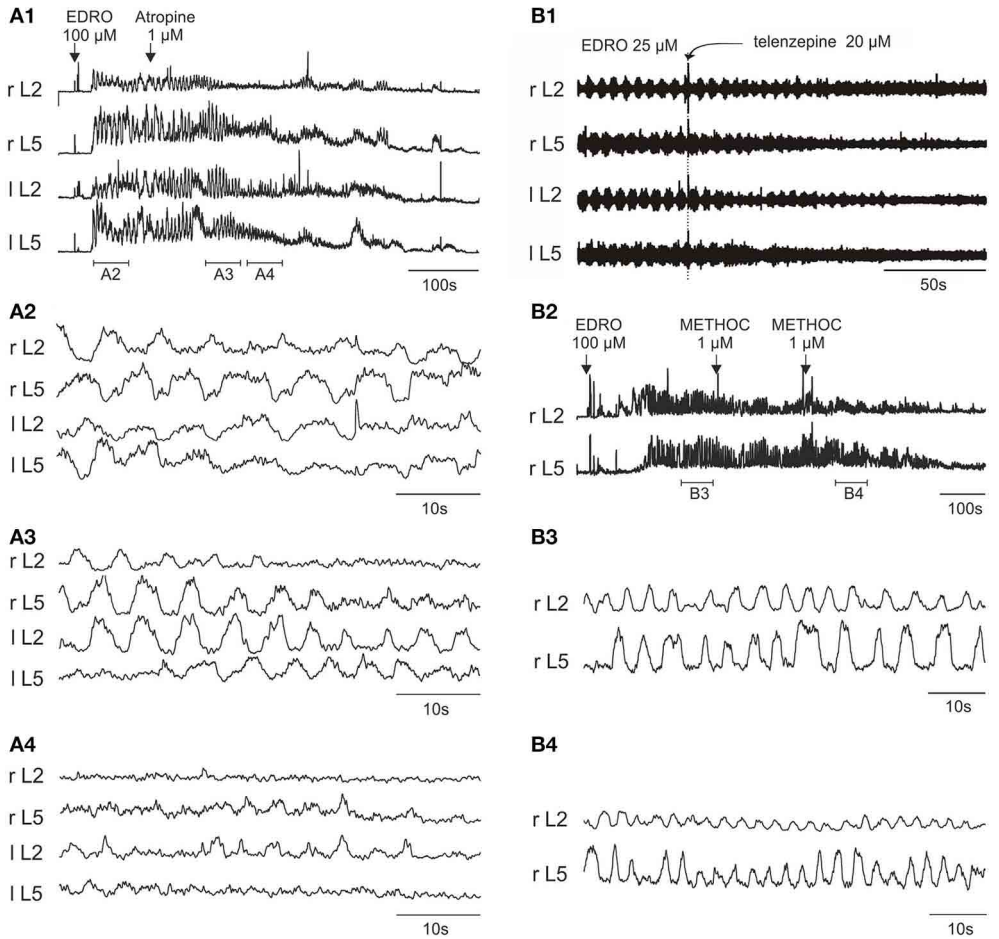

FIGURE 3 | Atropine, telenzepine, and methoctramine effects on the EDRO-induced locomotor rhythm. (A1) Rectified and filtered waveforms showing atropine block of EDRO-induced locomotion. **(A2)** Baseline locomotor-like activity. **(A3)** Transient increase in frequency and a decrease in amplitude after 1 µM atropine. **(A4)** Progressive reduction of EDRO-induced activity by atropine. **(B1)** Telenzepine (20 µM), an M_1 receptor antagonist, blocks EDRO-induced locomotor-like activity only at high doses, decreasing the amplitude but not the frequency of the ENG activity. **(B2–B4)** Methoctramine (METHOC, M2 receptor antagonist) produces an increase in frequency of EDRO-induced locomotion at 1–2 µM. **(B2)** Rectified and filtered waveforms recorded from the right L2 and L5 ventral roots produced by 100 µM EDRO. **(B3)** Baseline locomotor-like activity recorded during the period indicated by the horizontal bar below the ENG trace in **(B1)**. **(B4)** Increase in frequency of the ENG bursts resulting from a cumulative dose of two 1 µM METHOC (period indicated in **B2**).

to 50 µM. When applied to the chamber in concentrations from 1 to 2 µM (100–200 fold higher than K_i) METHOC seldom blocked locomotion. At these doses, METHOC produced a striking increase in the frequency of the locomotor bursts (**Figures 3B2–B4**). This effect was observed in 6 of 9 trials. The mean frequency prior to METHOC was 0.19 ± 0.03 Hz, while after METHOC treatment the frequency increased to a mean of 0.33 ± 0.04 Hz. This is a highly significant change ($p < 0.001$). These results suggest that METHOC relieves some form of muscarinic action that suppresses locomotion, perhaps through an inhibitory action on locomotor neurons, or through an excessive excitatory drive to CPG neurons that would serve to prolong each phase of the locomotor cycle. This is very likely through actions on the M_2 receptors, because METHOC has a rather low affinity for M_1, M_3, and M_4 receptors. These results are consistent with M_2 receptor mediated suppression of CPG activity. At a concentration of 2–50 µM (200–3000 fold higher than K_i) METHOC was able to suppress locomotion (5 of 9 trials). As with telenzepine, in every case where METHOC blocked EDRO-induced locomotion, ENG amplitude was decreased (**Figure 3B4**). This is consistent with an action directly on motoneurons, which are known to possess M_2 receptors (Hellstrom et al., 2003). The importance of these receptors has been recently reviewed (Brownstone, 2006; Miles and Sillar, 2011; Witts et al., 2014).

M_3 receptor antagonist

The muscarinic antagonist 4-DAMP was chosen due to a reported high affinity for the M_3 receptor subtype (Michel et al., 1989). In 12 experiments, 4-DAMP blocked locomotion at concentrations of 3, 10, 100, 500 nM, and 1 µM. **Figure 4A** shows a typical example of EDRO-induced locomotor activity. In this example, the

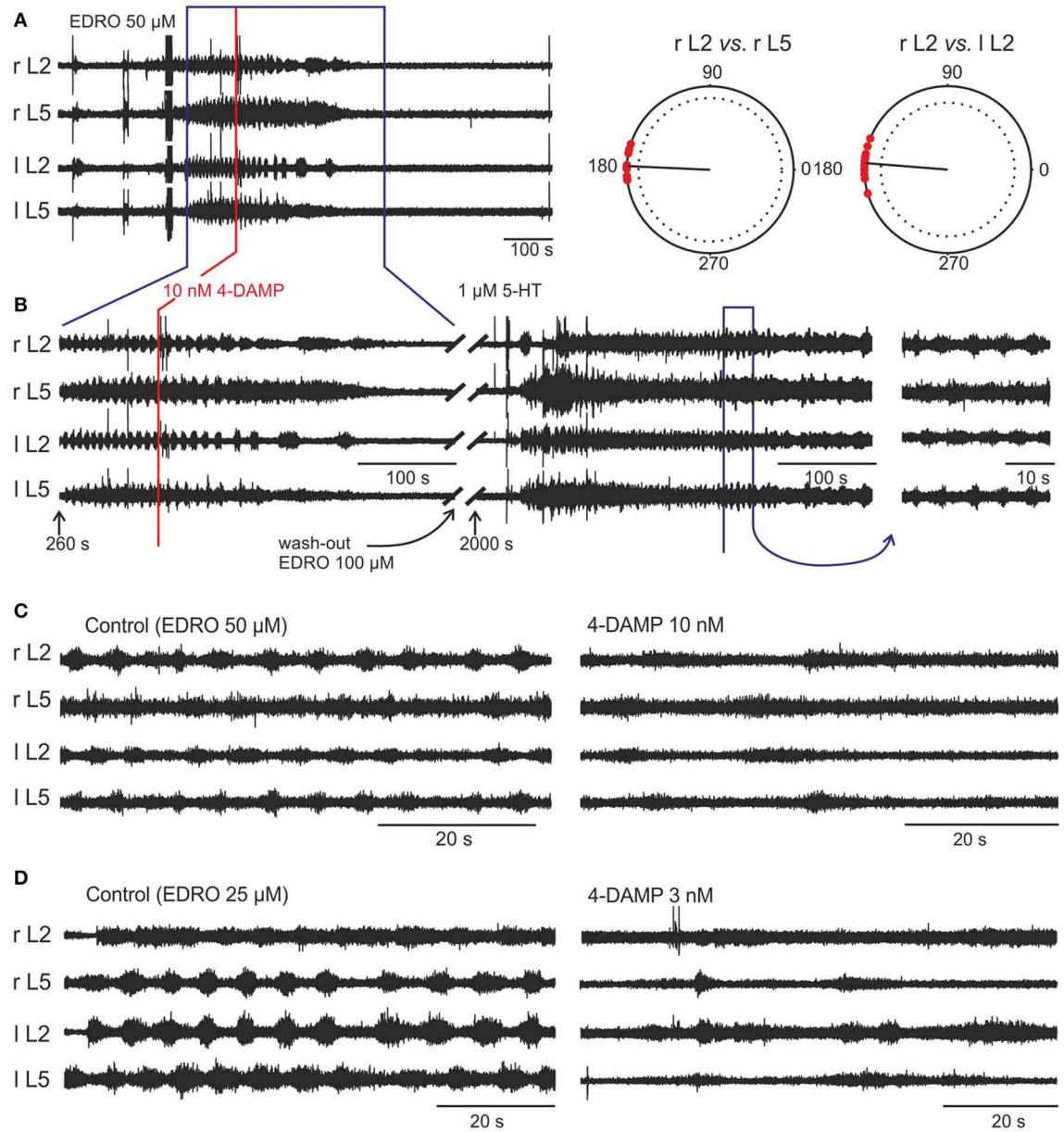

FIGURE 4 | EDRO-induced locomotion is blocked by 4-DAMP. (A) 50 µM EDRO induced a sustained pattern of locomotion. **(A**, right panel**)** Polar Plots of flexor-extensor and left/right alternation show highly coordinated locomotion prior to the application of 4-DAMP. **(B)** 4-DAMP applied to the bath decreased rhythm frequency from 0.178 to 0.063 Hz followed by complete blockade of rhythmic activity. An additional dose of EDRO (100 µM) failed to induce locomotion (data not shown), but adding 1 µM 5-HT to the bath produced tonic firing followed by locomotor-like activity. The fact that 5-HT can "rescue" locomotor-like activity despite continued block of the EDRO effect shows that the preparation remained viable and that M_3 receptor blockade did not interfere with locomotion induced by 5-HT. **(C,D)** Comparison of the effects of 4-DAMP on EDRO-induced locomotion with hind limbs attached **(C)** or removed **(D)**. The right panels show the results from the application of 4-DAMP in the two preparations.

hindlimbs were attached and moved during episodes of locomotor activity. Subsequent addition of 4-DAMP (10 nM) to the bath slowed the locomotor rhythm and eventually blocked it completely (**Figure 4B**). After wash-out, a subsequent dose of EDRO (100 µM) had no effect. In order to demonstrate that the preparation was still capable of producing locomotion, 1 µM 5-HT was then added to the bath (**Figure 4B**). Well-coordinated locomotion was induced, indicating that the locomotor CPG could still be activated in the absence of recovery of the EDRO effect. In each of the experiments where 4-DAMP blocked locomotion, subsequent application of EDRO, at its maximum concentration of 100 µM, failed to re-establish locomotion or induce any rhythmic activity.

In experiments where the hindlimbs were removed and 4-DAMP was applied to the isolated spinal cord alone ($n = 8$), the outcomes of 4-DAMP application on the locomotor cycle characteristics (frequency and amplitude) were similar to those

obtained with hindlimbs attached ($n = 4$). Examples of 4-DAMP effects on locomotor frequency in these two preparations are shown in **Figures 4C,D**. The mean step frequency of EDRO-induced locomotion before 4-DAMP with hindlimbs attached (**Figure 4C**, left panel) was 0.176 Hz and the mean step frequency after 4-DAMP was applied and before complete cessation of locomotion (**Figure 4C**, right panel) was 0.068 Hz. The mean step frequency of EDRO-induced locomotion before 4-DAMP with hindlimbs removed (**Figure 4D**, left panel) was 0.134 Hz and the mean step frequency after 4-DAMP was applied (**Figure 4D**, right panel) was 0.055 Hz. ENG amplitude decreased in all experiments using 4-DAMP. This suggests that the effect of 4-DAMP may be on neurons intrinsic to the spinal cord, rather than on the relay of sensory signals from the moving limb. The decrease in step frequency is consistent with M_3 receptor involvement in the cholinergic activation of cells within the locomotor network. Unlike atropine and METHOC, 4-DAMP did not produce even a transient increase in the frequency of locomotion.

Importantly, 4-DAMP was effective at doses as low as 3 nM. This finding demonstrates that 4-DAMP, in contrast to the M_1 and M_2 antagonists, blocks EDRO-induced locomotion near its K_i, thus implicating the M_3 receptor as the most likely muscarinic receptor subtype responsible for EDRO-induced locomotion in the *in vitro* neonatal rat preparation.

M_4 receptor antagonist

MT-3 was tested in three experiments with concentrations from 5, 10, and 300 nM (1.6 fold lower and 35 fold higher than K_i). It had no effect on EDRO-induced locomotion in any of these cases (data not shown). It is unlikely, therefore, that the M_4 receptor is involved in the locomotor activity produced by enhancing the effects of the endogenous cholinergic propriospinal system with EDRO.

These results show that the endogenous cholinergic propriospinal system is capable of producing coordinated locomotor activity, and that under the conditions of our experiments the control of motoneuron excitability is mediated by M_2 receptors, while the rhythm generating component is controlled by M_3 receptors. This raises the possibility that this endogenous spinal system might contribute to the locomotion in the intact animal and to recovery of locomotion after SCI.

INTRAVENOUS CHOLINERGIC ANTAGONISTS—DECEREBRATE CATS

In order to test the importance of both the brainstem cholinergic nuclei implicated in the initiation of locomotion and the endogenous cholinergic propriospinal system we used intravenous application of cholinergic antagonists in decerebrate cats. We elicited locomotion with stimulation of the MLR. Because both nicotinic and muscarinic receptors have been implicated in the relay of the locomotor command from the MLR to reticulospinal neurons (Ryczko and Dubuc, 2013), we tested antagonists to both types of receptors. These experiments also provided a test of whether the intrinsic cholinergic propriospinal system is normally recruited as a component of the locomotor CPG in adult animals.

Previous work on decerebrate cats showed that MLR-evoked locomotion could be abolished with injections of cholinergic antagonists into a specific site in the medulla that receives cholinergic input from the MLR (Garcia-Rill and Skinner, 1987). Whether this is an obligatory route for evoking locomotion has not been tested, however. Here we show (**Figure 5**) that intravenous infusion of atropine and/or mecamylamine did not abolish MLR-evoked treadmill locomotion in any of the experiments ($n = 15$). Administration of mecamylamine alone produced slight increases in the threshold for electrically induced locomotion that were within the range of normal variability for lengthy experiments on decerebrate animals (114, 116, 119 and 133% of control) in 4/7 experiments. No change was observed in two experiments and a decrease in the threshold (to 63% of control) was observed in the remaining one. Atropine given alone produced a transient increase in threshold (111% of the projected baseline) in one experiment and had no effect in one other. The effect of a combination of atropine and mecamylamine on the threshold for electrically induced locomotion was examined in 5 experiments. Aside from a transient increase (5–8 min) in locomotor threshold coinciding with a drop in blood pressure, no change in threshold outside of the normal variability was observed (100, 104, 130, 131, and 131% of control trials) after administration of both drugs. In four animals brief periods of spontaneous locomotion were present both prior to and after infusion of atropine and/or mecamylamine. The threshold for electrically-induced locomotion (outside of the normal variability) was transiently increased (5–8 min) in 5 animals after administration of mecamylamine or both drugs together. Since intravenous administration of atropine or mecamylamine (at the doses used in the present study) has a prolonged effect (Ueki et al., 1961; McCance et al., 1968b; Lake, 1973; Ryall and Haas, 1975; Noga et al., 1987) these brief increases in threshold are attributed to the transient decreases in blood pressure observed following administration of these drugs. Thus, no evidence for blockage of either spontaneous or MLR-induced locomotion by mecamylamine or atropine was obtained in the present experiments.

Figure 5 illustrates the EMG activity recorded in a typical experiment prior to and after administration of mecamylamine and atropine. The lowest threshold MLR stimulation site (**Figure 5A**) located in the cuneiform/subcuneiform nucleus was in close proximity to the cholinergic cell group hypothesized to be functionally relevant to the production of locomotion (Garcia-Rill and Skinner, 1987; Garcia-Rill et al., 1987). The muscle activity persisted with the normal timing relationships among the muscles maintained in spite of the administration of mecamylamine (**Figure 5C**) and atropine (**Figure 5D**) at doses appropriate for the blockage of nicotinic and muscarinic transmission. In this example, the thresholds for initiation of locomotion by electrical stimulation of the MLR did not increase but rather decreased after drug administration.

In order to determine the effect of the drugs on the overall levels of motor unit activity produced during locomotion, averaged EMG records obtained following drug administration were compared to those recorded during control trials. In 285 trials where TA and GL muscle activity was examined, mecamylamine produced an increase in EMG burst activity in 3 (1.1%), a decrease in 20 (7.0%) and no change in EMG burst activity in 262 (91.9%). Of the 38 trials with atropine an increase

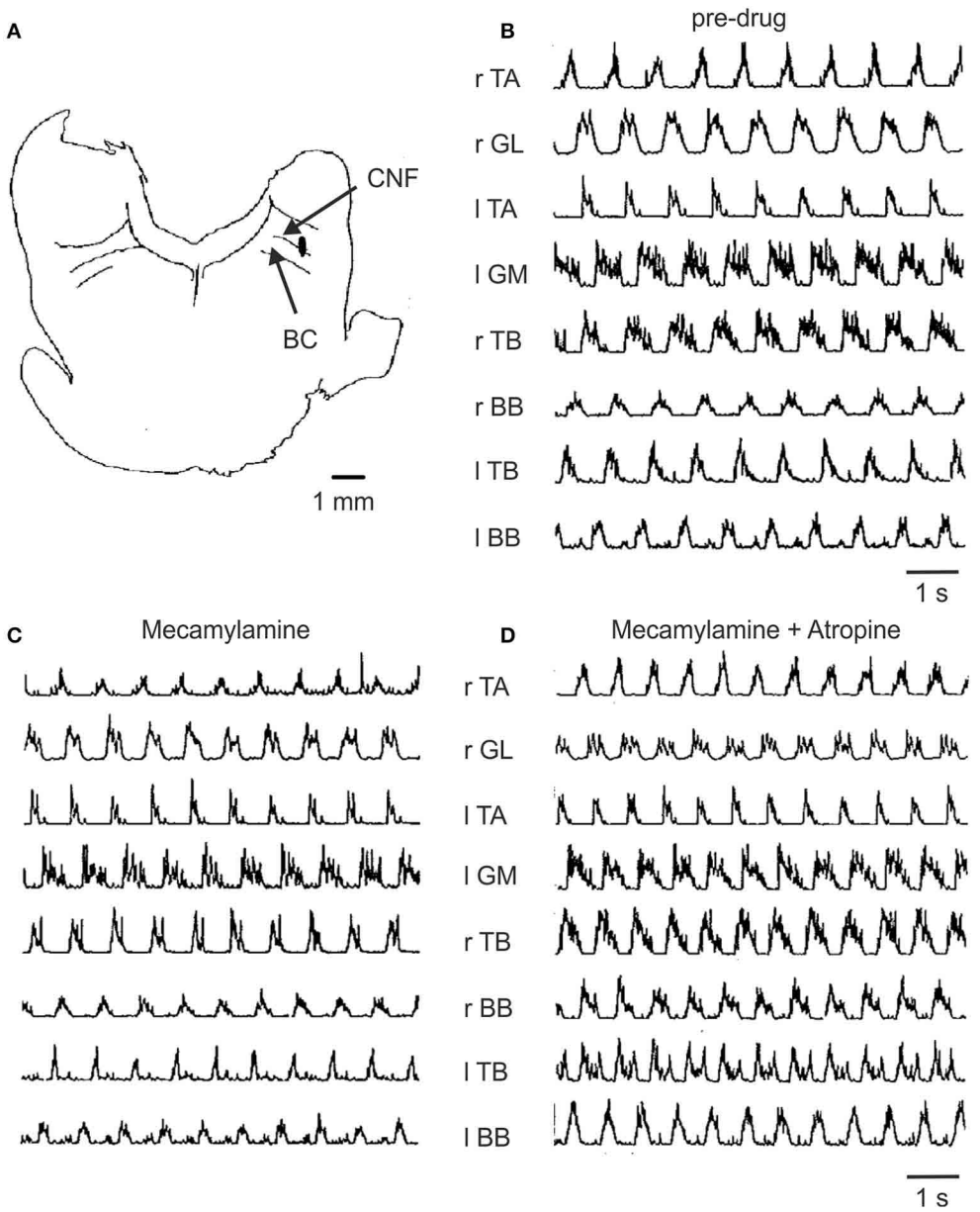

FIGURE 5 | Effect of mecamylamine and atropine on locomotion induced by electrical stimulation of the MLR. (A) Site of stimulation was localized to the ventrolateral border of the cuneiform nucleus (CNF) near the brachium conjunctivum (BC) at P2, L4, H-1.5 (coordinates according to Berman, 1968). **(B)** Treadmill locomotion evoked from this brainstem site prior to any drugs (stimulus strength of 135 μA); **(C)** following the infusion of mecamylamine (3 mg/kg, stimulation strength 60 μA) and **(D)** following atropine (2.5 mg/kg, stimulation strength 70 μA). Thresholds for the initiation of locomotion for **(B–D)** were 150, 150, and 75 μA, respectively. The gain of each muscle's EMG is the same throughout all trials.

of activity in 6 (15.8%), a decrease in 3 (7.9%) and had no effect in 29 (76.3%) was observed. Combined administration of atropine and mecamylamine produced an increase in muscle activity in 14/142 trials (9.9%), a decrease in 5/142 trials (3.5%) and had no effect on muscle activity in 123/142 trials (86.6%). It is clear from these data that the amount of muscle activity as measured from the area of the burst during the normalized step cycle was not altered in any consistent manner by cholinergic blockade.

We examined the effects of the drugs on the burst duration and times of onset and termination of EMG activity during the step cycle. In 315 trials on 6 muscles (TA, GL, IP, AF, TB, and BB), mecamylamine produced an increase in burst duration in 81 (25.7%), a decrease in 60 (19.1%) and no change in 174 (55.2%). For 88 trials, atropine produced an increase in burst duration in 33 (37.5%), a decrease in 11 (12.5%), and had no effect on burst duration in 44 (50.0%). It is evident from this analysis that cholinergic blockage did not consistently

alter the duration of EMG activity in any of the muscles examined.

These data reveal that in adult decerebrate cats there appears to be no requirement for cholinergic neuron participation in the initiation or coordination of locomotion at either the brainstem or the spinal cord levels, using i.v. application of drugs known to cross the blood-brain-barrier for testing the actions of muscarinic and nicotinic receptors. In agreement with this, it appears that the cholinergic C-boutons on motoneurons do not provide a major component of the locomotor drive (Witts et al., 2014), consistent with our finding that blockage of muscarinic inputs to motoneurons in adult cats does not alter the amplitude of EMGs recorded from hindlimb motoneurons. It is still possible, however, that cholinergic neurons of the spinal cord participate in the recovery of locomotor activity after SCI. Since this propriospinal system is a major intrinsic system known to facilitate locomotion that is spared by SCI, we hypothesized that it could be responsible for locomotor recovery. To establish this, we examined the effects of intrathecal cholinergic agonists and antagonists on locomotor recovery in cats and rats after spinal cord transection.

SPINAL CATS

An initial set of experiments was conducted in 2 spinal cats prepared so that intrathecal (i.t.) injections of various agonists (carbachol) or the AChE inhibitor EDRO, as well as antagonists such as atropine, 4-DAMP and dihydro-β-erythroidine could be injected in volumes of 100 μl (unless specified otherwise) in both cats on alternate days, thus keeping the time sequence of injections after spinalization approximately the same.

Early chronic period (5–14 days)

On Day 5 and 6 post lesion, the animal was placed on a treadmill running at 0.3 m/s. As expected, only faint movements were evoked by strong perineal stimulation and sporadic tonic hyperflexion of both hindlimbs but without any locomotion. This is illustrated in **Figure 6A** where stick figure reconstructions of the movements over consecutive seconds is shown (top panel), as well as the corresponding angular excursion of the joints (middle panel) and EMGs (bottom panel).

EDRO was then given i.t. (each application in a bolus of 100 μl) in two cats (in one 0.5 mM then another 0.5 mM 13 min later, in another 1 dose of 2 mM directly) and the effect monitored each 10 min over a 30–40 min period by holding the cat over the treadmill belt and by applying various skin or perineal stimulation to facilitate locomotor movements. No obvious effect was observed after EDRO (compare **Figures 6A,B**). Acetylcholine was then given (in one cat 1 mM; in the other 2 mM) 45 min after EDRO and again, no locomotor movements were observed (**Figure 6C**) for the next 1:30 h. To test the ability of the spinal cat to generate locomotion, 100 μg of clonidine was given i.t. and vigorous stepping was triggered for the next 2 h, from 0.3 to 0.7 m/s. As shown in **Figure 6D**, 6 min after clonidine, alternation between extensor and flexor muscles in each limb and alternation of the activity of right and left homologous muscles

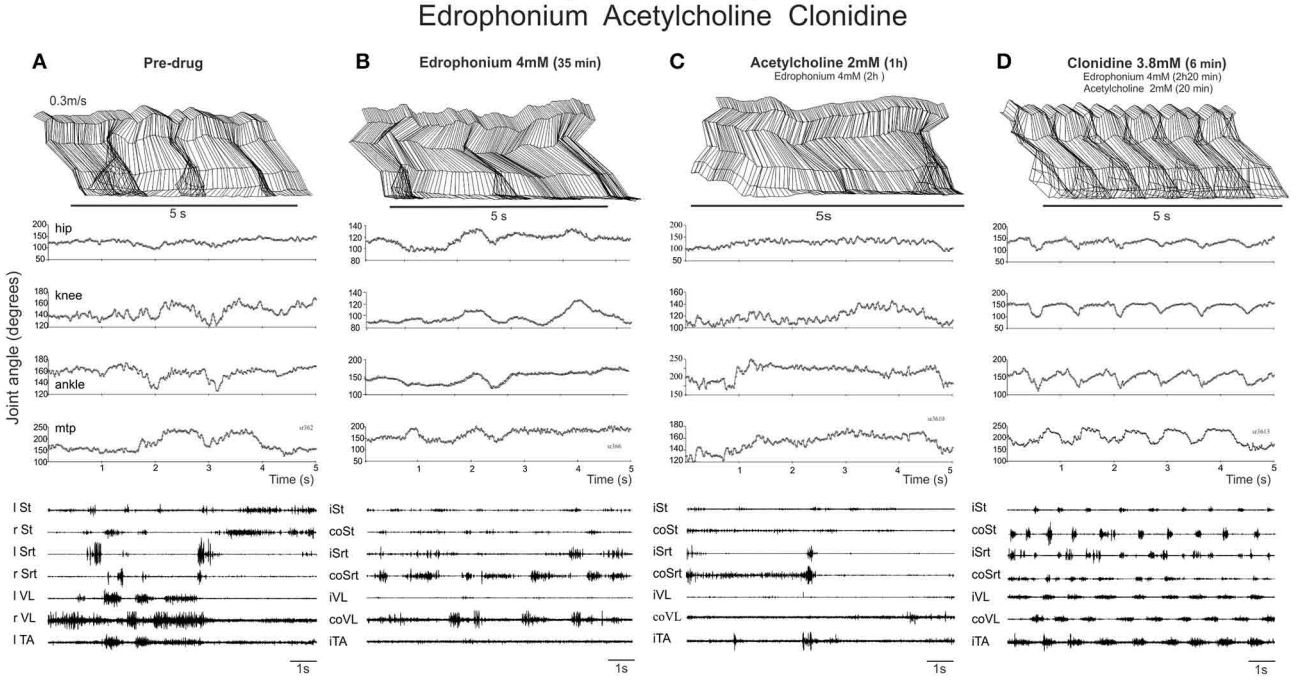

FIGURE 6 | Neither EDRO nor ACh in the presence of EDRO facilitated the onset of locomotor activity in a spinal cat. (A) Pre-drug records of a cat walking on a treadmill with strong exteroceptive stimulation used to activate the locomotor CPG. Stick figures of the limb trajectory over 5 s of activity, along with the corresponding joint angles and EMG recordings are illustrated. **(B)** Similar records taken 35 min. after 4 mM EDRO administration. **(C)** Recordings taken 1 h after administration of 2 mM ACh and 2 h after the original dose of edrophonium. **(D)** Subsequent dose of clonidine (3.8 mM) elicited typical stepping movements on the treadmill, as illustrated by joint angles and EMG, showing that the animal was capable of producing locomotion.

lead to a regular pattern of locomotion on the treadmill involving all 4 joints. The cat walked with the limb somewhat in extension as should be expected only 5 days after spinalization. This was seen in both cats and was repeated the day after (6 days post lesion) in one cat with a higher dose of EDRO (4 mM) and higher doses of acetylcholine (4 mM). In the other cat, higher dose of EDRO (4 mM) only, without acetylcholine, was tested to observe the effect of the acetylcholinesterase alone and again, no obvious effect was observed while clonidine could still trigger locomotion when injected after all the other drugs. It is thus clear that the AChE inhibitor produces no significant effect on the initiation of locomotion in spinal cats, unlike its effects in the isolated neonatal rat spinal cord. ACh deteriorates the already poor stepping and clonidine evokes a sustained locomotor pattern indicating that cats were capable of walking and suggesting that the negative effects obtained with cholinergic stimulation was not due to the inability of the spinal cats to walk.

On Day 8, after only 1 day of training (3 times for 5 min), the cholinergic agonist carbachol (1 mM) was tested in both cats. Before drug administration, one cat could make a few steps on the treadmill with foot contact on the dorsum of the foot at 0.3 m/s (not shown). After carbachol, standing was exaggerated with hyper-extensions of the joints and stepping was completely blocked within 7 min. Thirty minutes later, perineal stimulation evoked hyperflexions and a few steps with prolonged extension but this type of movement was far from the locomotor pattern observed before drug. In the other cat, locomotion was still not possible before carbachol (**Figure 7A**). With carbachol, perineal stimulation produced long duration discharges (several seconds) of several muscles often simultaneously (**Figure 7B**). Overall, carbachol administration was tested 4 times in the two cats and it is clear that carbachol could not trigger locomotion and was even detrimental to the faint spontaneous locomotor pattern in the cat when present.

On days 12–13, the cats began to show some recovery of locomotion (a few steps at 0.3–0.4 m/s) when the perineum was stimulated. At a higher speed, the animal showed some hyperflexion of both hindlimbs even with stronger perineal stimulation. As illustrated in **Figure 8A**, at a treadmill speed of 0.4 m/s, the cat cannot place its paw in front of the hip at the end of the swing phases and there is a paw drag in the first part of the swing phase (**Figure 8A**). The averaged EMGs show that although there is a good alternation between flexor and extensor muscles, like St and VL, Srt muscles on both sides have a very weak level of activity.

With this background of spontaneous activity, atropine was injected with the hypothesis that the deterioration of walking seen with ACh, carbachol or EDRO early after spinalization might be due to an already extant hyper-cholinergic state. Twelve minutes after drug administration (**Figure 8B**), the cat not only showed a better pattern of locomotion, it could express locomotion without the need of perineal stimulation. It could walk up to speed of 0.6 m/s. After 36 min the cat could reach treadmill speeds up to 0.8 m/s (**Figure 8C**). These effects could still be seen after 3 hours (**Figure 8D**). **Figure 8E** illustrates the speeds that could be reached by the cat at various time points after atropine injections. The averaged EMGs show a clear increase in the Srt muscles resulting in a greater flexion of the knee and a better elevation of the foot in the second portion of the swing phases before the paw contact, as illustrated. When carbachol was added, the spontaneous stepping was degraded although a few steps could be triggered with strong perineal stimulation at a max speed of 0.5 m/s without adequate limb extension or placement of the paws (data not shown).

Thus, at 12 days the cat is already capable of performing a few spontaneous steps. With atropine locomotion became more vigorous and 36 min later the cat could walk up to 0.8 m/s. Carbachol dramatically degraded this locomotor activity.

Day 14: carbachol followed by atropine

At Day 14, the spinal cats could walk spontaneously (without drugs) up to a treadmill speed of 0.7 m/s. After carbachol (**Figure 9**) the steps deteriorated with less weight support and no

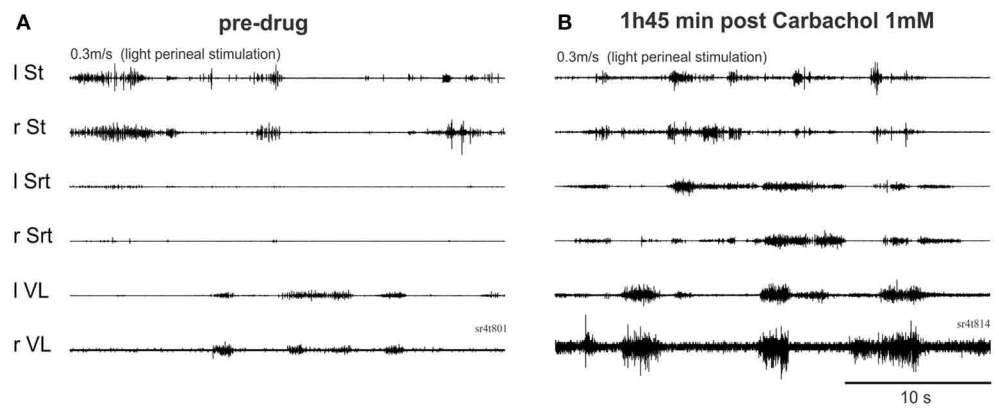

FIGURE 7 | Carbachol, a cholinergic agonist, produced co-contractions and interfered with locomotor activity early after injury. (A) Pre-drug trial with EMG activity produced by perineal stimulation. **(B)** 1 h 45 after i.t. carbachol (1 mM, 100 μl) administration, perineal stimulation produced uncoordinated rhythmic activity.

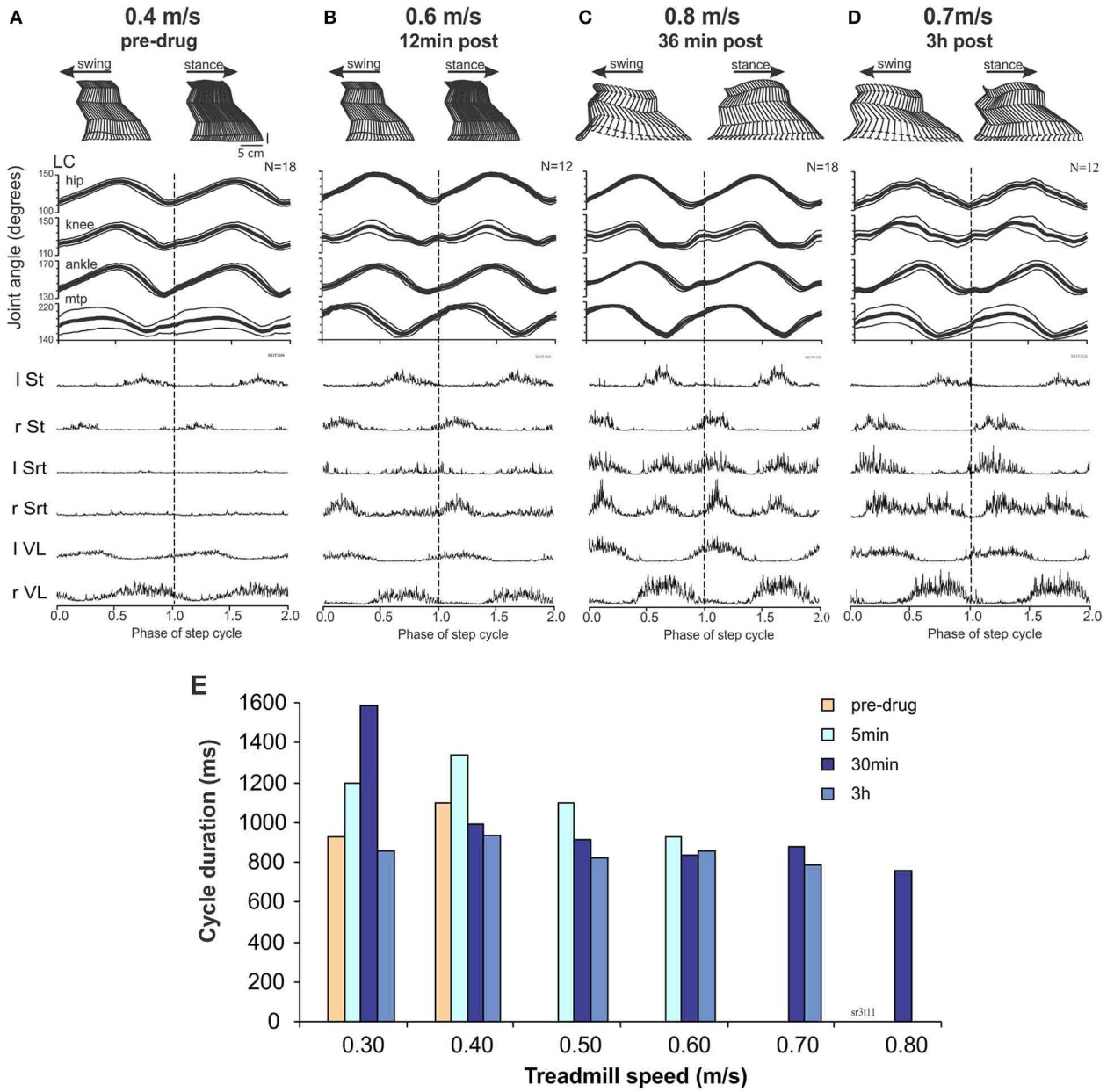

FIGURE 8 | Intrathecal atropine facilitated locomotion in spinal cats. **(A–D)** show kinematic and EMG recordings of a cat walking on a treadmill at increasing time after atropine (1 mM, 100 μl) administration: stick figures of one step cycle (top), averaged angular excursion of the four joints over $N > 12$ step cycles (middle) and corresponding averaged rectified EMG traces (bottom). The cycle is normalized to 1 and is repeated twice (separated by doted vertical lines) for clarity at turning points. The average is synchronized with the contact of the left foot (LC). **(A)** Pre-drug recordings at a treadmill speed of 0.4 m/s; **(B)** 12 min after atropine administration at a treadmill speed of 0.6 m/s; **(C)** 36 min after atropine at a treadmill speed of 0.8 m/s; **(D)** 3 h after Atropine at a treadmill speed of 0.7 m/s. **(E)** Cycle duration as a function of treadmill speed, showing a maximal increase in treadmill speed the cat could follow after atropine at approximately 30 min, with a slight decrease at 3 h after drug administration.

foot placement at 0.3 m/s and perineal stimulation was required to improve stepping at speeds ranging from 0.3 to 0.7 m/s. Twenty minutes after carbachol the cats could walk from 0.3 to 0.7 m/s with perineal stimulation but could not walk without it at 0.4 m/s.

This deterioration of locomotion by carbachol could be reversed by atropine. The cat could indeed walk from 0.4 to 0.6 without perineal stimulation with more forward movements of the hindlimbs. Twenty minutes later the cat could walk as fast as

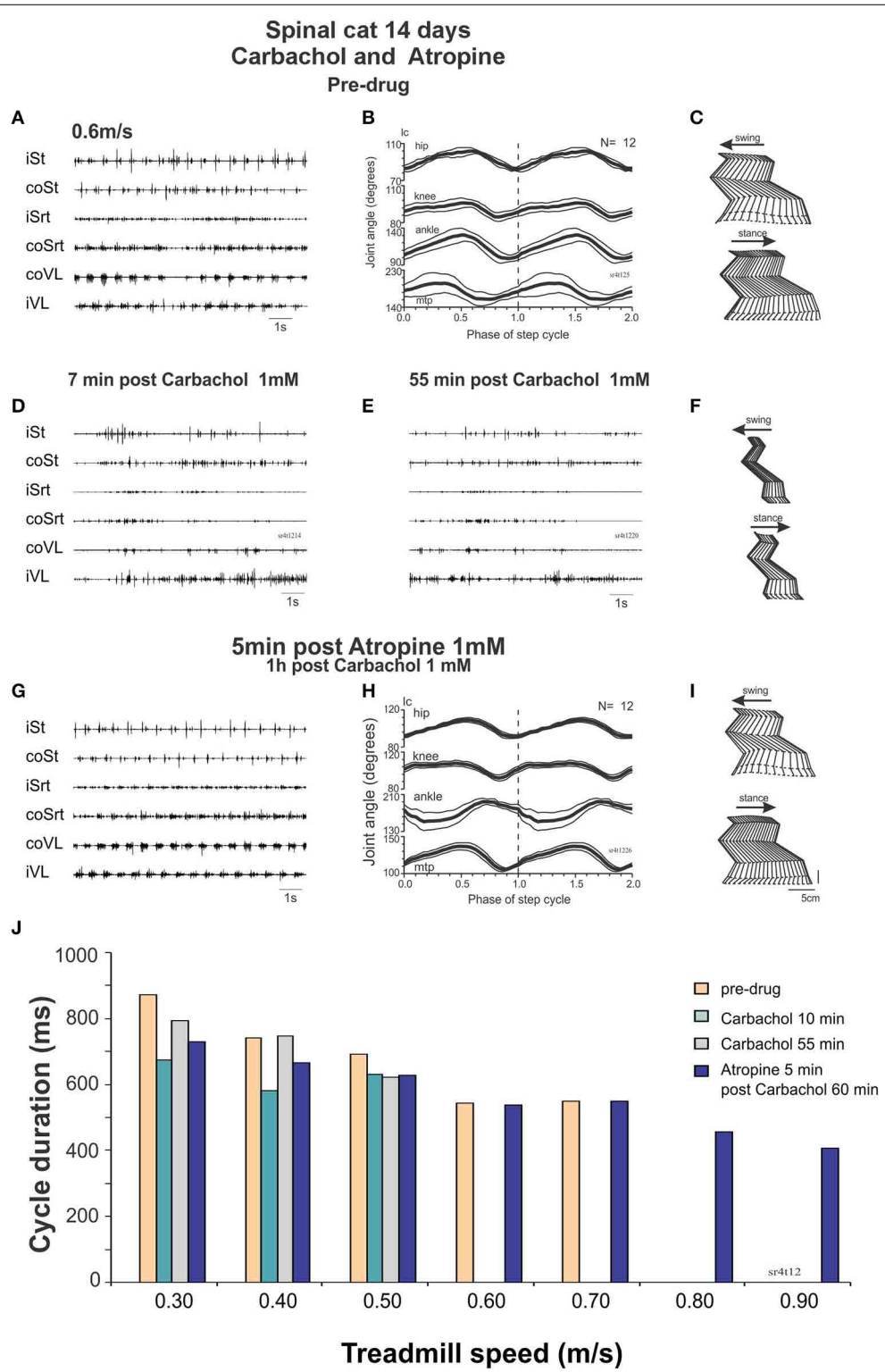

FIGURE 9 | Carbachol disruption of locomotion in spinal cats is reversed by atropine. (A–C) regular stepping movements before drug administration 14 days after the lesion represented by raw EMG traces **(A)**, averaged ($N = 12$ cycles) joint angular displacements **(B)** and stick figures **(C)**. **(D)** Raw EMG recordings after intrathecal carbachol (1 mM, 100 μl) showing deterioration of stepping as soon as 7 min post drug administration even with strong perineal stimulation. **(E,F)** 55 min post carbachol, the EMG is still not organized to produce a good stepping pattern **(E)**. There is no foot placement as represented by the stick figures **(F)**. **(G–I)** Five minutes after intrathecal administration of atropine (1 mM, 100 μl), a good pattern of stepping was observed without perineal stimulation as was the case before carbachol administration [compare **(G–I)** with **(A–C)** same display]. **(J)** Cycle duration expressed in terms of treadmill speed, showing disruption of locomotion by carbachol, and facilitation of locomotion with atropine.

0.9 m/s without perineal stimulation (**Figure 9J**). Thus, in a walking spinal cat, carbachol disrupts locomotion but the locomotor ability could be rescued by injecting atropine.

Late chronic period (>2 months)

In the late period (around 2 months) we injected a muscarinic blocker, 4-DAMP or a nicotinic antagonist, dihydro-β-erythroidine. At this time, the cat still had appreciable locomotor activity in both hindlimbs, however, they needed more perineal stimulation. After injecting 4-DAMP (45 μg in 100 μl, M_3 muscarinic receptor antagonist), the locomotor activity was much improved, several sequences without perineal stimulation could be obtained, and the cat could often walk without any external weight support. Similar results were obtained using dihydro-β-erythroidine (26 μg in 100 μl i.t.), which produced long sequences of unaided spinal stepping. This was observed in 5 trials in 2 chronic spinal cats (40–97 days post SCI). Thus, the cholinergic suppression of spinal locomotion involves both muscarinic and nicotinic receptors (data not shown).

CHRONIC SPINAL RATS

The above results indicate that despite the ability of endogenous release of ACh in the neonatal rat cord to give rise to well-coordinated locomotion, our hypothesis that locomotor recovery after SCI could be mediated by increased activity of the intrinsic cholinergic propriospinal system was not confirmed. In fact, the data suggest that a hyper-cholinergic state might develop after SCI that somehow interferes with locomotion. In order to test whether this is a consistent feature of SCI across species, and to rule out the possibility that our results in adult spinal cats might deviate from our expectations based on the neonatal rat data due to a species difference, we investigated the effects of intrathecal applications of cholinergic drugs in adult spinal rats 10 weeks after injury. **Figure 10** shows a typical example of the effects of i.t. carbachol (1 mM, 20 μl) followed by an application of atropine (1 mM, 20 μl). We consistently observed a dramatic and immediate cessation of treadmill locomotion induced by tail pinching when carbachol was administered ($n = 3$) that lasted for several hours. A subsequent i.t. application of atropine (in 10–15 min after the carbachol) reversed this effect and actually increased the efficacy of tail stimulation to elicit locomotion. Plantar stepping was occasionally induced (**Figure 10C**), and rhythmic activity persisted even when there was no tail stimulation. Doses of 20 μl of carbachol of 0.5 and 0.25 mM were also effective. Thus, the effects of carbachol and atropine in the spinal rat were remarkably similar to the effects of these drugs in the adult spinal cat (**Figure 9**).

Therefore, our data provide evidence for a hyper-cholinergic state in both cats and rats after SCI, with no evidence for a species difference that might account for the unexpected absence of the locomotion promoting action of the cholinergic propriospinal system observed in neonatal rat preparations. An obvious difference between these two preparations is the presence of cutaneous receptor feedback (Bouyer and Rossignol, 2003a,b; Sławińska et al., 2012) in the spinal rats and cats walking on a treadmill. The proprioceptive feedback from the moving limb (Pearson, 2004) in neonatal rat preparations with the hindlimbs attached is not sufficient to account for the differences, because the results were not distinguishable from preparations with no hindlimbs attached. The other difference between these two preparations might be that some plasticity in the cholinergic system and the changes in the receptors induced by total transection in spinal chronic rats are not present in neonatal spinal cord preparations.

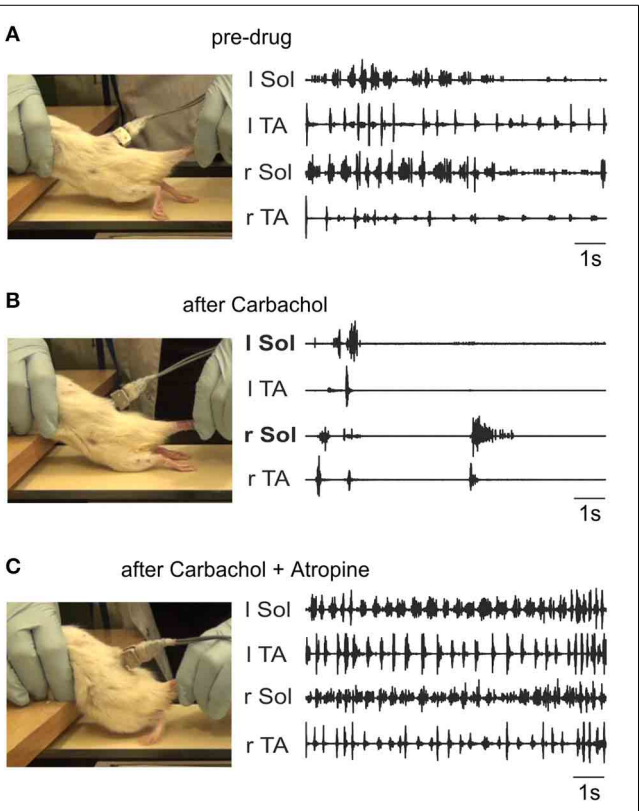

FIGURE 10 | Carbachol disruption of locomotion in spinal rats is reversed by atropine. The left panels show representative frames from the videos taken during the time of the corresponding EMG activity (right panels). **(A)** Irregular stepping movements produced with tail stimulation prior to drug administration. As shown in the left panel, the EMG activity did not give rise to plantar stepping. The toes were always dragging on the treadmill surface during rhythmic alternating activity. **(B)** Intrathecal carbachol (1 mM, 20 μl) eliminated all stepping movements immediately, and only a few sporadic episodes of largely synchronous bursting occurred (recording taken 1 min after administration). **(C)** The intrathecal administration of atropine (1 mM, 20 μl) 10 min after carbachol treatment restored alternating movement and led to occasional plantar stepping (left panel). The EMG activity was more consistent, and occurred even in the absence of tail stimulation. The recording was made 30 min after atropine was given.

ACUTE SPINAL CATS

Our experiments on chronic spinal animals led us to attempt to determine whether the atropine effect could be observed acutely after spinalization, or whether a period of time was necessary for it to develop. We also wished to examine the actions of cholinergic drugs on sensory input, since our results suggest that a decreased response to sensory inputs may be one explanation for the detrimental effects of cholinergic agonists and the facilitation of locomotion produced by cholinergic antagonists.

Effect of atropine alone

In all cats, atropine alone, whether given intrathecally (1 mM, 70 µl) at mid lumbar segments or in a bath covering L3-L4 spinal segments, failed to evoke locomotion in acutely spinalized cats or in 1-week spinal cats decerebrated on the day of the experiment. This suggests that some plasticity in the cholinergic system is required for the effects of cholinergic drugs in chronic spinal animals (data not shown).

Effect of atropine on sensory input

The effect of atropine on the SP reflex was tested in 4 cats: acute ($n = 2$), 24 h ($n = 1$) and 7 days ($n = 1$) after complete section of the spinal cord at T13. The amplitude of the reflex evoked by a stimulation of the SP nerve increased after atropine (i.t., 70 µg in 100 µl) compared to control values. **Figure 11A** illustrates the averaged ($n = 25$) rectified response in St, GL, and TA muscles after stimulation of SP 1.5T, 3 pulses, 300 Hz

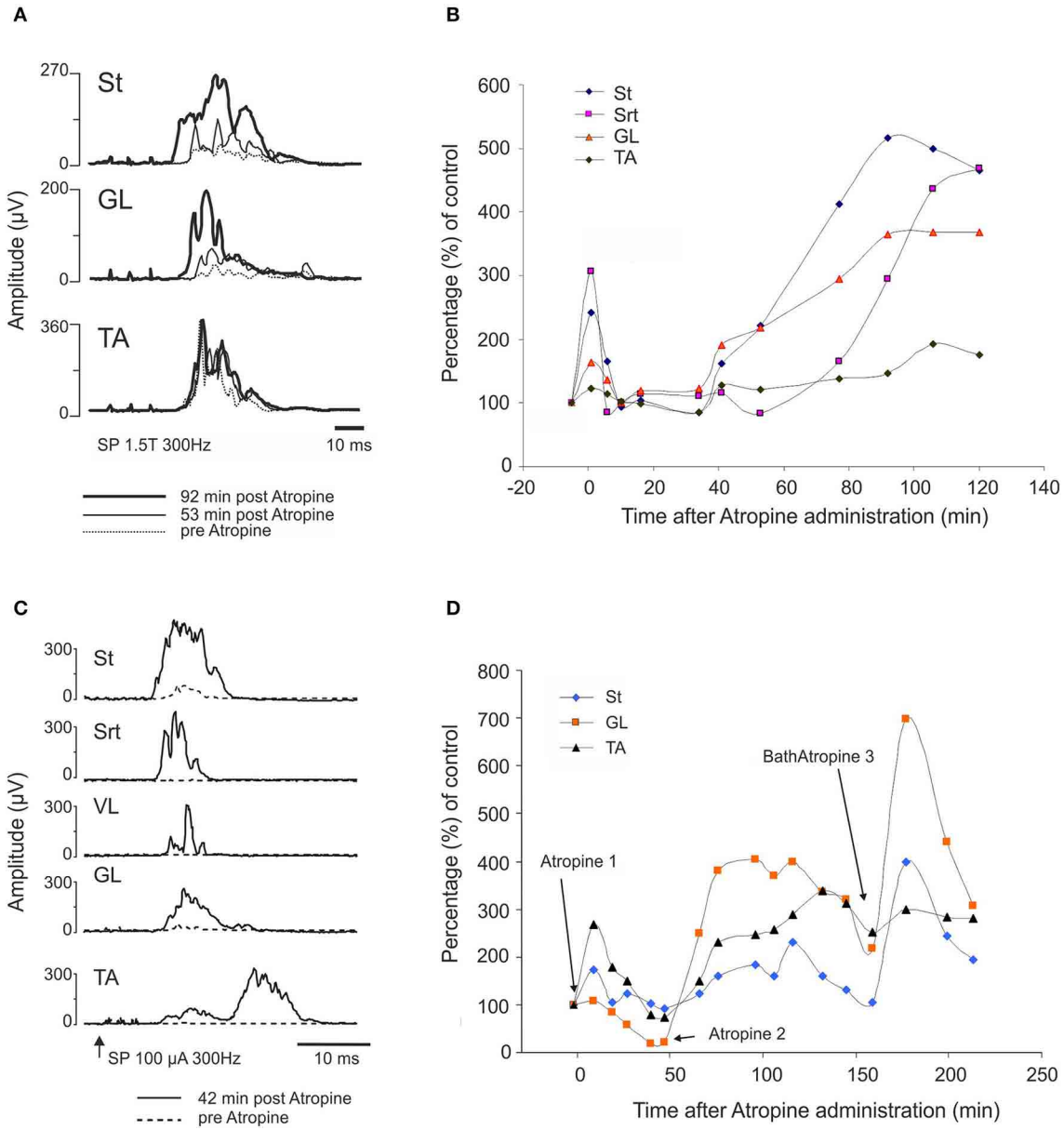

FIGURE 11 | Atropine facilitates the cutaneous reflex responses evoked by stimulation of superficial peroneal (SP) nerve. (A,B) SP facilitation by intrathecal atropine (70 µg, 100 µl) 1 day after the complete section of the spinal cord: **(A)** averaged ($n = 25$) rectified response in ST, GL, and TA muscles after stimulation of SP (1.5T, 3 pulses, 300 Hz) 300 Hz before (dotted line), 53 (thin gray line) and 92 (thick black line) min post atropine; **(B)** illustrates the evolution of the response amplitude of the SP reflexes as a function of control responses in 4 muscles on the side of the stimulation. **(C,D)** SP facilitation by atropine 1 week after SCI. Atropine 1 and 2 correspond to intrathecal atropine (70 µg, 100 µl) whereas Bath Atropine 3 corresponds to a bath application of 1.5 ml of a solution containing 1 mg/ml of atropine.

before (dotted line), 53 (thin gray line) and 92 (thick black line) min post atropine in a cat spinalized 24 h before. Not only did the reflex increase in amplitude, but the latency decreased in St and GL.

Figure 11B illustrates the evolution of the response amplitude of cutaneous reflexes as a function of control responses in 4 muscles on the side of the stimulation. Amplitude of St and Srt responses significantly increased 200–300% above control level early after injection of atropine. There was clearly also a second phase of increased amplitude starting at about 40 min and lasting for at least 2 h after atropine injection.

Similar results were obtained in a 1 week spinal cat in which atropine was applied over the L3-L4 segments. In this case, the increase in reflex amplitude was obtained earlier, i.e., 42 min after intrathecal administration of atropine (**Figure 11C**). In the other 1 week spinal cat, the increase was obtained within the same delay as in the 24 h spinal cat, i.e., around 90 min (**Figure 11D**). We tested whether locomotion could be evoked by administration of a second dose of atropine in the lumbar bath. As illustrated in **Figure 11D**, each time atropine was given, the reflex amplitude increased (see at 180 min). However, as was the case in all cats detailed above, even this larger dose of atropine and with strong exteroceptive stimulation, no locomotion could be evoked. To test again whether this spinal cat was able to walk at all, clonidine (150 μg/kg, i.v.) was given and within a short time the usual nice locomotor pattern was evoked and reflex threshold increased from 170 to 500 μA as seen before with clonidine (Barbeau et al., 1987).

Atropine facilitates clonidine induction of locomotion

The previous results suggest that perhaps atropine and clonidine could have a synergistic effect, even at times after transection when atropine alone is ineffective. In one cat bath application of atropine was followed, 40 min later, by a small dose of i.v. clonidine. As was the case with the 4 above mentioned cats, atropine at L3-L4 did not evoke locomotion but, a small dose of clonidine (60 μg/kg, i.v.) 40 min after the atropine triggered a vigorous pattern of locomotion. It thus seems that even if atropine alone does not evoke locomotion, it acts synergistically with clonidine since a minimal dose of clonidine that normally fails to evoke locomotion can trigger the locomotor rhythm.

Effect of clonidine followed by atropine

To further validate the facilitation of locomotion produced by atropine, an even smaller sub-threshold dose of clonidine was first given (20 μg/kg, i.v.) and then atropine was given through a bath over L3-L4 in a 4 day spinal cat, a 5 day spinal cat as well as in an acute spinal cat. In all cases the dose of clonidine was insufficient to evoke locomotor rhythm by itself. **Figure 12** shows the EMG activity 80 min after clonidine injection and 40 min after atropine application. At 40 and 60 min post atropine some bursts of activity appear, but strong locomotor activity was observed only 110 min post atropine (160 min post clonidine). Similar results were found in another semi-chronic cat and in one acute spinal cat (**Figure 13**). This late activation of locomotion corresponds to

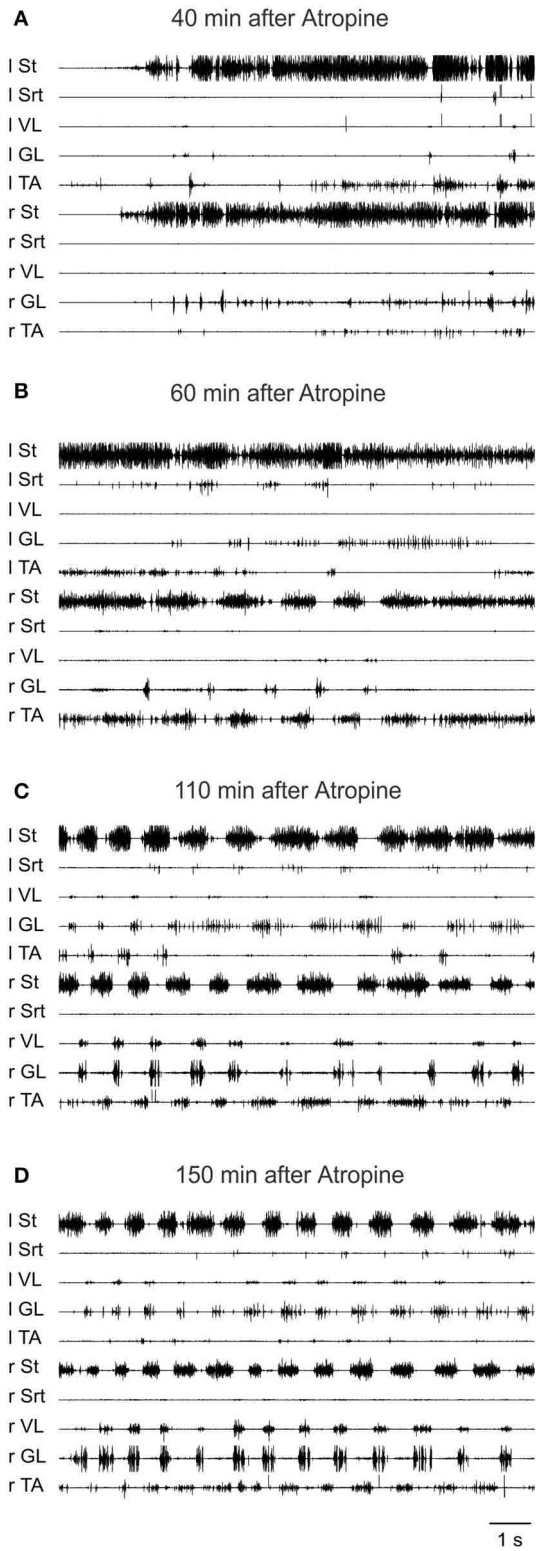

FIGURE 12 | The effect of a sub-threshold dose of clonidine is facilitated by atropine. Clonidine was first given i.v. (20 μg/kg) and then atropine (1.5 ml of a solution containing 1 mg/ml of atropine) was given through a bath covering the L3-L4 segments of the spinal cord 40 min later

(Continued)

FIGURE 12 | Continued
in a 4 days spinal cat. **(A)** EMG signals from hindlimbs muscles 80 min after clonidine injection and 40 min after atropine application. Some bursts of activity appear after atropine especially in the hip flexors (St); **(B)** 60 min after atropine some alternating activity appears between flexor and extensor muscles but it was not organized enough to trigger strong locomotion; **(C,D)** strong pattern of locomotion was observed 110 and 150 min post atropine.

the time of the peak reflex amplitude observed in other cats with atropine.

A plausible explanation for these results is that early after SCI atropine is ineffective alone due to the absence of some plasticity that increases muscarinic control of cutaneous input after chronic injury. In the presence of low-dose clonidine, however, we propose that a tonic cholinergic control of afferent input that suppresses feedback necessary for locomotion induction is already present. Atropine blocks this suppression and thus facilitates locomotion.

DISCUSSION

We first demonstrated in neonatal rat preparations that facilitation of the endogenous cholinergic propriospinal system can result in coordinated locomotor activity, suggesting it may be a substrate for recovery of locomotion after SCI. We demonstrated that specific muscarinic but not nicotinic receptors are involved in this process. We tested the importance of brainstem and spinal cholinergic systems in the initiation of locomotion from the MLR in adult decerebrate cats, and showed that systemic application of neither nicotinic nor muscarinic antagonists could interfere with MLR-evoked locomotion. We tested the hypothesis that cholinergic propriospinal cells contribute to the recovery of locomotor activity in spinal cats, and found that this hypothesis was not supported. Surprisingly, both nicotinic and muscarinic cholinergic antagonists facilitated locomotion, suggesting the development of a hyper-cholinergic state after SCI. The effects of carbachol and atropine were confirmed in chronic spinal rats, showing that the results obtained in the adult spinal cat were not due to a species difference. Finally, we provided evidence that an underlying change in cholinergic contributions to spinal control of locomotion was facilitation of the control of afferent input, so that cholinergic antagonists were effective facilitators of spinal locomotion. Different populations of cholinergic neurons that may contribute to these effects are illustrated in cartoon form in **Figure 14**. The actions of the various pharmacological agents on the different preparations used in this study are summarized in **Table 1**.

WELL-COORDINATED LOCOMOTION IS INDUCED *IN VITRO* BY FACILITATION OF THE ENDOGENOUS CHOLINERGIC PROPRIOSPINAL SYSTEM

The predominance of well-coordinated locomotion induced by EDRO documented in these experiments provides the first clear demonstration that facilitation of the effects of ACh released from the endogenous cholinergic propriospinal system is sufficient to activate the locomotor CPG in the isolated neonatal

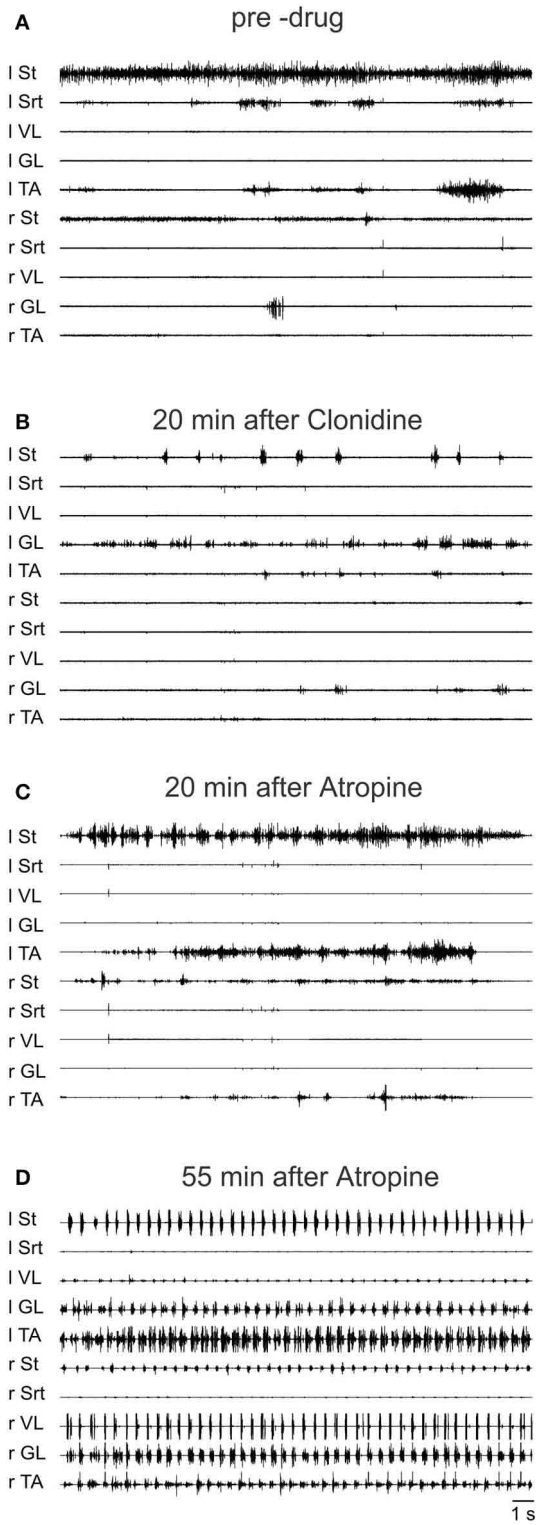

FIGURE 13 | Sub-threshold clonidine is facilitated by atropine in an acute spinal cat. (A) Pre-drug records of EMG signals from hindlimbs muscles; **(B)** 20 min after administration of a small volume of clonidine i.v. (20 μg/kg); **(C)** 20 min after bath atropine (1.5 ml of a solution containing 1 mg/ml of atropine) over L3-L4 and 40 min post clonidine; **(D)** 55 min after atropine application, a vigorous pattern of locomotion is represented by the EMG signals.

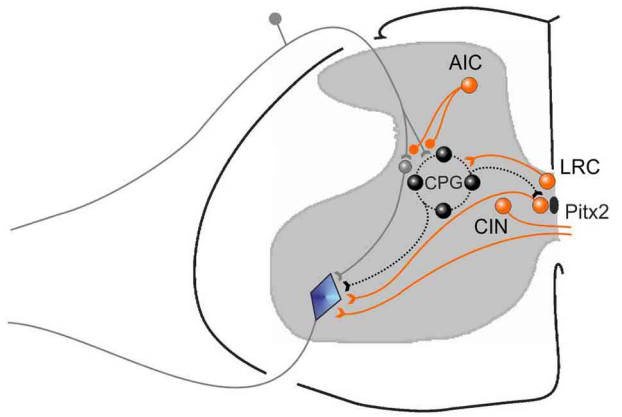

FIGURE 14 | Cartoon showing different functional groups of cholinergic neurons that are suggested by our results. A population for afferent input control (AIC) is illustrated, some of which are known to have direct presynaptic terminals on cutaneous afferents, as well as terminals on GABAergic interneruons (not shown) that may also be responsible for suppressing afferent input. AIC neurons act on their targets through both muscarinic and nicotinic receptors. A population of cholinergic neurons (LRC) that can control the locomotor rhythm by activating the central pattern generator for locomotion (CPG) are also shown. Their precise positions are unknown. They act on their target neurons via M3 receptors. Pitx2 neurons give rise to C terminals on motoneurons and increase motoneruron excitability by reducing the AHP amplitude via M2 receptors. They are also known as V0c neurons. Commissural cholinergic interneurons (CIN) are illustrated, many of which terminate on contralateral motoneurons and produce EPSPs in motoneurons via M-currents blocked by atropine. Other partition or central canal cluster cells with unknown function are likely also present in the spinal cord.

rat preparation. Previous experiments with application of exogenous ACh led to the conclusion that ACh-induced rhythmic activity was seldom locomotor-like, due to the predominance of ipsilateral flexor-extensor co-activation observed (Cowley and Schmidt, 1994). Other studies also suggested that cholinergic activation does not induce locomotor-like activity in the neonatal rat (Atsuta et al., 1991) or the mudpuppy (Fok and Stein, 2002) spinal cord. In the "motor functionally mature" mouse spinal cord, application of muscarine evoked irregular bursting in the ventral roots, and no locomotion (Jiang et al., 1999). According to Anglister et al. (2008), periods of well-coordinated locomotor activity can be revealed in the neonatal rat in the presence of EDRO and ACh, but only when coherent power analysis is used to analyze the variable activity produced by the network. Our detailed analysis of EDRO-induced locomotion presented here, however, clearly establishes that endogenous cholinergic activation is sufficient for the full expression of fictive locomotion. Similar results with another inhibitor of acetylcholine esterase (NEO) confirm this conclusion. Sacral application of EDRO has been shown to elicit locomotor activity in the lumbar cord (Finkel et al., 2014), and this component of a sacrocaudal afferent system capable of eliciting locomotor activity may have contributed to the results reported here. Our results suggest that using cholinesterase inhibitors allows the expression of cholinergic-induced rhythms triggered by release from the intrinsic cholinergic neurons, rather than by application of an exogenous agonist, to give rise to coordinated locomotion. This suggests that certain actions of exogenous cholinergic agonists may produce conflicting effects that actually interfere with the production of coordinated locomotion including flexor-extensor antagonist activity and right-left alternation. This could result for instance from conflicting effects of agonists on afferent inputs and central drive mechanisms. Locomotion induced by other means, such as MLR stimulation (this study) or exogenous neurochemicals such as 5-HT, dopamine and NMDA, is not antagonized by cholinergic blockers (Fok and Stein, 2002; Bertrand and Cazalets, 2011) indicating some specificity of the cholinergic antagonists used.

There is evidence for strong cholinergic influences on commissural cells (Carlin et al., 2006), and for cholinergic commissural cells that are active during locomotion (Huang et al., 2000). Some commissural cells with excitatory actions on contralateral motoneurons are cholinergic (Bertrand and Cazalets, 2011), and sacral cholinergic neurons modulate locomotor activity recorded from the lumbar segments *in vitro* (Finkel et al., 2014). A portion of these cholinergic neurons are commissural. Exogenous cholinergic agonists may have the effect of activating commissural cholinergic influences that predominate, provided the ipsilateral coordinating interneurons controlling flexor/extensor alternation are not so strongly activated.

The cholinergic input to spinal neurons includes C-terminals on motoneurons, which are now known to originate from V0c neurons that are characterized by Pitx2 expression (reviewed in Witts et al., 2014). They are active during locomotion, and they increase motoneuron firing by reducing the AHP amplitude *via* M_2 muscarinic receptors (Miles et al., 2007; Zagoraiou et al., 2009). Pitx2 V0c neurons provide cholinergic terminals on Ia inhibitory interneurons (IaINs) (Siembab et al., 2010), the neurons involved in reciprocal inhibition from primary afferent fibers (Jankowska and Roberts, 1972). It is clear, based on the size of the terminals, that those on IaINs are not C-terminals (Siembab et al., 2010).

M_2 RECEPTORS CONTROL MOTONEURON EXCITABILITY AND LOCOMOTOR FREQUENCY

The reduction of ENG amplitude observed in the cases of antagonists with high affinities for M_2 receptors (atropine, methoctramine, and to a lesser extent telenzepine, and 4-DAMP) is consistent with the presence of M_2 receptors at cholinergic terminals on motoneurons and with the finding that muscarinic receptor activation increases motoneuron excitability. The effects of various antagonists on the control of locomotion derived from the present results are summarized in **Table 1**. In adult mammalian motoneurons, cholinergic agents produce plateau-like responses (Zieglgansberger and Reiter, 1974), consistent with the muscarinic effects in the turtle spinal cord slice (Svirskis and Hounsgaard, 1998; Guertin and Hounsgaard, 1999). In other studies, cholinergic agonists have been shown to depolarize rat motoneurons (Evans, 1978; Jiang and Dun, 1986) and in mouse motoneurons, muscarinic receptor activation leads to an increase in excitability, manifested by an increase in f-I slope and a reduction in the post-spike afterhyperpolarization

Table 1 | Summary of the effects of drugs altering cholinergic function in the various preparations used in this study.

Pharmacological agents	Neonatal rat spinal cord preparation	Decerebrate cat preparation	Acute spinal cat	Early chronic spinal cat	Late chronic spinal cat	Late chronic spinal rat
Edrophonium (EDRO) AChE inhibitor	↑↑			↔		
Neostigmine (NEO) AChE inhibitor	↑↑					
EDRO + ACh AChE inhibitor + acetylocholine				↔		
EDRO + tubocurarine AChE inhibitor + nicotinic antagonist	↔					
Clonidine NA agonist			↑↑			
EDRO + clonidine AChE inhibitor+ NA agonist				↑↑		
Clonidine (low dose) NA agonist			↔			
Atropine muscarinic antagonist		↔	↔	↑↑		
Atropine + clonidine (low dose) muscarinic antagonist + NA agonist			↑↑			
Clonidine (low dose) + atropine NA agonist + muscarinic antagonist			↑↑			
Carbachol cholinergic agonist				↓↓		↓↓
Carbachol + atropine cholinergic agonist + muscarinic antagonist				↑↑		↑↑
Atropine + carbachol muscarinic antagonist + cholinergic agonist				↓↓		
EDRO + atropine AChE inhibitor + muscarinic antagonist	↑↓↓					
EDRO + telenzepine AChE inhibitor + M_1 antagonist	↔					
EDRO + telenzepine (high dose) AChE inhibitor + M_1 antagonist	↓↓					
EDRO + methoctramine (METHOC) AChE inhibitor + M_2 antagonist	↑↓					
EDRO + METHOC (high dose) AChE inhibitor + M_2 antagonist	↓↓					
EDRO + 4-diphenylacetoxy-N-methylpiperidine metiodide (4-DAMP) AChE inhibitor + M_3 antagonist	↓↓				↑↑	
MT-3 M_4 antagonist	↔					
Mecamylamine nicotinic antagonist		↔				
Mecamylamine + atropine nicotinic antagonist + muscarinic antagonist		↔				
Dihydro-β-erythrpidine nicotinic antagonist					↑↑	

↑↑, *induced or enhanced locomotion;* ↓↓, *blocked locomotion;* ↑, *increased frequency;* ↓, *decreased amplitude;* ↔, *no effect.*

(Miles et al., 2007). This effect is due to M_2 receptor activation at C-terminals on motoneurons (Evans, 1978; Witts et al., 2014). Other neurons involved in controlling the CPG for locomotion, currently unidentified, are implicated by the increase in locomotor frequency produced by these drugs, consistent with some tonic cholinergic suppression of CPG activity. Importantly, M_2 receptors, but not M_1 or M_3 receptors, reduce glutamate release from primary afferents (Chen et al., 2014).

M_3 RECEPTORS ARE INVOLVED IN CHOLINERGIC ACTIVATION OF THE SPINAL LOCOMOTOR CPG

The ability of the M_3 receptor antagonist 4-DAMP (**Table 1**) to initially reduce the frequency of locomotion induced by EDRO and then to block it entirely at doses as low as 3 nM suggests that the cells of the locomotor network that are activated by the endogenous spinal action of ACh possess M_3 receptors. The distribution of M_3 receptors in the spinal cord has not been extensively described, although cells with M_3 receptors have been observed in laminae VII, VIII and X (Wilson et al., 2004). Whether any of the cholinergic propriospinal neurons are among those with M_3 receptors is not known. There is evidence, however, that cholinergic cells in the dorsal horn, lamina X, and the lateral intermediate zone (laminae VI) are densely labeled with M_2 receptors (Stewart and Maxwell, 2003). The distribution of neurons in the ventral horn with M_2 receptors has not been examined in detail, except in the case of motoneurons (Wilson et al., 2004).

In summary, the *in vitro* data presented here establish that well-coordinated locomotion can be produced in the isolated neonatal rat spinal cord, and suggest that an endogenous propriospinal cholinergic system could be a potential contributor to the recovery of locomotion after SCI due to its ability to activate the CPG for locomotion. The cholinergic propriospinal system and glutamatergic propriospinal cells endogenous to the spinal cord are among the remaining groups that might be responsible for the ability of most animal species to regain locomotor function after complete SCI (Jordan and Schmidt, 2002; Rossignol et al., 2002; Rossignol, 2006).

DOES THE CHOLINERGIC PROPRIOSPINAL SYSTEM CONTRIBUTE TO NORMAL LOCOMOTION OR TO RECOVERY OF LOCOMOTION AFTER SCI?

Our experiments using MLR-evoked locomotion in adult cats with i.v. application of both nicotinic and muscarinic antagonists (summarized in **Table 1**) suggest that there is very little contribution to normal locomotion by cholinergic systems, either at the spinal or the brainstem level. This finding is not consistent with the report by Garcia-Rill et al. (1987) who showed that muscarinic receptor blockade by injecting antagonists directly into the caudal brainstem could abolish MLR-evoked locomotion. Our results show that even in cases where the stimulus site is optimum for activation of the cholinergic cells in the vicinity of the MLR (i.e., pedunculopontine nucleus: cf. Rye et al., 1987), no decrement is observed in the ability of MLR stimulation to evoke locomotion. It would appear, therefore, that the frequently observed overlap of cholinergic cells with the lowest threshold stimulation sites within the MLR (Garcia-Rill et al., 1987; Garcia-Rill and Skinner, 1988) is coincidental. Destruction of cholinergic cells in the dorsolateral tegmentum-cholinergic cell area of cats (in the region corresponding to the MLR) does not abolish the locomotor capacity of the animal once recovery from the surgery is complete (Webster and Jones, 1988). We conclude that the cholinergic contribution to the induction of locomotion is not obligatory in mammals.

The absence of effects at the spinal level is consistent with the failure of cholinergic antagonists to alter motoneuron activity in cases where the activity is not produced by cholinergic activation. The possibility that these results differ from those with intrathecal drug application because of a reduced ability of atropine and/or mecamylamine to enter the spinal cord in sufficient concentrations to alter locomotion was discussed in the Methods section of this paper, where we pointed out that the doses of these drugs chosen were equivalent to those used previously to effectively alter CNS effects. Clearly these drugs cross the blood-brain barrier in sufficient quantities to produce effects on CNS processes.

In experiments on spinal cats we found that ACh, or EDRO or carbachol, could not initiate locomotion soon after spinalization (**Table 1**) but rather induced an increased tonic excitability so that upon exteroceptive stimulation sustained contractions could be evoked but no locomotion. This may have been due to an excitatory effect on motoneurons. Our experiments with i.t. applications of carbachol in chronic spinal rats confirmed that carbachol dramatically interferes with spinal stepping, and this effect is reversed by atropine. To our surprise, atropine could improve the spontaneous emerging locomotor pattern in the second postspinal week in spinal cats. We also found a synergy between clonidine and atropine at this stage after SCI, so that a small dose of clonidine which normally does not trigger locomotion could now evoke a clear locomotor pattern. Dihydro-β-erythroidine, a nicotinic antagonist, was also capable of facilitating locomotion in chronic spinal cat, suggesting both muscarinic and nicotinic control of the cholinergic propriospinal system.

This work suggests that in early days after spinalization, there is a hyper-cholinergic state of the cord that interferes with the early production of rhythmic activity. It indeed appears that adding ACh stimulation just further disrupts the ability to walk. This is consistent with observations using high dose intrathecal cholinergic agonist applications in intact rats (Yaksh et al., 1985; Gillberg et al., 1990). The increased excitability might be attributable to a direct action of cholinergic agonists on motoneurons, which can be excited by ACh acting at M_2 receptors (Witts et al., 2014). This is consistent with our finding *in vitro* that motoneuron activity during fictive locomotion is reduced by activation of cholinergic M_2 receptors. In addition to powerful suppression of cutaneous afferent input, possibly via M_2 receptors (Chen et al., 2014), the cholinergic system appears to reorganize after SCI so that there is an increased cholinergic suppression of CPG activation. This would explain the absence of a locomotor facilitation by atropine early after SCI while facilitating afferent input, and the efficacy of clonidine to elicit locomotion at this stage. We interpret the fact that clonidine and atropine have a synergistic effect at very low doses at this stage as evidence that some means of activating the CPG is required at this stage, but a few weeks after SCI development of cholinergic control of some portion of the CPG allows atropine to elicit locomotor activity without the need for another means to facilitate CPG activity.

An important finding from these studies is the contrast between the locomotor promoting action of cholinergic activation in the neonatal preparation and the reversal of this effect to one that suppresses locomotion in the adult. There is no obvious explanation for this, except to speculate that there may be features of the cholinergic control that are present in the adult but absence in the neonate, and vice versa. These might include differential effects of descending pathways on the cholinergic propriospinal system, or the later development of tonic control of afferent input.

There is evidence that the cholinergic terminals of the dorsal horn do not appear until sometime in the post-natal period (Phelps et al., 1984), so their influence would not be exerted in neonatal preparations.

Control of afferent input in adult animals is a mechanism revealed by our results with injections of atropine. ACh exerts both muscarinic and nicotinic control over sensory inputs (Gillberg et al., 1990; Zhuo and Gebhart, 1991; Li et al., 2002; Zhang et al., 2007; Chen et al., 2010, 2014; Hochman et al., 2010) and it is possible that early on after spinalization a powerful inhibition is exerted on afferent pathways that can be reversed by cholinergic antagonists. It is known that after spinalization, cats and rats depend much more on sensory inputs, having lost all descending inputs, particularly relying on cutaneous afferents from the foot (Bouyer and Rossignol, 2003b; Sławińska et al., 2012). Our results showing atropine effects on the recovery of locomotion and atropine enhancement of cutaneous input from the SP nerve suggest that such a mechanism underlies these results. This is also consistent with the differences between *in vitro* locomotion, which is without cutaneous input from load receptors on the foot pad, and the effects of the cholinergic agonists and antagonists during treadmill stepping in spinal cats. Such cutaneous input is an absolute requirement for locomotion in spinal cats (Bouyer and Rossignol, 2003b).

The possible role of ACh in the control of afferents that influence locomotion has not been investigated, although there is evidence for cholinergic presynaptic inhibition (Ribeiro-Da-Silva and Cuello, 1990; Hochman et al., 2010), including control of non-nociceptive cutaneous afferents. Consistent with this, M_2 receptors are found on primary afferent fibers, and they are eliminated with dorsal rhizotomy (Li et al., 2002). It is also clear that there is postsynaptic control of sensory transmission by cholinergic neurons of the dorsal horn. For example, there is evidence for nicotinic modulation of GABAergic control of sensory input (Genzen and McGehee, 2005). The relative contributions of nicotinic and muscarinic receptors to the control of the sensory input necessary for enhancement of locomotion by cholinergic antagonists in our study is unknown, but it is clear that both types of receptors are involved in the control of sensory input to the spinal cord.

IS THE CHOLINERGIC PROPRIOSPINAL SYSTEM MODIFIED AFTER SCI?

Chartlon et al. (1981) and Faden et al. (1986) found that muscarinic cholinergic receptor binding is reduced after spinal cord transection. A dramatic decrease in cholinergic boutons on tail motoneurons was observed after sacral SCI (Kitzman, 2006). Skup et al. (2012) showed VAChT + boutons on TA MNs increased slightly after SCI and locomotor training, while VAChT boutons decreased dramatically on Sol MNs after spinalization, and this was partly restored with training. Kapitza et al. (2012) observed a progressive decrease in cholinergic input onto motoneuron soma, and shrinkage of cholinergic interneuron cell bodies located around the central canal after SCI. They suggested that "... reduced cholinergic input on motoneurons is assumed to result in the rapid exhaustion of the central drive required for the performance of locomotor movements in animals and humans..." These observations predict that the effect of SCI should be a reduced excitability of motoneurons and other targets of the cholinergic propriospinal cells. Our results directly contradict these predictions, and show evidence for a hyper-cholinergic state after SCI. They suggest the use of cholinergic antagonists as a potential treatment of recovery of function after SCI. They further suggest that the suppression of afferent input to the spinal cord by ACh *via* both muscarinic and nicotinic receptors is the key to understanding the role of ACh in motor control after SCI.

CONCLUSIONS

Our results show a potent cholinergic control of locomotor activity emerges after spinal cord injury, but in contrast to expectations, it was neither a decrease in motoneuron excitability, as predicted by recent findings, nor a recruitment of cholinergic propriospinal cells to account for stepping in spinal animals. Instead, a hyper-cholinergic state that supressed locomotor activity emerged, and this could be reduced with cholinergic antagonists to facilitate locomotion. **Figure 14** summarizes the cholinergic neurons that might account for our results. V0c (Pitx2) and commissural cholinergic neurons (CIN) contact motoneurons directly and increase their excitability, while unknown cholinergic interneurons, likely from the partition cell and central canal cluster cell groups, project to CPG neurons and account for the cholinergic locomotor rhythm control (LRC). Dorsal horn cholinergic interneurons known to inhibit cutaneous afferent input and produce analgesia, either by presynaptic inhibition or by controlling other interneurons (not shown) that are responsible for afferent input control (AIC) are also illustrated. The AIC cholinergic cells are the most likely ones to account for the ability of cholinergic antagonists to facilitate locomotion. We believe such a population of AIC cholinergic neurons must tonically inhibit afferent feedback necessary for locomotor activity in spinal animals. It is likely that controlling the activity of these neurons will provide a new opportunity for restoring locomotion after injury.

AUTHOR CONTRIBUTIONS

Serge Rossignol and Larry M. Jordan conceived and designed the experiments on neonatal rats and spinal cats. B. R. Noga designed and carried out the experiments on decerebrate cats with systemic drug applications. J. R. McVagh carried out the *in vitro* experiments on neonatal rat spinal cord. J. Provencher, H. Leblond, Larry M. Jordan, and Serge Rossignol performed the experiments on chronic spinal cats, while A. M. Cabaj, H. Majczyński, Urszula Sławińska, and Larry M. Jordan carried out the intrathecal drug application experiments on chronic spinal rats. Larry M. Jordan, Serge Rossignol, Urszula Sławińska, J. R. McVagh, H. Leblond and B. R. Noga wrote various aspects of the paper. All the authors discussed the results and accepted the final version of this manuscript.

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REFERENCES

Anglister, L., Etlin, A., Finkel, E., Durrant, A. R., and Lev-Tov, A. (2008). Cholinesterases in development and disease. *Chem. Biol. Interact.* 175, 92–100. doi: 10.1016/j.cbi.2008.04.046

Atsuta, Y., Abraham, P., Iwahara, T., Garcia-Rill, E., and Skinner, R. D. (1991). Control of locomotion *in vitro*: II. Chemical stimulation. *Somatosens. Mot. Res.* 8, 55–63. doi: 10.3109/08990229109144729

Barbeau, H., Julien, C., and Rossignol, S. (1987). The effects of clonidine and yohimbine on locomotion and cutaneous reflexes in the adult chronic spinal cat. *Brain Res.* 437, 83–96. doi: 10.1016/0006-8993(87)91529-0

Barber, R. P., Phelps, P. E., Houser, C. R., Crawford, G. D., Salvaterra, P. M., and Vaughn, J. E. (1984). The morphology and distribution of neurons containing choline acetyltransferase in the adult rat spinal cord: an immunocytochemical study. *J. Comp. Neurol.* 229, 329–346. doi: 10.1002/cne.902290305

Berman, S. L. (1968). *The Brain Stem of the Cat*. Madison, WI: University of Wisconsin Press.

Belanger, M., Drew, T., Provencher, J., and Rossignol, S. (1996). A comparison of treadmill locomotion in adult cats before and after spinal transection. *J. Neurophysiol.* 76, 471–491.

Bertrand, S. S., and Cazalets, J. R. (2011). Cholinergic partition cells and lamina x neurons induce a muscarinic-dependent short-term potentiation of commissural glutamatergic inputs in lumbar motoneurons. *Front. Neural. Circuits* 5:15. doi: 10.3389/fncir.2011.00015

Borges, L. F., and Iversen, S. D. (1986). Topography of choline acetyltransferase immunoreactive neurons and fibers in the rat spinal cord. *Brain Res.* 362, 140–148. doi: 10.1016/0006-8993(86)91407-1

Bouyer, L. J., and Rossignol, S. (2003a). Contribution of cutaneous inputs from the hindpaw to the control of locomotion. I. Intact cats. *J. Neurophysiol.* 90, 3625–3639. doi: 10.1152/jn.00496.2003

Bouyer, L. J., and Rossignol, S. (2003b). Contribution of cutaneous inputs from the hindpaw to the control of locomotion. II. Spinal cats. *J. Neurophysiol.* 90, 3640–3653. doi: 10.1152/jn.00497.2003

Bouyer, L. J., Whelan, P. J., Pearson, K. G., and Rossignol, S. (2001). Adaptive locomotor plasticity in chronic spinal cats after ankle extensors neurectomy. *J. Neurosci.* 21, 3531–3541.

Brownstone, R. M. (2006). Beginning at the end: repetitive firing properties in the final common pathway. *Prog. Neurobiol.* 78, 156–172. doi: 10.1016/j.pneurobio.2006.04.002

Carlin, K. P., Dai, Y., and Jordan, L. M. (2006). Cholinergic and serotonergic excitation of ascending commissural neurons in the thoraco-lumbar spinal cord of the neonatal mouse. *J. Neurophysiol.* 95, 1278–1284. doi: 10.1152/jn.00963.2005

Carr, P. A., Huang, A., Noga, B. R., and Jordan, L. M. (1995). Cytochemical characteristics of cat spinal neurons activated during fictive locomotion. *Brain Res. Bull.* 37, 213–218. doi: 10.1016/0361-9230(94)00271-2

Carr, P. A., Noga, B. R., Nance, D. M., and Jordan, L. M. (1994). Intracellular labeling of cat spinal neurons using a tetramethylrhodamine-dextran amine conjugate. *Brain Res. Bull.* 34, 447–451. doi: 10.1016/0361-9230(94)90017-5

Chartlon, G., Brennan, M. J. W., and Ford, D. M. (1981). Alterations in neurotransmitter receptor binding in the spinal cord after transection. *Brain Res.* 218, 372–375.

Chau, C., Barbeau, H., and Rossignol, S. (1998). Effects of intrathecal alpha1- and alpha2-noradrenergic agonists and norepinephrine on locomotion in chronic spinal cats. *J. Neurophysiol.* 79, 2941–2963.

Chen, S. R., Chen, H., Yuan, W. X., Wess, J., and Pan, H. L. (2010). Dynamic control of glutamatergic synaptic input in the spinal cord by muscarinic receptor subtypes defined using knockout mice. *J. Biol. Chem.* 285, 40427–40437. doi: 10.1074/jbc.M110.176966

Chen, S. R., Chen, H., Yuan, W. X., Wess, J., and Pan, H. L. (2014). Differential Regulation of Primary Afferent Input to Spinal Cord by Muscarinic Receptor Subtypes Delineated Using Knockout Mice. *J. Biol. Chem.* 289, 14321–14330. doi: 10.1074/jbc.M114.550384

Cowley, K. C., and Schmidt, B. J. (1994). A comparison of motor patterns induced by N-methyl-D-aspartate, acetylcholine and serotonin in the *in vitro* neonatal rat spinal cord. *Neurosci. Lett.* 171, 147–150. doi: 10.1016/0304-3940(94)90626-2

Cowley, K. C., Zaporozhets, E., Maclean, J. N., and Schmidt, B. J. (2005). Is NMDA receptor activation essential for the production of locomotor-like activity in the neonatal rat spinal cord? *J. Neurophysiol.* 94, 3805–3814. doi: 10.1152/jn.00016.2005

Crawford, J. M., and Curtis, D. R. (1966). Pharmacological studies on feline Betz cells. *J. Physiol. (Lond.)* 186, 121–138.

Dai, Y., Carlin, K. P., Li, Z., Mcmahon, D. G., Brownstone, R. M., and Jordan, L. M. (2009). Electrophysiological and pharmacological properties of locomotor activity-related neurons in cfos-EGFP mice. *J. Neurophysiol.* 102, 3365–3383. doi: 10.1152/jn.00265.2009

Dai, Y., and Jordan, L. M. (2010). Multiple effects of serotonin and acetylcholine on hyperpolarization-activated inward current in locomotor activity-related neurons in Cfos-EGFP mice. *J. Neurophysiol.* 104, 366–381. doi: 10.1152/jn.01110.2009

Dubuc, R., Brocard, F., Antri, M., Fenelon, K., Gariepy, J. F., Smetana, R., et al. (2008). Initiation of locomotion in lampreys. *Brain Res. Rev.* 57, 172–182. doi: 10.1016/j.brainresrev.2007.07.016

Eisenach, J. C. (1999). Muscarinic-mediated analgesia. *Life Sci.* 64, 549–554. doi: 10.1016/S0024-3205(98)00600-6

English, A. W. (1984). An electromyographic analysis of compartments in cat lateral gastrocnemius muscle during unrestrained locomotion. *J. Neurophysiol.* 52, 114–125.

English, A. W., and Weeks, O. I. (1989). Electromyographic cross-talk within a compartmentalized muscle of the cat. *J. Physiol. (Lond.)* 416, 327–336.

Evans, R. H. (1978). Cholinoceptive properties of motoneurons of the immature rat spinal cord maintained *in vitro*. *Neuropharmacology* 17, 277–279. doi: 10.1016/0028-3908(78)90112-0

Faden, A. I., Pilotte, N. S., and Burt, D. R. (1986). Experimental spinal cord injury: effects of trauma or ischemia on TRH and muscarinic receptors. *Neurology* 36, 723–726. doi: 10.1212/WNL.36.5.723

Finkel, E., Etlin, A., Cherniak, M., Mor, Y., Lev-Tov, A., and Anglister, L. (2014). Neuroanatomical basis for cholinergic modulation of locomotor networks by sacral relay neurons with ascending lumbar projections. *J. Comp. Neurol.* 522, 3437–3455. doi: 10.1002/cne.23613

Fok, M., and Stein, R. B. (2002). Effects of cholinergic and noradrenergic agents on locomotion in the mudpuppy (*Necturus maculatus*). *Exp. Brain Res.* 145, 498–504. doi: 10.1007/s00221-002-1125-6

Garcia-Rill, E. (1986). The basal ganglia and the locomotor regions. *Brain Res.* 396, 47–63. doi: 10.1016/0165-0173(86)90009-3

Garcia-Rill, E., Houser, C. R., Skinner, R. D., Smith, W., and Woodward, D. J. (1987). Locomotion-inducing sites in the vicinity of the pedunculopontine nucleus. *Brain Res. Bull.* 18, 731–738. doi: 10.1016/0361-9230(87)90208-5

Garcia-Rill, E., Simon, C., Smith, K., Kezunovic, N., and Hyde, J. (2011). The pedunculopontine tegmental nucleus: from basic neuroscience to neurosurgical applications: arousal from slices to humans: implications for DBS. *J. Neural. Trans.* 118, 1397–1407. doi: 10.1007/s00702-010-0500-x

Garcia-Rill, E., and Skinner, R. D. (1987). The mesencephalic locomotor region. I. Activation of a medullary projection site. *Brain Res.* 411, 1–12. doi: 10.1016/0006-8993(87)90675-5

Garcia-Rill, E., and Skinner, R. D. (1988). Modulation of rhythmic function in the posterior midbrain. *Neuroscience* 27, 639–654. doi: 10.1016/0306-4522(88)90295-3

Genzen, J. R., and McGehee, D. S. (2005). Nicotinic modulation of GABAergic synaptic transmission in the spinal cord dorsal horn. *Brain Res.* 1031, 229–237. doi: 10.1016/j.brainres.2004.10.042

Gillberg, P. G., Hartvig, P., Gordh, T., Sottile, A., Jansson, I., Archer, T., et al. (1990). Behavioral effects after intrathecal administration of cholinergic receptor agonists in the rat. *Psychopharmacology (Berl.)* 100, 464–469. doi: 10.1007/BF02243997

Guertin, P. A., and Hounsgaard, J. (1999). L-type calcium channels but not N-methyl-D-aspartate receptor channels mediate rhythmic activity induced by

cholinergic agonist in motoneurons from turtle spinal cord slices. *Neurosci. Lett.* 261, 81–84. doi: 10.1016/S0304-3940(99)00013-0

Hellstrom, J., Oliveira, A. L., Meister, B., and Cullheim, S. (2003). Large cholinergic nerve terminals on subsets of motoneurons and their relation to muscarinic receptor type 2. *J. Comp. Neurol.* 460, 476–486. doi: 10.1002/cne.10648

Hochman, S., Shreckengost, J., Kimura, H., and Quevedo, J. (2010). Presynaptic inhibition of primary afferents by depolarization: observations supporting nontraditional mechanisms. *Ann. N.Y. Acad. Sci.* 1198, 140–152. doi: 10.1111/j.1749-6632.2010.05436.x

Houser, C. R., Barber, R. P., Crawford, G. D., Matthews, D. A., Phelps, P. E., Salvaterra, P. M., et al. (1984). Species-specific second antibodies reduce spurious staining in immunocytochemistry. *J. Histochem. Cytochem.* 32, 395–402. doi: 10.1177/32.4.6368679

Huang, A., Noga, B. R., Carr, P. A., Fedirchuk, B., and Jordan, L. M. (2000). Spinal cholinergic neurons activated during locomotion: localization and electrophysiological characterization. *J. Neurophysiol.* 83, 3537–3547.

Jakeman, L. B., Wei, P., Guan, Z., and Stokes, B. T. (1998). Brain-derived neurotrophic factor stimulates hindlimb stepping and sprouting of cholinergic fibers after spinal cord injury. *Exp. Neurol.* 154, 170–184. doi: 10.1006/exnr.1998.6924

Jankowska, E., and Roberts, W. J. (1972). Synaptic actions of single interneurones mediating reciprocal Ia inhibition of motoneurones. *J. Physiol. (Lond.)* 222, 623–642.

Jiang, Z., Carlin, K. P., and Brownstone, R. M. (1999). An *in vitro* functionally mature mouse spinal cord preparation for the study of spinal motor networks. *Brain Res.* 816, 493–499. doi: 10.1016/S0006-8993(98)01199-8

Jiang, Z. G., and Dun, N. J. (1986). Presynaptic suppression of excitatory postsynaptic potentials in rat ventral horn neurons by muscarinic agonists. *Brain Res.* 381, 182–186. doi: 10.1016/0006-8993(86)90710-9

Jordan, L. M. (1998). Initiation of locomotion in mammals. *Ann. N.Y. Acad. Sci.* 860, 83–93. doi: 10.1111/j.1749-6632.1998.tb09040.x

Jordan, L. M., and Schmidt, B. J. (2002). Propriospinal neurons involved in the control of locomotion: potential targets for repair strategies? *Prog. Brain Res.* 137, 125–139. doi: 10.1016/S0079-6123(02)37012-2

Kapitza, S., Zorner, B., Weinmann, O., Bolliger, M., Filli, L., Dietz, V., et al. (2012). Tail spasms in rat spinal cord injury: changes in interneuronal connectivity. *Exp. Neurol.* 236, 179–189. doi: 10.1016/j.expneurol.2012.04.023

Khan, I. M., Yaksh, T. L., and Taylor, P. (1994). Ligand specificity of nicotinic acetylcholine receptors in rat spinal cord: studies with nicotine and cytisine. *J. Pharmacol. Exp. Ther.* 270, 159–166.

Kiehn, O., Johnson, B. R., and Raastad, M. (1996). Plateau properties in mammalian spinal interneurons during transmitter-induced locomotor activity. *Neuroscience* 75, 263–273. doi: 10.1016/0306-4522(96)00250-3

Kitzman, P. (2006). Changes in vesicular glutamate transporter 2, vesicular GABA transporter and vesicular acetylcholine transporter labeling of sacrocaudal motoneurons in the spastic rat. *Exp. Neurol.* 197, 407–419. doi: 10.1016/j.expneurol.2005.10.005

Kjaerulff, O., and Kiehn, O. (1996). Distribution of networks generating and coordinating locomotor activity in the neonatal rat spinal cord *in vitro*: a lesion study. *J. Neurosci.* 16, 5777–5794.

Kriellaars, D. J., Brownstone, R. M., Noga, B. R., and Jordan, L. M. (1994). Mechanical entrainment of fictive locomotion in the decerebrate cat. *J. Neurophysiol.* 71, 2074–2086.

Lake, N. (1973). Studies of the habenulo-interpeduncular pathway in cats. *Exp. Neurol.* 41, 113–132. doi: 10.1016/0014-4886(73)90184-2

Li, D. P., Chen, S. R., Pan, Y. Z., Levey, A. I., and Pan, H. L. (2002). Role of presynaptic muscarinic and GABA(B) receptors in spinal glutamate release and cholinergic analgesia in rats. *J. Physiol. (Lond.)* 543, 807–818. doi: 10.1113/jphysiol.2002.020644

Liu, J., and Jordan, L. M. (2005). Stimulation of the parapyramidal region of the neonatal rat brain stem produces locomotor-like activity involving spinal 5-HT7 and 5-HT2A receptors. *J. Neurophysiol.* 94, 1392–1404. doi: 10.1152/jn.00136.2005

Majczyński, H., Cabaj, A., and Górska, T. (2005). Intrathecal application of cyproheptadine impairs locomotion in intact rats. *Neurosci. Lett.* 381, 16–20. doi: 10.1016/j.neulet.2005.01.075

Majczyński, H., Cabaj, A., Sławińska, U., and Górska, T. (2006). Intrathecal administration of yohimbine impairs locomotion in intact rats. *Behav. Brain Res.* 175, 315–322. doi: 10.1016/j.bbr.2006.08.040

Marcoux, J., and Rossignol, S. (2000). Initiating or blocking locomotion in spinal cats by applying noradrenergic drugs to restricted lumbar spinal segments. *J. Neurosci.* 20, 8577–8585.

Martin, B. R., Onaivi, E. S., and Martin, T. J. (1989). What is the nature of mecamylamine's antagonism of the central effects of nicotine? *Biochem. Pharmacol.* 38, 3391–3397. doi: 10.1016/0006-2952(89)90106-8

McCance, I., and Phillis, J. W. (1968). Cholinergic mechanisms in the cerebellar cortex. *Int. J. Neuropharmacol.* 7, 447–462. doi: 10.1016/0028-3908(68)90044-0

McCance, I., Phillis, J. W., Tebecis, A. K., and Westerman, R. A. (1968a). The pharmacology of acetylcholine-excitation of thalamic neurones. *Br. J. Pharmacol. Chemother.* 32, 652–662. doi: 10.1111/j.1476-5381.1968.tb00464.x

McCance, I., Phillis, J. W., and Westerman, R. A. (1968b). Acetylcholine-sensitivity of thalamic neurones: its relationship to synaptic transmission. *Br. J. Pharmacol. Chemother.* 32, 635–651. doi: 10.1111/j.1476-5381.1968.tb00463.x

Michel, A. D., Stefanich, E., and Whiting, R. L. (1989). Direct labeling of rat M3-muscarinic receptors by [^{3}H]4DAMP. *Eur. J. Pharmacol.* 166, 459–466.

Miles, G. B., Hartley, R., Todd, A. J., and Brownstone, R. M. (2007). Spinal cholinergic interneurons regulate the excitability of motoneurons during locomotion. *Proc. Natl. Acad. Sci. U.S.A.* 104, 2448–2453. doi: 10.1073/pnas.0611134104

Miles, G. B., and Sillar, K. T. (2011). Neuromodulation of vertebrate locomotor control networks. *Physiology (Bethesda)* 26, 393–411. doi: 10.1152/physiol.00013.2011

Noga, B. R., Shefchyk, S. J., Jamal, J., and Jordan, L. M. (1987). The role of Renshaw cells in locomotion: antagonism of their excitation from motor axon collaterals with intravenous mecamylamine. *Exp. Brain Res.* 66, 99–105. doi: 10.1007/BF00236206

Pearson, K. G. (2004). Generating the walking gait: role of sensory feedback. *Prog. Brain Res.* 143, 123–129. doi: 10.1016/S0079-6123(03)43012-4

Phelps, P. E., Barber, R. P., Houser, C. R., Crawford, G. D., Salvaterra, P. M., and Vaughn, J. E. (1984). Postnatal development of neurons containing choline acetyltransferase in rat spinal cord: an immunocytochemical study. *J. Comp. Neurol.* 229, 347–361. doi: 10.1002/cne.902290306

Proakis, A. G., and Harris, G. B. (1978). Comparative penetration of glycopyrrolate and atropine across the blood–brain and placental barriers in anesthetized dogs. *Anesthesiology* 48, 339–344. doi: 10.1097/00000542-197805000-00007

Ribeiro-Da-Silva, A., and Cuello, A. C. (1990). Choline acetyltransferase-immunoreactive profiles are presynaptic to primary sensory fibers in the rat superficial dorsal horn. *J. Comp. Neurol.* 295, 370–384. doi: 10.1002/cne.902950303

Rossignol, S. (2006). Plasticity of connections underlying locomotor recovery after central and/or peripheral lesions in the adult mammals. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 361, 1647–1671. doi: 10.1098/rstb.2006.1889

Rossignol, S., Bouyer, L., Barthelemy, D., Langlet, C., and Leblond, H. (2002). Recovery of locomotion in the cat following spinal cord lesions. *Brain Res. Brain Res. Rev.* 40, 257–266. doi: 10.1016/S0165-0173(02)00208-4

Ryall, R. W., and Haas, H. L. (1975). "On the physiological significance of muscainic receptors on Renshaw Cells: a hypothesis," in *Cholinergic Mechanisms,* ed P. G. Waser (New York, NY: Raven Press), 335–341.

Ryczko, D., and Dubuc, R. (2013). The multifunctional mesencephalic locomotor region. *Curr. Pharm. Des.* 19, 4448–4470. doi: 10.2174/1381612811319240011

Rye, D. B., Saper, C. B., Lee, H. J., and Wainer, B. H. (1987). Pedunculopontine tegmental nucleus of the rat: cytoarchitecture, cytochemistry, and some extrapyramidal connections of the mesopontine tegmentum. *J. Comp. Neurol.* 259, 483–528. doi: 10.1002/cne.902590403

Shik, M. L., Severin, F. V., and Orlovskii, G. N. (1966). Control of walking and running by means of electric stimulation of the midbrain. *Biofizika* 11, 659–666.

Sholomenko, G. N., Funk, G. D., and Steeves, J. D. (1991). Avian locomotion activated by brainstem infusion of neurotransmitter agonists and antagonists. I. Acetylcholine excitatory amino acids and substance P. *Exp. Brain Res.* 85, 659–673. doi: 10.1007/BF00231752

Siembab, V. C., Smith, C. A., Zagoraiou, L., Berrocal, M. C., Mentis, G. Z., and Alvarez, F. J. (2010). Target selection of proprioceptive and motor axon synapses on neonatal V1-derived Ia inhibitory interneurons and Renshaw cells. *J. Comp. Neurol.* 518, 4675–4701. doi: 10.1002/cne.22441

Skup, M., Gajewska-Wozniak, O., Grygielewicz, P., Mankovskaya, T., and Czarkowska-Bauch, J. (2012). Different effects of spinalization and locomotor training of spinal animals on cholinergic innervation of the soleus and tibialis anterior motoneurons. *Eur. J. Neurosci.* 36, 2679–2688. doi: 10.1111/j.1460-9568.2012.08182.x

Sławińska, U., Majczyński, H., Dai, Y., and Jordan, L. M. (2012). The upright posture improves plantar stepping and alters responses to serotonergic drugs in spinal rats. *J. Physiol.* 590, 1721–1736. doi: 10.1113/jphysiol.2011.224931

Sławińska, U., Majczyński, H., and Djavadian, R. (2000). Recovery of hindlimb motor functions after spinal cord transection is enhanced by grafts of the embryonic raphe nuclei. *Exp. Brain Res.* 132, 27–38. doi: 10.1007/s002219900323

Sławińska, U., Miazga, K., Cabaj, A. M., Leszczyńska, A. N., Majczyński, H., Nagy, J. I., et al. (2013). Grafting of fetal brainstem 5-HT neurons into the sublesional spinal cord of paraplegic rats restores coordinated hindlimb locomotion. *Exp. Neurol.* 247, 572–581. doi: 10.1016/j.expneurol.2013.02.008

Sławińska, U., Miazga, K., and Jordan, L. M. (2014). The role of serotonin in the control of locomotor movements and strategies for restoring locomotion after spinal cord injury. *Acta Neurobiol. Exp. (Wars.)* 74, 172–187.

Smetana, R., Juvin, L., Dubuc, R., and Alford, S. (2010). A parallel cholinergic brainstem pathway for enhancing locomotor drive. *Nat. Neurosci.* 13, 731–738. doi: 10.1038/nn.2548

Smith, J. C., and Feldman, J. L. (1987). *In vitro* brainstem-spinal cord preparations for study of motor systems for mammalian respiration and locomotion. *J. Neurosci. Methods* 21, 321–333. doi: 10.1016/0165-0270(87)90126-9

Smith, J. C., Feldman, J. L., and Schmidt, B. J. (1988a). Neural mechanisms generating locomotion studied in mammalian brain stem-spinal cord *in vitro*. *FASEB J.* 2, 2283–2288.

Smith, J. C., Liu, G., and Feldman, J. L. (1988b). Intracellular recording from phrenic motoneurons receiving respiratory drive *in vitro*. *Neurosci. Lett.* 88, 27–32. doi: 10.1016/0304-3940(88)90310-2

Stewart, W., and Maxwell, D. J. (2003). Distribution of and organisation of dorsal horn neuronal cell bodies that possess the muscarinic m2 acetylcholine receptor. *Neuroscience* 119, 121–135. doi: 10.1016/S0306-4522(03)00116-7

Svirskis, G., and Hounsgaard, J. (1998). Transmitter regulation of plateau properties in turtle motoneurons. *J. Neurophysiol.* 79, 45–50.

Takakusaki, K., Habaguchi, T., Ohtinata-Sugimoto, J., Saitoh, K., and Sakamoto, T. (2003). Basal ganglia efferents to the brainstem centers controlling postural muscle tone and locomotion: a new concept for understanding motor disorders in basal ganglia dysfunction. *Neuroscience* 119, 293–308. doi: 10.1016/S0306-4522(03)00095-2

Takakusaki, K., Tomita, N., and Yano, M. (2008). Substrates for normal gait and pathophysiology of gait disturbances with respect to the basal ganglia dysfunction. *J. Neurol.* 255(Suppl. 4), 19–29. doi: 10.1007/s00415-008-4004-7

Tillakaratne, N. J., Duru, P., Fujino, H., Zhong, H., Xiao, M. S., Edgerton, V. R., et al. (2014). Identification of interneurons activated at different inclines during treadmill locomotion in adult rats. *J. Neurosci. Res.* 92, 1714–1722. doi: 10.1002/jnr.23437

Ueki, S., Koketsu, K., and Domino, E. F. (1961). Effects of mecamylamine on the Golgi recurrent collateral - Renshaw - cell synapse in the spinal cord. *Exp. Neurol.* 3, 141–148. doi: 10.1016/0014-4886(61)90064-4

Umana, I. C., Daniele, C. A., and McGehee, D. S. (2013). Neuronal nicotinic receptors as analgesic targets: it's a winding road. *Biochem. Pharmacol.* 86, 1208–1214. doi: 10.1016/j.bcp.2013.08.001

Webster, H. H., and Jones, B. E. (1988). Neurotoxic lesions of the dorsolateral pontomesencephalic tegmentum-cholinergic cell area in the cat. II. Effects upon sleep-waking states. *Brain Res.* 458, 285–302. doi: 10.1016/0006-8993(88)90471-4

Wess, J., Duttaroy, A., Gomeza, J., Zhang, W., Yamada, M., Felder, C. C., et al. (2003). Muscarinic receptor subtypes mediating central and peripheral antinociception studied with muscarinic receptor knockout mice: a review. *Life Sci.* 72, 2047–2054. doi: 10.1016/S0024-3205(03)00082-1

Wilson, J. M., Rempel, J., and Brownstone, R. M. (2004). Postnatal development of cholinergic synapses on mouse spinal motoneurons. *J. Comp. Neurol.* 474, 13–23. doi: 10.1002/cne.20089

Witts, E. C., Zagoraiou, L., and Miles, G. B. (2014). Anatomy and function of cholinergic C bouton inputs to motor neurons. *J. Anat.* 224, 52–60. doi: 10.1111/joa.12063

Yaksh, T. L., Dirksen, R., and Harty, G. J. (1985). Antinociceptive effects of intrathecally injected cholinomimetic drugs in the rat and cat. *Eur. J. Pharmacol.* 117, 81–88. doi: 10.1016/0014-2999(85)90474-1

Zagoraiou, L., Akay, T., Martin, J. F., Brownstone, R. M., Jessell, T. M., and Miles, G. B. (2009). A cluster of cholinergic premotor interneurons modulates mouse locomotor activity. *Neuron* 64, 645–662. doi: 10.1016/j.neuron.2009.10.017

Zhang, H. M., Chen, S. R., and Pan, H. L. (2007). Regulation of glutamate release from primary afferents and interneurons in the spinal cord by muscarinic receptor subtypes. *J. Neurophysiol.* 97, 102–109. doi: 10.1152/jn.00586.2006

Zhuo, M., and Gebhart, G. F. (1991). Tonic cholinergic inhibition of spinal mechanical transmission. *Pain* 46, 211–222. doi: 10.1016/0304-3959(91)90078-C

Zieglgansberger, W., and Reiter, C. (1974). A cholinergic mechanism in the spinal cord of cats. *Neuropharmacology* 13, 519–527. doi: 10.1016/0028-3908(74)90141-5

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Anatomical and functional evidence for trace amines as unique modulators of locomotor function in the mammalian spinal cord

Elizabeth A. Gozal, Brannan E. O'Neill, Michael A. Sawchuk, Hong Zhu, Mallika Halder, Ching-Chieh Chou and Shawn Hochman*

Physiology Department, Emory University, Atlanta, GA, USA

Edited by:
Brian R. Noga, University of Miami, USA

Reviewed by:
Ronald M. Harris-Warrick, Cornell University, USA
Mark D. Berry, Brandon University, Canada

****Correspondence:***
Shawn Hochman, Physiology Department, Room 601, Whitehead Biomed Res Bldg, 615 Michael St., Atlanta, GA 30322, USA
e-mail: shawn.hochman@emory.edu

The trace amines (TAs), tryptamine, tyramine, and β-phenylethylamine, are synthesized from precursor amino acids via aromatic-L-amino acid decarboxylase (AADC). We explored their role in the neuromodulation of neonatal rat spinal cord motor circuits. We first showed that the spinal cord contains the substrates for TA biosynthesis (AADC) and for receptor-mediated actions via trace amine-associated receptors (TAARs) 1 and 4. We next examined the actions of the TAs on motor activity using the *in vitro* isolated neonatal rat spinal cord. Tyramine and tryptamine most consistently increased motor activity with prominent direct actions on motoneurons. In the presence of N-methyl-D-aspartate, all applied TAs supported expression of a locomotor-like activity (LLA) that was indistinguishable from that ordinarily observed with serotonin, suggesting that the TAs act on common central pattern generating neurons. The TAs also generated distinctive complex rhythms characterized by episodic bouts of LLA. TA actions on locomotor circuits did not require interaction with descending monoaminergic projections since evoked LLA was maintained following block of all Na^+-dependent monoamine transporters or the vesicular monoamine transporter. Instead, TA (tryptamine and tyramine) actions depended on intracellular uptake via pentamidine-sensitive Na^+-independent membrane transporters. Requirement for intracellular transport is consistent with the TAs having much slower LLA onset than serotonin and for activation of intracellular TAARs. To test for endogenous actions following biosynthesis, we increased intracellular amino acid levels with cycloheximide. LLA emerged and included distinctive TA-like episodic bouts. In summary, we provided anatomical and functional evidence of the TAs as an intrinsic spinal monoaminergic modulatory system capable of promoting recruitment of locomotor circuits independent of the descending monoamines. These actions support their known sympathomimetic function.

Keywords: tyramine, tryptamine, β-phenylethylamine, locomotion, TAAR, dopa decarboxylase

INTRODUCTION

The classical monoamine neuromodulatory transmitters, dopamine (DA), noradrenaline (NA), and serotonin (5-HT), play an important role in modulating spinal cord sensory, autonomic and motor function (Jacobs and Fornal, 1993; Rekling et al., 2000; Schmidt and Jordan, 2000; Hochman et al., 2001; Millan, 2002; Clarac et al., 2004; Zimmerman et al., 2012; Garcia-Ramirez et al., 2014). Their actions are thought to occur largely via descending monoaminergic neurons that project to the spinal cord (Gerin et al., 1995).

Another group of endogenous monoamines called the trace amines (TAs), include tryptamine, tyramine, and β-phenylethylamine (PEA). The TAs have structural, metabolic, physiologic, and pharmacologic similarities to the classical monoamine transmitters and are synthesized from the same precursor amino acids (Saavedra, 1989). Unlike the classical monoamines, aromatic-L-amino acid decarboxylase (AADC; also called dopa decarboxylase) is the only enzyme required to produce them. Conversion from the TAs to the monoamines does not appear to occur (Berry, 2007).

The TAs have a heterogeneous central nervous system (CNS) distribution with concentrations ranging from 0.1 to 13 ng/g (Durden et al., 1973; Philips et al., 1974a,b, 1975; Boulton, 1976, 1978; Juorio, 1988; Nguyen and Juorio, 1989; Boulton et al., 1990). There are mixed reports on whether the concentrations are higher in spinal cord or brain (Spector et al., 1963; Boulton et al., 1977; Juorio, 1979, 1980; Karoum et al., 1979). While often viewed as metabolic by-products (Boulton, 1976; Berry, 2004), the TAs are clearly neuroactive. For example, in spinal cord PEA enhanced while tyramine depressed monosynaptic reflexes (Kitazawa et al., 1985; Ono et al., 1991). Tyramine also depressed flexion and crossed-extension reflexes (Bowman et al., 1964) consistent with

reported antinociceptive actions (Reddy et al., 1980). Tyramine also directly depolarized motoneurons with an EC_{50} comparable to DA (~50 μM) (Kitazawa et al., 1985).

The recent discovery of trace amine-associated receptors (TAARs) establishes a mechanism by which TAs can produce effects of their own (Borowsky et al., 2001; Bunzow et al., 2001). Tyramine and PEA activate TAAR1, while PEA and tryptamine activate TAAR4 (Borowsky et al., 2001). Observations using selective TAAR1 agonists and antagonists and TAAR1 knockout mice demonstrate a clear role for TA actions on CNS TAAR1 receptors. TAAR1 activity appears to depress monoamine transport and limit dopaminergic and serotonergic neuronal firing rates via interactions with presynaptic D_2 and 5-HT_{1A} autoreceptors, respectively (Wolinsky et al., 2007; Lindemann et al., 2008; Xie and Miller, 2008; Xie et al., 2008; Bradaia et al., 2009; Revel et al., 2011; Leo et al., 2014). This supports a large classical literature on the role of TAs as endogenous neuromodulators of monoaminergic excitability and neurotransmission (Boulton, 1991; Berry, 2004).

TA-induced modulatory actions in the CNS have yet to be linked with specific trace aminergic neuronal systems. Candidate neurons include 16 anatomically segregated collections of D cells (Jaeger et al., 1984). D cells contain the essential synthesis enzyme AADC but no other monoamine synthesis enzymes, and can only synthesize the TAs from their precursor amino acids (Jaeger et al., 1983, 1984; Nagatsu et al., 1988). D1 cells associate with the lumen of the spinal cord central canal (Jaeger et al., 1983). While the function of these neurons is unknown, a morphologically similar population activates locomotor circuits in larval zebrafish (Wyart et al., 2009).

As TAAR1 mRNA is found in the spinal cord (Borowsky et al., 2001), we hypothesize that the TAs and their receptors represent an intrinsic neuromodulatory system. Here, we characterize spinal expression patterns for AADC, tyramine, TAAR1 and TAAR4, then make use of an isolated neonatal rat spinal cord preparation to examine the role of applied TAs on spinal motor circuits (Kiehn, 2006). Overall we show that the TAs can activate complex rhythmic motor behaviors, including locomotor-like activity (LLA), independent of classical monoaminergic mechanisms. Some of these results have been presented in abstract form (Gieseker et al., 2004; Gozal et al., 2006, 2007).

MATERIALS AND METHODS

All experimental procedures complied with the NIH guidelines for animal care and the Emory Institutional Animal Care and Use Committee. Sprague-Dawley rats aged post-natal day (P) 0–5, P14, and adult were used.

TAAR PCR

Total RNA was extracted from male and female rat whole spinal cords (P2) using QIAGEN RNeasy Mini Kit (Cat No: 74104). We performed on-column DNase digestion using the same QIAGEN kit. Total RNA was quantified by NanoDrop 2000 (Thermo Scientific). DNase-treated total RNA (1 μg) was retrotranscribed using the iScript cDNA synthesis kit according to the instructions of the manufacturer (Bio-Rad Laboratories, Hercules, CA, USA). Platinum PCR SuperMix was used for PCR reactions following the instructions of the manufacturer (Invitrogen, Cat No: 11306-016). PCR was conducted with the following cycling program: 2 min at 95°C of initial denaturation, followed by 37 cycles of denaturation at 95°C for 30 s, annealing at 60°C for all TAAR primers for 30 s (except TAAR7 and TAAR9 which were at 55°C), and extension at 72°C for 30 s. PCR products were size-separated by electrophoresis in ethidium bromide-stained 2% agarose gels with bands visualized by ultraviolet transillumination.

Primer sequence information for TAARs 1–6, 8a, and 9 were obtained from Chiellini et al. (2007). We also used one of introns of *Rattus norvegicus* strain BN/SsNHsdMCW chromosome 1 (RGSC_v3.4) as a negative control to make sure that the total RNA from the whole spinal cord was not contaminated by the genomic DNA. Primer sequences for GAPDH and RGSC_v3.4 are: GAPDH (NM017008): forward primer, 5′-GCAACTCC CATTCTTCCACCTTTGA-3′; reverse primer, 5′-TTGGAGGC CATGTAGGCCATGA-3′ (139 bp). RGSC_v3.4 intron (NC_0051 00.2): forward primer, 5′-AGAGTGGTCTGTTGCAAGTGGTCT-3′; reverse primer, 5′-AAGGGTCTCCAGAAACACCCAAGT (723 bp).

In situ hybridization

Complete rat spinal cords were dissected out and the whole cords were stored in RNAlater (Qiagen, Valencia, CA) at −80° until use. Total RNA was extracted by using Qiagen RNeasy Mini kits (Qiagen, Valencia, CA). Five micrograms of total RNA was subject to cDNA synthesis with oligo-dT15 primer and SuperScript II Reverse transcriptase (Invitrogen, Carlsbad, CA) for 1 h at 42°C. The reverse transcriptase was inactivated, and RNA was degraded by heating at 95°C for 5 min. Of the 20 μl of cDNA obtained from the synthesis reaction 5 μl were directly added to the PCR reaction using a PCR Mastermix kit (Eppendorf, Hamburg, Germany) containing 1 μM gene-specific primers. The primer used in this study was designed by the Invitrogen-OligoPerfect™ Designer program (Invitrogen, Carlsbad, CA). Non-radioactive single-stranded digoxigenin cRNA probes were used for *in situ* hybridization using methodology reported previously (Zhu et al., 2007). Briefly, single stranded, digoxigenin-labeled antisense and sense probes are transcribed *in vitro* using T7 and Sp6 RNA polymerase (Promega). The probe sequence for AADC is 523–927 bp (GenBank U31884), 404 bp product. The probe sequence used for TAAR1 is 400 bp long (GenBank AF380186). Hybridization was carried out at 68°C overnight with 3 μg/ml digoxigenin-labeled antisense cRNA probe. Sense probes were used at identical concentrations and development reaction as a negative control. Sections were washed with concentrated standard saline citrate (and then incubated with anti-digoxigenin-AP Fab fragments (1:5000, Roche) in blocking buffer overnight at 4°C. The color development reaction was carried out in the dark and neutralized with color stop buffer (10 mM Tris, pH 5, 1 mM EDTA). Slides were then dehydrated through a series of alcohol washes, coverslipped with Vectamount (Vector Labs) and images were captured on a Nikon E800 light microscope (Nikon ACT-1 software).

Immunohistochemistry

Sprague-Dawley rats were anesthetized with urethane (1.5 mg/kg), perfused with 1:3 volume/body weight of prefix

(0.9%NaCl, 0.1%$NaNO_2$, 1 units/1 ml heparin) followed by equal volume/body weight of Lana's fixative (4% paraformaldehyde, 0.2% picric acid, 0.16 M PO_3); pH 6.9. In a small subset of experiments, P25 rats with and without a transection were used. In many of the experiments, Fluorogold, which does not cross the blood brain barrier, was injected intraperitoneally 24 h prior to sacrifice to retrogradely label most spinal motoneurons (Ambalavanar and Morris, 1989; Merchenthaler, 1991). The spinal cords were then isolated and post-fixed for 1 h in Lana's fixative then cryoprotected in 10% sucrose, 0.1 M PO_3 until sectioned into 10 um thick sections on a cryostat and processed for immunohistochemistry. All incubations and washes were performed in 0.1 M PO_3-buffered saline containing 0.3% triton X-100 (PBS-T). Tissue was washed overnight in PBS-T at 4°C followed by incubation in primary antibody for 48–72 h. Slides were then washed three times for 30 min and incubated in secondary antibody. The concentrations for the antibodies can be found in **Table 1**. In all experiments, omission controls were used for the primary antibodies. In addition, tyramine, TAAR1, and TAAR4 pre-absorption controls were performed with used antibody concentrations (1:100 and 1:1000) absorbed with 20 μg/100 μl of antigen (from respective antibody suppliers) for 1 h prior to incubation. As reported by the supplier (Chemicon) the tyramine antibody has >50,000-fold selectivity over its precursor amino acid (tyrosine), >40,000 selectivity over dopamine, and 800-fold selectivity over the trace amine octopamine. Images were photographed with a Nikon (Tokyo, Japan) digital camera through a Nikon E800 microscope or using an Olympus FV1000 inverted confocal microscope. Images were processed using Corel Draw (Corel, Ottawa, Ontario, Canada).

Table 1 | Antibodies used for immunohistochemistry expression.

Primary antibody	Secondary antibody	Tertiary
Rabbit anti-tyramine 1:1000 (Chemicon) Rabbit anti-TAAR1 1:1000 (Lifespan Biosciences)	Biotin anti-rabbit 1:250 (Jackson Immunoresearch)	Extravidin Cy3 1:1000 (Sigma)
Rabbit anti-DDC 1:00 (Biomol Sciences) Rabbit anti-tyramine 1:100 or 1:1000 (Chemicon) Rabbit anti-TAAR1 1:1000 (Lifespan Biosciences)	cy3 anti-rabbit 1:250 (Jackson Immunoresearch)	
Sheep anti-DDC 1:100 (Biomol Sciences)	FITC anti-sheep 1:100 (Jackson Immunoresearch)	
Goat anti-TAAR4 1:100 (Santa Cruz Biotechnology)	cy3 anti-goat 1:250 (Jackson Immunoresearch)	
Mouse anti-NeuN 1:50 (Chemicon)	FITC anti-mouse 1:100 (Jackson Immunoresearch)	

Lipophilic dye labeling

A P7 rat was anesthetized with isoflurane via inhalation for thoracic cord transection. Following dorsal laminectomy to expose lower-thoracic segments of the cord, one section of the cord between T8 and T12 was removed using surgical microdissection scissors. One week later the cord was isolated and preserved in 2% paraformaldehyde fixative, then suspended in agarose gel and labeled with the carbocyanine dye DiI. Crystals of DiI were placed at the cut surface of ventral funiculi. The dye was allowed to diffuse to identify gray matter projections sites.

Electrophysiology

Sprague-Dawley rats P0-5 were decapitated, eviscerated, and placed in a bath containing oxygenated (95% O_2, 5% CO_2) artificial cerebral spinal fluid (aCSF) containing the following (in mM): 128 NaCl, 1.9 KCl, 1.2 KH_2P0_4, 26 $NaHCO_3$, 2.4 $CaCl_2$, 1.3 $MgSO_4$, and 10 glucose (pH of 7.4). The spinal cord was exposed by a ventral vertebrectomy and carefully dissected out of the body cavity leaving the dorsal and ventral roots attached. The spinal cord was secured with insect pins to a chamber with Sylgard (Dow) on the bottom. Glass suction electrodes were applied to dorsal and/or ventral roots, after which the preparation was allowed to recover for at least 1 h before experimentation at room temperature. The ventral root electroneurographic activity was amplified (10,000x), band-pass filtered at 10–3000 Hz, and digitized at 5 kHz (Digidata 1321A, 16-bit; Axon Instruments). Data was captured on a computer with the pCLAMP acquisition software (v8-9, Molecular Devices; Union City, CA). Electrophysiological data analysis was performed using pCLAMP analysis software (Clampfit) or software written in-house using MATLAB. Statistical comparisons were made using ANOVA, Student's *t*-test, or paired *t*-test. The means is reported as mean ± SD.

Motor activity was monitored using glass suction electrodes attached to ventral lumbar roots, typically bilaterally to L2 and L5. Changes in levels of motor activity were quantified by first applying a RC high-pass filter at 1 Hz to reduce drift, then calculating the root mean square of a representative ventral root signal, and comparing 100 s periods before application of the TAs and monoamines and during their period of maximal response. Changes were expressed as a percent increase over baseline. L2 ventral root activity primarily indicates activity in flexors, while L5 ventral root activity primarily indicates activity in extensors (Kiehn and Kjaerulff, 1998). LLA was defined as an alternation between right and left L2 ventral roots, with each L2 ventral root alternating with the L5 root on the same side. LLA was analyzed using the in-house MATLAB software, SpinalMOD (Hochman et al., 2012), which calculated the frequency, peak amplitude, and phase, which was calculated using the middle of the burst (Matsushima and Grillner, 1992).

To evoke the reflexes, constant current stimuli were applied to the dorsal roots while motor activity was recorded from ventral lumbar roots, typically L5. Stimulus intensities were 500 μA and durations ranging from 100 to 500 μs. For experiments examining motoneuron activity in the absence of synaptic transmission, reflexes were abolished after switching from regular aCSF to

with high Mg^{2+} (6.5 mM)/low Ca^{2+} (0.85 mM) aCSF or zero Ca^{2+} aCSF.

Neurochemicals, which were stored in 10 or 100 mM stock solutions at −20°C, were added to a directly oxygenated static bath (typically 30 mL) with drug equilibration achieved via rapid recirculation using several syringe-based fluid removal/replacement events. Solution exchange was achieved by replacing the bath aCSF at least three times. Neurochemicals were obtained from Sigma-Aldrich (St. Louis, MO). N-methyl-D-aspartate (NMDA) (3–5 μM), 5-HT (50 μM), noradrenaline (50 μM), and dopamine (50 μM) were applied at concentrations comparable to those used previously in neonatal rat (Cazalets et al., 1995; Kiehn and Kjaerulff, 1996; Kjaerulff and Kiehn, 1996; Cowley and Schmidt, 1997; Kremer and Lev, 1997; Kiehn et al., 1999; Sqalli-Houssaini and Cazalets, 2000; Barriere et al., 2004). The following TAs were used: tryptamine (50 μM), tyramine (1–100 μM), and PEA (50–100 μM). TA doses were chosen to match those of the monoamines under the assumption that they have equivalent transporter uptake and degradation following exogenous application. Due to the efficiency of the monoamine transporters, it has been estimated that the actual dose at receptor sites is ∼1/30th that applied (Murray et al., 2011). The aromatic amino acids tyrosine, tryptophan, and phenylalanine were applied at doses between 100 and 200 μM. The non-specific 5-HT receptor antagonist methysergide was applied at 1–10 μM. The serotonin (SERT), dopamine (DAT), and noradrenaline (NET) transport inhibitors applied were: citalopram (1 μM; a SERT inhibitor), buproprion (1 μM; a DAT inhibitor), and clomipramine (5 μM; a SERT and NET inhibitor). The vesicular monoamine transport (VMAT) inhibitor, reserpine, was applied at 10 μM. The organic cation transport (OCT) inhibitors, pentamidine was applied at 200 μM. The protein synthesis inhibitor, cycloheximide, was applied at 100 μM.

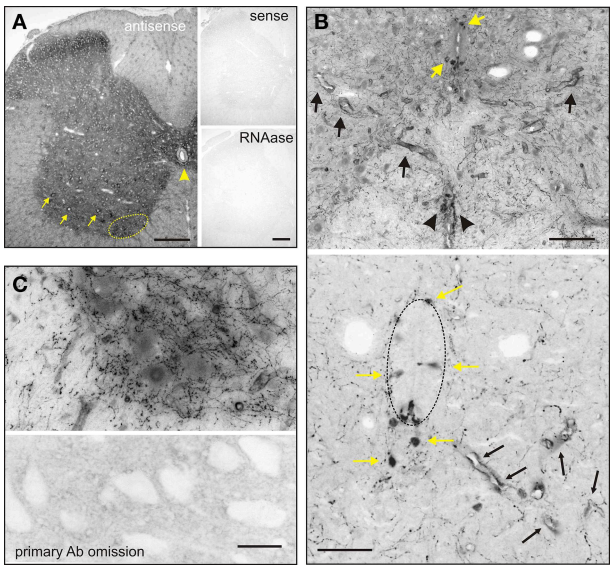

FIGURE 1 | *In situ* hybridization (A) and immunohistochemistry (B,C) reveal AADC labeling throughout the adult spinal gray matter. (A) Left Low power magnification of AADC antisense *in situ* hybridization. **Right** Sense and RNAase treatment at right confirms specificity of antisense probe. Arrows point to putative motoneurons with perinuclear labeling, and putative D cells below the central canal. **(B) Top** Intense AADC immunolabeling is found in blood vessels (black arrows) and in the D cells (yellow arrows) and in the ventromedial white matter (black arrowheads). Note that some $AADC^+$ neurons are also found in the dorsal central canal. **Bottom** Higher power image of central canal region (outlined). Blood vessels are identified with black arrows and $AADC^+$ neurons with yellow arrows. **(C)** $AADC^+$ terminal arborizations surrounding motoneurons. Most of these arborizations are presumably from descending monoaminergic systems. Bottom panel is a negative control omission of primary antibody. Scale bars: **(A)**, 250 μm; **(B)**, 100 μm top, 50 μm, bottom; **(C)**, 25 μm.

RESULTS

ANATOMICAL SUBSTRATE FOR TA ACTIONS IN THE SPINAL CORD

AADC and tyramine are widely expressed in the spinal cord

In situ hybridization and immunohistochemistry carried out in adult rat lumbar spinal cord showed that AADC was detected with widespread labeling throughout the spinal cord (**Figure 1**). AADC expression was most notable around the central canal, the ventral funiculus, and in ventral neurons, including motoneurons. There was also some nuclear labeling as seen before (Mann and Bell, 1991). AADC immunolabeling around the central canal included the previously reported D cells (Jaeger et al., 1983; Li et al., 2014). Immunohistochemical staining was undertaken as shown in **Figures 1B,C**. We observed clear AADC immunolabeling in neurons associated with the central canal, in axonal projections in the ventral funiculus, and in blood vessel endothelia where AADC activity is known to be high (Hardebo et al., 1979; Nagatsu et al., 1988; Li et al., 2014; Pfeil et al., 2014).

Next, immunolabeling studies on AADC and tyramine, were conducted in the neonatal spinal cord (**Figure 2**). This age was chosen to match the age at which the subsequent electrophysiological studies were undertaken. AADC labeling was similar to that found in the adult. Labeled spinal neurons were most notable adjacent and ventral to the central canal, the ventral funiculus, and in ventral neurons, including putative motoneurons (**Figure 2A**). As observed in the adult, but more strikingly, central canal $AADC^+$ neurons projected ventrally in a stream of cells with subsequent termination of presumed axonal projections in the most medial portion of the ventral funiculus (**Figure 2A**, right). $AADC^+$ neurons were also found in the lateral regions near the central canal as well as in the dorsal horn (not shown), although more rarely.

To determine whether cells at the central canal could have axonal projections to the ventral funiculus, DiI crystals were applied to various ventral funicular regions in the fixed lumbar spinal cord of a P14 rat 1 week after thoracic spinalization. When dye placement contacted the midline tract, central canal-associated cells were retrogradely labeled, confirming that cells consistent with the location of D cells can project to the ventral funicular white matter tract (**Figure 2B**).

Tyramine immunolabeling was characterized by enormous variability between animals, ranging from widespread to near-absent. This variability may reflect the dynamic sensitivity of tyramine to rates of synthesis and degradation. Expression patterns included preferential ventral horn labeling, particularly associated

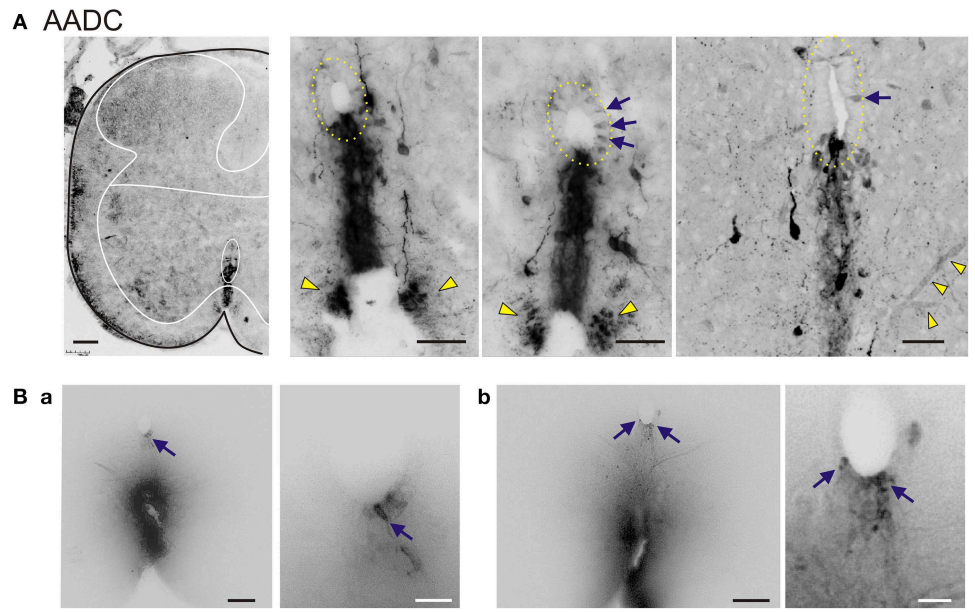

FIGURE 2 | AADC neuronal subpopulations in the neonatal rat lumbar spinal cord. (A) Left Low power transverse spinal cord section (10 μm) of AADC immunolabeling. Superimposed is an outline of the spinal cord (black) with interior white lines approximately identifying dorsal and ventral gray matter and central canal region. Note strongest labeling is associated with D cells intermingled with epithelial cells surrounding the central canal (top arrow). Also, note the associated vertical row of cellular labeling projecting ventrally, and bilateral white matter labeling in the ventral funiculus (bottom arrow). There is also weak AADC immunolabeling in putative motoneurons (in lamina IX below dotted lines). **Right** Three panels showing higher power images from separate sections illustrate the diversity of AADC labeling in the ventral medial gray matter region. Epithelial cell layer surrounding central canal is outlined while blue arrows point to example D cells. Common to all is the vertical stream of projections with intermingled cells ventral to the central canal. These appear to end in a white matter tract in the ventral funiculus (yellow arrowheads). Last panel at right is a z-stack of 25, 0.4 μm consecutive images. Note the AADC$^+$ blood vessel at bottom right identified by yellow arrowheads. **(B)** Central canal cells project to the ventral funiculus. DiI crystals were placed on fixed lumbar spinal cords at the medial ventral funiculus or just lateral to it and allowed to diffuse to identify gray matter projections sites. Shown are two 70 μm thick transverse sections **(a,b)** from a P14 rat 1 week after with a mid-thoracic spinalization. Images are presented as grayscale with contrast enhancement. Note that when dye placement contacts the midline tract, neurons associated with the central canal are retrogradely labeled. Panels at right are magnified images to highlight retrogradely-labeled neurons in the central canal (corresponding arrows). Scale bars are: **(A)**, 100 μm (left) 50 μm (right three); **(B_a,B_b)**, 100 and 25 μm for left and right images, respectively.

with the central canal and in motoneurons (**Figures 3A,B**). To increase detectability and further demonstrate that tyramine immunolabeling was neuronal, we preincubated spinal cords in 100 μM tyramine for 2 h. Tyramine labeling co-localized with the neuron specific marker NeuN, including in retrogradely-labeled Fluorogold$^+$ motoneurons (**Figure 3C**).

Tyramine commonly co-expressed with AADC in central canal D cells, in the ventral stream of cells at the midline, and in the ventral funiculus (**Figure 3D**). These results are consistent with the notion that D cells and related midline neurons synthesize tyramine. We also observed co-labeling in putative spinal motoneurons (**Figure 3E**).

Trace amine-associated receptor expression in the spinal cord

Given that 17 TAAR rat genes have been identified, we tested for the presence of TAAR gene expression in the spinal cord. RT-PCR was undertaken on total RNA prepared from neonatal rat spinal cord of both sexes. We observed detectable transcripts for TAARs 1–6, 7a, 8a, and 9 (**Figures 4A,B**). Because TAAR1 is the only receptor for which there has been detailed study, we also undertook *in situ* hybridization for TAAR1 in the neonate. Widespread spinal cord labeling was observed including around the central canal and in the ventral horn region associated with motoneurons (**Figure 4C**).

Immunolabeling for both TAAR1 and TAAR4 was found throughout the neonatal spinal cord, including in motoneurons and in neurons around the central canal (**Figures 4D,E**). TAAR1 and TAAR4 labeling in all neurons appeared intracellular, consistent with previous reported results for TAAR1 (Miller, 2011). A cytoplasmic location of ligand and receptor (e.g., tyramine and TAAR1) supports intracellular activation of signal transduction pathways, as suggested previously (Miller, 2011). The presence of TAAR1 and TAAR4 provides for actions mediated by tyramine and PEA, and tryptamine and PEA, respectively (Borowsky et al., 2001; Bunzow et al., 2001).

TA ACTIONS ON MOTOR AND LOCOMOTOR ACTIVITY *IN VITRO*

To examine whether tyramine, tryptamine, and PEA increased motor excitability, population motor activity was recorded from a lumbar ventral root before and after their application. As shown in **Figure 5A**, tryptamine and tyramine increased motor activity while PEA did not. To test whether the TAs acted directly on motoneurons, chemical synaptic transmission was minimized by replacing the regular aCSF with one having high

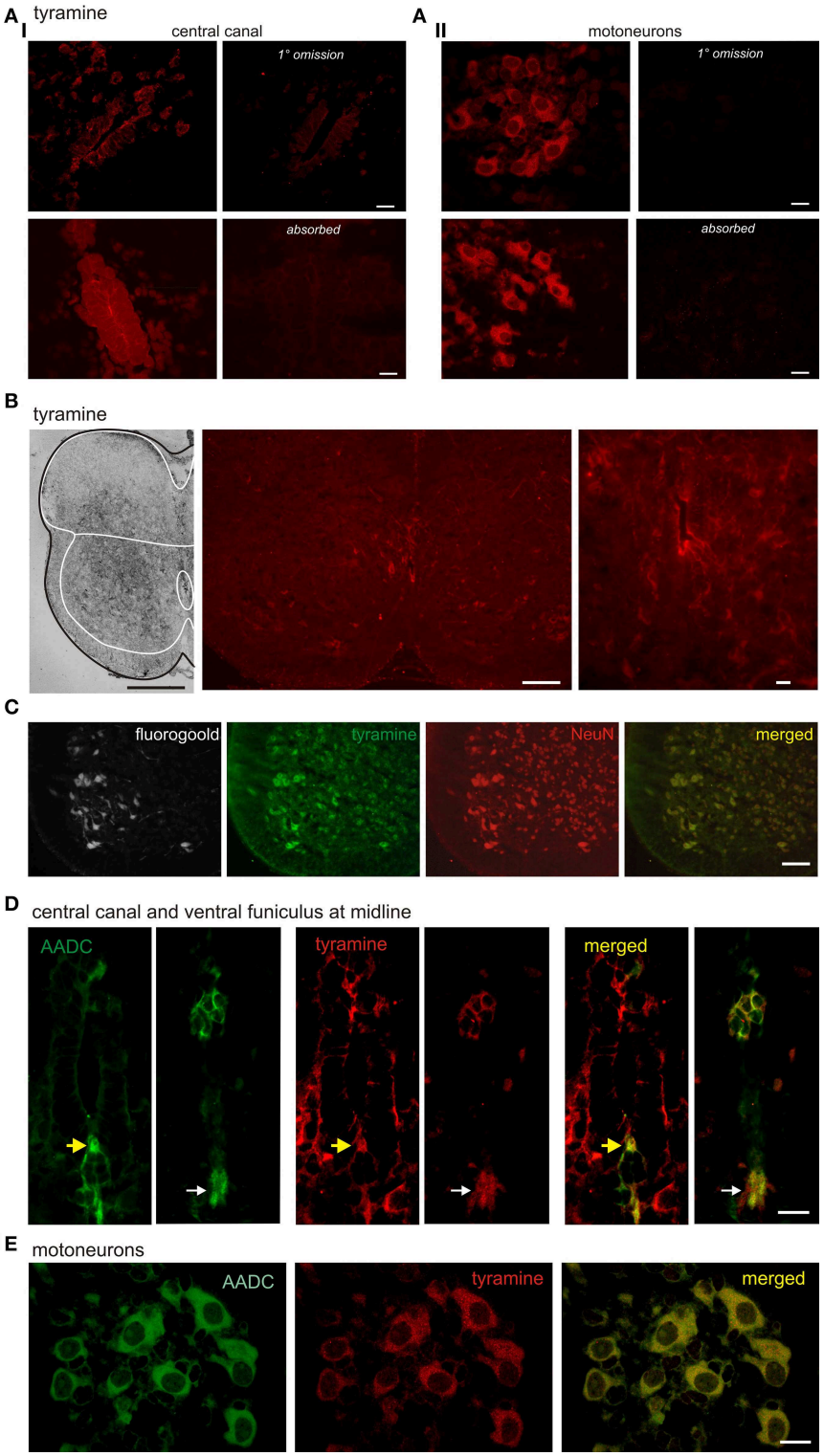

FIGURE 3 | Tyramine expression in subpopulations of spinal cord neurons and co-expression with AADC. (A) Tyramine is expressed in cells around the central canal and in putative motoneurons. Fluorescence is minimal following exclusion of primary antibody and greatly reduced in the presence of pre-absorbed antigen. **(B)** Tyramine expression patterns are highly variable. Shown are examples of preferential labeling in ventral gray matter (left) and in association with the central canal (middle and right). **(C)** Preincubation in tyramine leads to widespread neuronal labeling. The isolated spinal cords of P2 rat were pre-incubated in tyramine for 2 h. The day before the treatments, the rat pups were injected with Fluorogold to retrogradely label motoneurons (left). Column 2 shows immunostaining for tyramine.

(Continued)

FIGURE 3 | Continued
Column 3 provides immunostaining for the neuron-specific marker, NeuN. Column 4 is a merge of the trace amine with NeuN to show that tyramine is observed in many neurons, including motoneurons. **(B)** AADC and tyramine labeling in D cells associated with the central canal (yellow arrows; left panels for each pair) and its ventral cellular projection stream appearing to terminate at a midline tract in the ventral funiculus (right panels in each pair). White arrows identify ventral funiculus. Note D cells, ventral midline cells, and ventral funiculus are co-labeled. **(C)** AADC and tyramine immunolabeling in putative motoneurons. While AADC produces more uniform cytoplasmic labeling (left), tyramine labeling includes larger puncta which are not co-labeled in merged image at right. All images in B and C are high power confocal images with an optical section of 0.4 μm. Scale bars are: **(A)**, 20 μm, **(B)**, 10 μm; **(C)**, 100 μm; **(D,E)**, 20 μm.

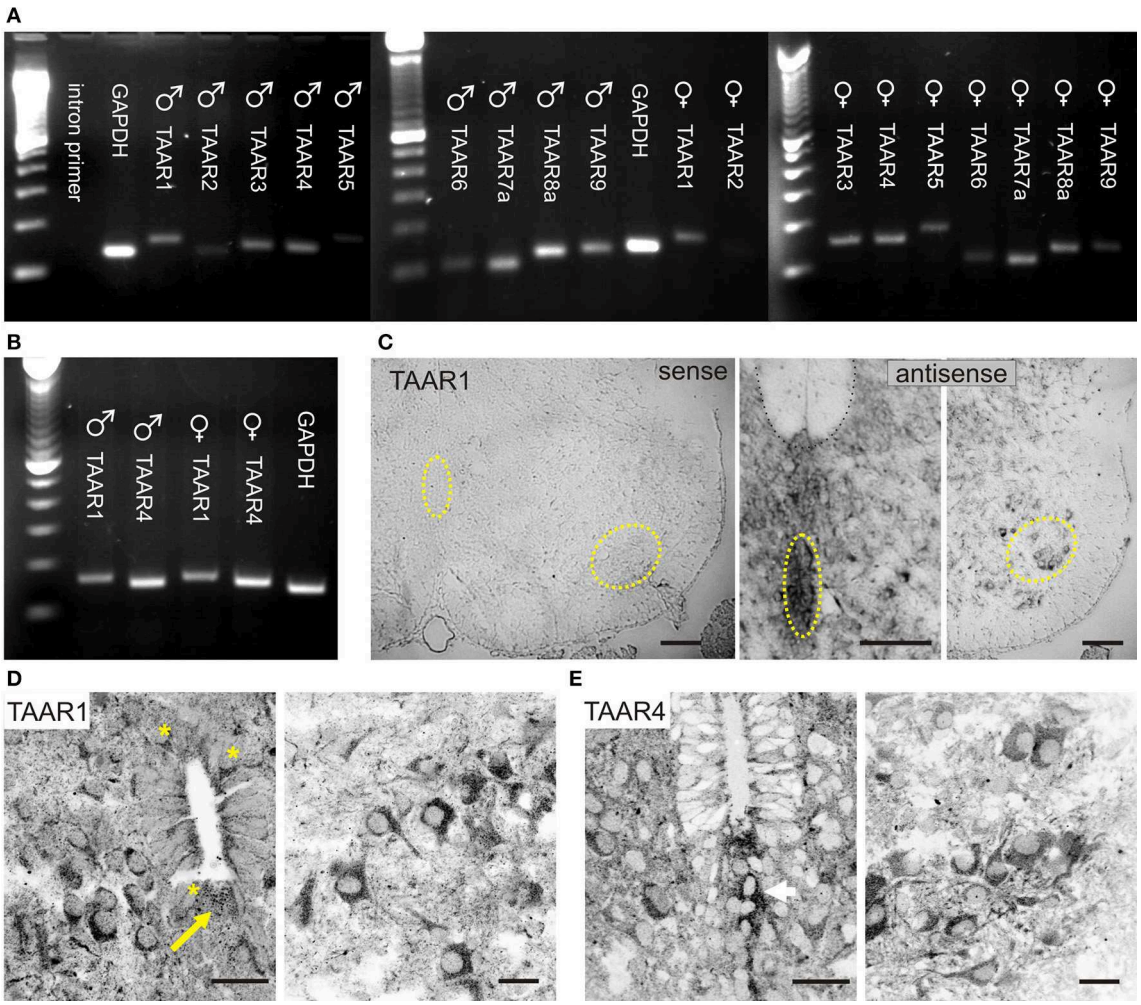

FIGURE 4 | TAARs are expressed in neonatal rat spinal cord. (A) RT-PCR evidence of TAAR expression in both sexes. In males, TAARs 2,5, and 6 expression was weakest, and in females, TAARs 2,6, and 9 were weakest. **(B)** RT-PCR series directly comparing TAAR1 and TAAR4 expression in both sexes as these receptors are known to be activated by trace amines. **(C)** TAAR1 mRNA expression in spinal neurons. *In situ* hybridization reveals TAAR1 labeling throughout the P2 rat spinal cord ventral horn (compare sense to antisense). Highlighted regions in sense panel approximately identify same central canal and motor nucleus location in antisense panels. **(D)** TAAR1 receptor immunolabeling is found in many spinal neurons, including those associated with the central canal (asterisks; left), and in the ventral horn, including putative motoneurons (right). Note thread-like projections adjacent to epithelial cells with expansion in central canal interior. **(E)** TAAR4 receptor immunolabeling is also found in many spinal neurons and appears to localize in cells ventral to the central canal (left) and in the gray matter ventral horn, including putative motoneurons (right). All images are grayscale negatives. Scale bars: **(C)**, 100 μm; **(D,E)**, 25 μm. Confocal images in **(D,E)** were taken at 0.30 μm optical section thickness.

Mg^{2+}/ low Ca^{2+} or in nominally zero Ca^{2+} (Shreckengost et al., 2010). Under these conditions, dorsal root stimulation-evoked reflexes were abolished (**Figure 5B$_1$**). Subsequently, tryptamine and tyramine still increased motor activity (**Figures 5B$_2$,C**).

NMDA is commonly co-applied with 5-HT to produce a stable locomotor-like rhythm in the isolated spinal cord maintained *in vitro* with a coordination comparable to normal locomotion when limbs remain attached (Sqalli-Houssaini et al., 1993; Kjaerulff and Kiehn, 1996; Hayes et al., 2009). This LLA can be

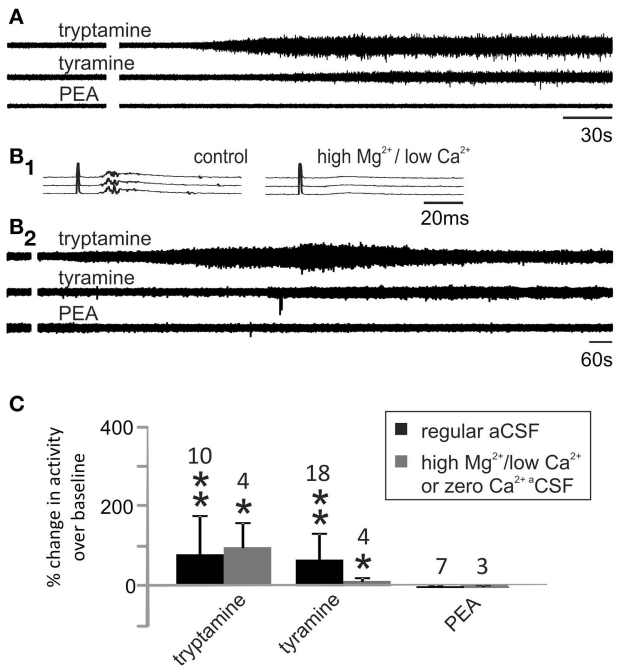

FIGURE 5 | Tyramine and tryptamine increase motor activity. (A) Tryptamine and tyramine increased lumbar ventral root activity while PEA was without effect. **(B_1)** Dorsal root stimulation evoked reflexes (left) were abolished when synaptic transmission was blocked in high Mg^{2+} (6.5 mM), low Ca^{2+} (0.85 mM) containing aCSF (right). **(B_2)** Under these conditions, direct excitatory actions were still observed for tryptamine and tyramine. **(C)** Changes in ventral root activity were quantified compared to baseline activity levels with and without synaptic transmission (* indicates $p < 0.05$ and ** indicates $p < 0.01$). Samples sizes are listed.

monitored from ventral roots as left/right and ipsilateral alternation of bursts between flexors (L2) and extensors (L5) (**Figure 6A**) (Kiehn and Kjaerulff, 1998). To test for locomotor promoting actions of the TAs and classical monoamines, we co-applied NMDA at concentrations that never produced stable LLA on its own (3–5 μM; $n = 15$). Tryptamine ($n = 14/19$), tyramine ($n = 24/26$), PEA ($n = 10/10$), NA ($n = 14/14$), DA ($n = 14/14$), and 5-HT ($n = 24/24$) reliably recruited rhythmic motor activity. Of those expressing rhythmic motor activity, a locomotor-like rhythm comparable to 5-HT was observed for DA, NA and the TAs with the following incidences: DA (5/14), NA (2/14), tryptamine (13/14), tyramine (12/24), and PEA (3/10). Examples are shown in **Figures 6B–G**. In these instances, all amines led to bursting at frequencies statistically indistinguishable from 5-HT (**Figure 7A**). Since the bursting patterns produced by the TAs are often similar to 5-HT, the TAs likely recruit the same pattern-generating circuits. Tyramine ($n = 5/24$; **Figure 6H**) and dopamine ($n = 3/14$) also produced a second rhythm that was significantly slower, but with comparable locomotor-like coordination (**Figure 7B**). DA ($n = 9/14$; 0.04 ± 0.02 Hz) and NA ($n = 12/14$; 0.02 ± 0.02 Hz) also produced rhythmic patterns of continuous bursting that were non-locomotor-like in coordination and significantly slower than 5-HT-evoked rhythms (**Figure 7C**).

Interestingly, tryptamine ($n = 1/14$), tyramine ($n = 8/24$), and PEA ($n = 7/10$) were able to generate a bursting pattern never seen with the monoamines in this preparation. The pattern was more complex with episodic bouts of bursting (**Figure 7D**). During these bouts, burst frequencies were statistically indistinguishable from 5-HT locomotion. Episodic bouts of bursting were variable but usually concurrent across roots and alternated with comparatively quiescent periods at very slow average frequencies (**Figure 8**). For tyramine, bursting patterns interconverted between continuous locomotion to episodic bouts in one experiment. Notably, bouts of episodic bursting were the dominant activity pattern observed for PEA (**Figure 7C**). Expressed bouts of episodic bursting were highly variable, containing up to 47 events within an episode and with quiescent periods varying from 5 to 230 s.

Frequently, there were progressive increases and decreases in the locomotor burst amplitude within each episode, which were noticeable in at least some bouts for all TAs tested (**Figure 8**). Similarly, locomotor frequency could be seen to wax and wane during these bouts, supporting a sinusoidal drive to the CPG. This is highlighted in **Figure 8A** with progressive increases then decreases in burst amplitude, and locomotor frequency are further quantified in **Figure 8D**.

EVIDENCE THAT THE TAs ACT AT UNIQUE BINDING SITES

Differences in receptor pharmacology

5-HT is thought to activate spinal locomotor circuits via 5-HT_2 and 5-HT_7 receptors (Madriaga et al., 2004; Liu and Jordan, 2005; Liu et al., 2009). Methysergide is a high affinity non-selective 5-HT_1, 5-HT_2, and 5-HT_7 receptor antagonist (Madriaga et al., 2004) and a tryptamine binding site antagonist (Martin et al., 1988). To determine if the TAs and other monoamines had a similar sensitivity to methysergide as 5-HT, methysergide was added to TA and monoamine–induced rhythms (with NMDA) at progressively increasing doses (**Table 2**). Tryptamine was more sensitive than 5-HT to methysergide block. Tyramine was variably sensitive to methysergide block, while PEA, NA, and DA-evoked LLA was methysergide-insensitive even at the highest dose tested (10 μM). Based on methysergide sensitivity, it appears likely that tyramine and PEA actions are not dependent on 5-HT receptor activation. Methysergide block of tryptamine-evoked LLA may be via competitive block at 5-HT receptors and/or at the methysergide-sensitive tryptamine binding site (Martin et al., 1988).

Temporal differences in activation onset

When time to burst onset after bath application was examined, 5-HT and NA had rapid burst onset (medians of 7.5 and 2.5 s, respectively). In comparison, burst onset took dramatically longer for DA, tryptamine, tyramine, and PEA (medians of 480, 300, 300, and 540 s, respectively; all are $p < 0.05$) (**Figure 9**). This suggests that the TAs and DA are not acting predominantly on plasma membrane metabotropic receptors.

Possible sites of action

TA-evoked locomotor-like activity is independent of the vesicular monoamine transporter. To test whether TA-evoked actions

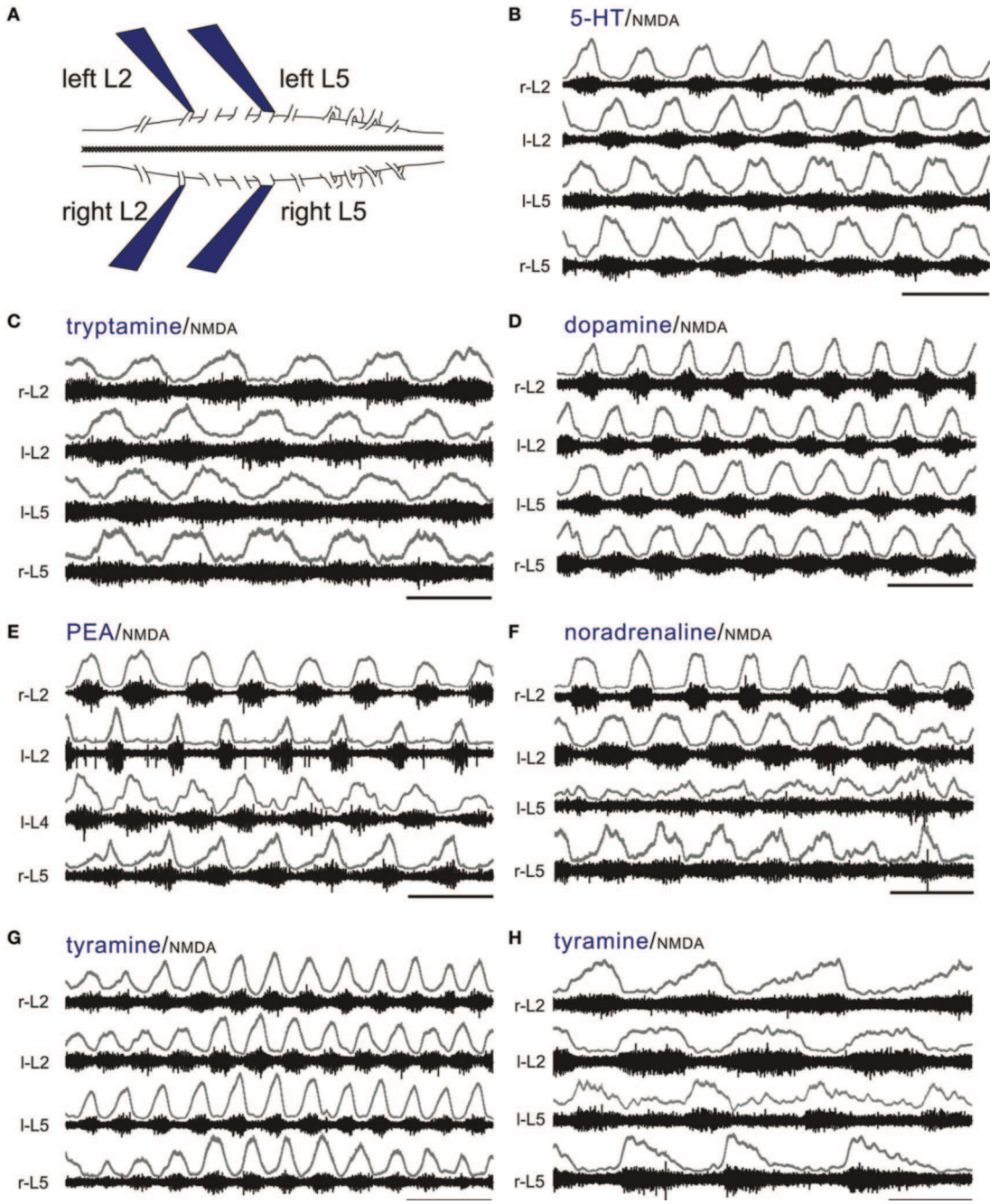

FIGURE 6 | The TAs and monoamines can all produce a continuous LLA pattern. (A) Experimental setup (left). All of the electrophysiological experiments use the thoraco-lumbar isolated neonatal rat spinal cord maintained *in vitro*. Suction electrodes are placed on lumbar L2 and L5 ventral roots bilaterally to monitor population motoneuron flexor and extensor activity, respectively. LLA is shown as an alternation between right and left L2 flexors, with each flexor rhythm alternating with the L5 extensor rhythm on the same side. **(B)** Typical locomotor rhythms observed with co-application of 5-HT and NMDA. **(C–G)** Tryptamine, dopamine, PEA, NA, and tyramine can also produce a continuous LLA pattern comparable to 5-HT in the presence of NMDA. **(H)** Tyramine can also produce a slower LLA pattern. The upper traces in gray at each lumbar root represent the rectified/low-pass filtered equivalent of the raw waveforms below them. Scale bars are 10 s in all panels.

required interactions with the vesicular monoamine transporter (VMAT), we compared the locomotor-inducing ability of the TAs to those of 5-HT (always in the presence of NMDA) in bath chambers pre-incubated with the VMAT inhibitor reserpine (>40 min) (10 μM; **Figure 10A**). In the presence of reserpine, 5-HT and tryptamine evoked LLA in all cords tested ($n = 5/5$). In comparison, tyramine- and PEA-evoked LLA was seen in 4.5 and 3/5 cords, respectively. As reserpine has moderate binding affinity

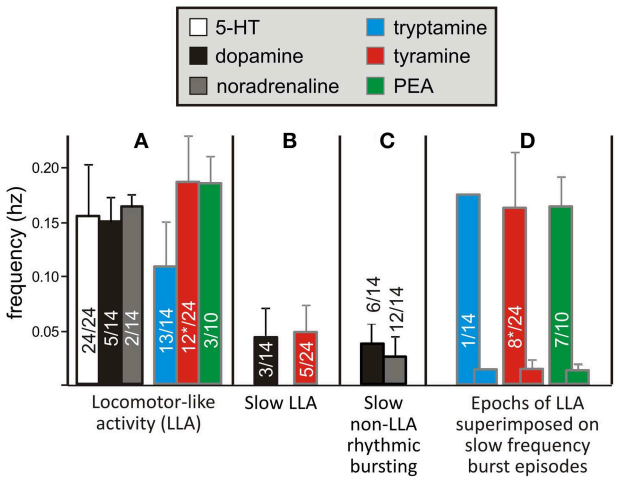

FIGURE 7 | Similarities and differences in motor burst phenotypes observed with the monoamines and TAs. Four characteristic categories **(A–D)** describe the range bursting patterns observed. Frequencies varied depending on the type of bursting. **(A)** In the neonatal rat, 5-HT/NMDA-induced LLA has a characteristic frequency range, which was also seen when NMDA was co-applied with the other amines. **(B)** Dopamine and tyramine also produced a much slower rhythm, but with the same flexor-extensor left-right locomotor-like coordination. **(C)** NA and tyramine could also produce a much slower rhythm, but without locomotor-like coordination. **(D)** Seen only with the TAs, this episodic pattern is described as "Bouts of LLA superimposed on slow frequency burst episodes." LLA and the slower episodic bursts are represented as overlapping bars in this grouping. The frequencies of slow LLA **(B)**, slow non-LLA rhythmic bursting **(C)**, and the slow frequency of episodic bursting **(D)** were statistically slower than LLA. Asterisks (*) denote inclusion of an animal for tyramine in two categories as both behaviors emerged at different times.

to D_2 and D_3 dopamine receptors (Toll et al., 1998) and also interferes with DAT and NET (Yamamoto et al., 2007; Mandela et al., 2010), non-specific actions including at a PEA binding site may have accounted for the reduced incidence of LLA with PEA. Overall, the VMAT inhibitor, reserpine, does not prevent the expression of TA-evoked LLA.

TA-evoked locomotor-like activity is independent of high-affinity monoamine transporters located on descending monoaminergic terminals. The high-affinity monoamine transporters for 5-HT (SERT), DA (DAT), and NA (NET) control the extracellular concentrations of monoamines and maintain presynaptic function (Torres et al., 2003). Since the monoamine transporters are also a substrate for transport of TAs (Xie et al., 2007), a mechanism by which the TAs could exert their action is by transporter-mediated presynaptic uptake at descending monoaminergic terminals. For example, the TAs could elevate extracellular monoamines by competing for uptake or displacing biologically active biogenic amines from their storage leading to efflux via reverse transport (Premont et al., 2001). Additionally, once transported intracellularly, they could act on presynaptic TAARs to alter basal activity (Miller, 2011).

To explore this, we compared the motor rhythm-inducing actions of the TAs or monoamines to those seen in the presence of co-applied SERT, DAT, and NET transport inhibitors (citalopram, 1 μM; bupropion, 1 μM; and clomipramine, 5 μM, respectively). Given the variability in expression of TA-evoked responses observed in earlier experiments, we undertook most subsequent studies using littermate pairs of spinal cords in the same experimental chamber (**Figure 10B**). 5-HT-evoked LLA was not significantly altered ($n = 12$; 0.16 ± 0.06 Hz without vs. 0.15 ± 0.04 Hz with inhibitors). Similarly, PEA-evoked LLA was unaffected by the transport inhibitors in 9 animals (0.17 ± 0.09 Hz vs. 0.15 ± 0.06 Hz). Tryptamine-induced motor rhythms were comparable in 7/8 animals and blocked in another (0.11 ± 0.03 Hz without vs. 0.08 ± 0.06 Hz with inhibitors). Tyramine-evoked actions were blocked in 2/11 animals. Interestingly, tyramine block occurred in both spinal cords of a single chamber, littermate-paired experiment (**Figure 10Bb$_2$**). In the remaining 9 neonates, LLA was not blocked (0.08 ± 0.07 Hz before vs. 0.12 ± 0.10 Hz in the presence of inhibitors). Thus, the TAs can activate motor rhythms independent of actions on high-affinity monoamine transporters in the isolated spinal cord. As no spinal neurons express NET, SERT, or DAT, direct actions on spinal circuits are strongly implicated.

Intracellular transport of tyramine and tryptamine are required for locomotor-like activity: dependence on low affinity Na^+-independent membrane transporters. OCTs belong to a family of Na^+ and Cl^- independent bidirectional solute carrier (SLC) transporters, SLC22A (Jonker and Schinkel, 2004). The family contains three subtypes (OCT1-3) and all appear widely expressed in the spinal cord (Allen_Spinal_Cord_Atlas, 2009). Tyramine was shown to be the best physiological substrate for OCT1 and OCT2 (Schomig et al., 2006). In comparison, DA, NA, and 5-HT clearance was too low for a primary role in transport while tryptamine and PEA actions were not tested. The plasma membrane monoamine transporter (PMAT) is another at Na^+-independent transporter with comparable pharmacological and transport properties to the OCTs. PMAT transports biogenic amines including tyramine and tryptamine (Engel and Wang, 2005) while PEA acts as a potent cis-transport inhibitor (Ho et al., 2011). PMAT has widespread expression in neurons in brain and spinal cord (Engel and Wang, 2005; Allen_Spinal_Cord_Atlas, 2009).

Pentamidine is an OCT inhibitor with IC_{50} values of 0.4 and 5.1 μM for OCT1 and OCT2, respectively (Jung et al., 2008). The effects of pentamidine were only tested on tyramine and tryptamine-evoked LLA (**Figure 11A**). Tyramine-induced LLA (0.19 ± 0.02 Hz) was blocked in all 8/8 cords tested ($p < 0.001$). Tryptamine was also tested in 5 of these animals. Pentamidine completely blocked tryptamine-induced LLA in 2/5 animals with reduced overall frequency in the other 3 animals (0.16 ± 0.02 to 0.07 ± 0.07 Hz; $p < 0.05$). Depressant actions were associated with dramatic reductions in the magnitude of observed activity (**Figure 11B**). When 5-HT was subsequently applied, LLA re-emerged in all 5 animals (0.17 ± 0.08 Hz) supporting an independence of pentamidine actions on 5-HT-evoked LLA (**Figure 11B**). It is noteworthy that pentamidine is also a NMDA receptor non-competitive antagonist, but did not prevent expression of 5-HT/NMDA LLA (Reynolds and Aizenman, 1992; Reynolds et al., 1993).

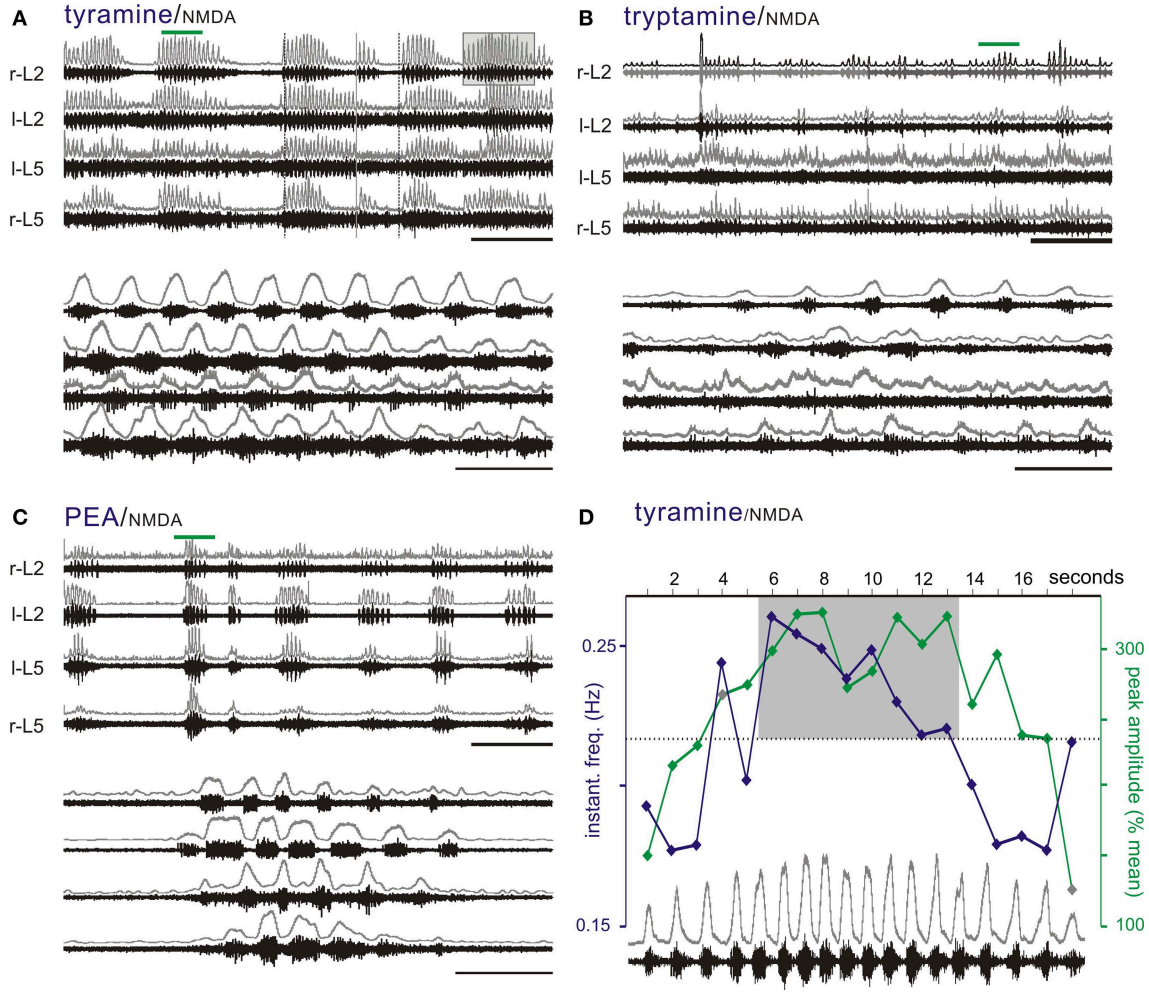

FIGURE 8 | The TAs produce episodic bursting patterns that are different than the regular pattern seen with 5-HT in the presence of NMDA. (A–C) Tyramine, tryptamine, and PEA can produce episodic rhythmic motor bursting patterns with bouts of locomotor-like bursting interrupted by quiescent periods. Bottom panels are expanded time scales of time periods identified by green bars to show in detail the locomotor-like coordination. Typically, episodic bursts are concurrent on all ventral roots. **(D)** Episodic bouts of locomotor-like activity are frequently associated with wax and wane changes in amplitude and frequency. Shown is the bout highlighted by the box on r-L2 in panel A with overlaid plots of instantaneous frequency (blue) and peak amplitude of the rectified response (green). Note the trend for amplitude and frequency to increase then decrease over the episodic bout of locomotion. Events within the shaded box emphasize the dominance of higher frequency/higher amplitude values in the middle of the episode of bursting. The upper traces in gray at each lumbar root represent the rectified/low-pass filtered equivalent of the raw waveforms below them. Scale bars are 100 s in top panels and 10 s in bottom panels.

Evidence that the TAs can be endogenously synthesized from their aromatic amino acid precursors to generate locomotor-like activity. Intrinsic spinal cord AADC activity should enable endogenous synthesis of the TAs from their precursor aromatic amino acids (AAAs), phenylalanine (for PEA), tyrosine (for tyramine), and tryptophan (for tryptamine). We therefore tested whether bath application of AAAs lead to the expression of rhythmic motor activity. Tyrosine ($n = 5$), tryptophan ($n = 3$), or phenylalanine ($n = 3$) were applied at doses between 100 and 200 μM with or without NMDA. In two additional experiments, the AAAs were co-applied. In all cases, no obvious maintained motor rhythms were observed (not shown).

As *in vitro* experiments are undertaken in the absence of essential amino acid containing media, we explored whether the lack of observed effect was related to lack of substrate availability. In the developing neonate, available cytoplasmic AAAs may be preferentially directed toward protein synthesis. We therefore tested the effects of complete arrest of protein synthesis using cycloheximide (100 μM). Cycloheximide is known to increase intracellular levels of AAA (Beugnet et al., 2003) and inhibition is mostly reversible within 1 h (Abbas, 2013).

Eight dual experiments were conducted with pairs of spinal cords from littermates recorded simultaneously in the same bath chamber ($n = 16$ total sample). In the presence of NMDA and cycloheximide, motor rhythms with epochs consistent with a locomotor-like coordination were observed in all 16 spinal cords. Activity was comparatively fast at 0.31 ± 0.09 Hz but appeared to be weaker and more variable than that seen with

Table 2 | Methysergide preferentially blocks tryptamine and 5-HT-induced locomotor like activity.

Drug	Methysergide dose			
	1 μM	2 μM	5 μM	10 μM
5-HT	2/8	5/5		
Tryptamine	6/6			
Tyramine	1/6	0/5	2/5	0/3
PEA	0/3	0/3	0/3	0/3
Noradrenaline	0/4	0/4	0/4	0/4
Dopamine	0/6	0/4	0/4	0/4

Following induced LLA, methysergide was applied in progressively increasing doses as shown to assess differential monoamines agonist sensitivity to block.

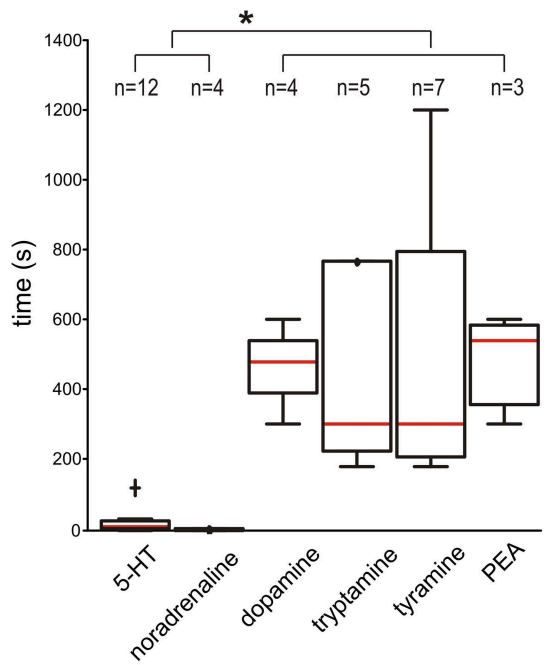

FIGURE 9 | Dopamine and the TAs take a significantly longer time to initiate bursting than 5-HT and NA. The box-and-whisker plots show the time it takes to initiate bursting (always in the presence of NMDA). The red line is the median and individual outliers, defined as more than 1.5 times the interquartile range were observed for 5-HT (cross) and for tryptamine at 1800 s (not shown). Asterisk (*) indicates $p < 0.05$. Samples sizes are shown at top.

the monoamines. Cycloheximide was applied before NMDA ($n = 2$), after NMDA ($n = 5$), or co-applied with NMDA ($n = 1$). Cycloheximide alone produced no observable action, but episodic LLA emerged with subsequent application of NMDA (**Figure 12A**). NMDA applied alone generated some epochs of weak bursting activity in at least one of the paired cords in 3/5 experiments with subsequent addition of cycloheximide leading to episodic LLA on spinal cord pairs (**Figure 12B**). In the 3 experiments with only bath applied NMDA, spontaneous bursting was also seen prior to NMDA application. Overall increases in initial cord excitability seen may have been due to residual cycloheximide from prior days. In one of these experiments, NMDA alone generated robust bursting though subsequent application of cycloheximide led to a clearly strengthened and regularized LLA (**Figure 12C**).

As described earlier, episodic tyramine- and PEA-evoked an episodic form of LLA never seen with the classical monoamines (in 33 and 70% of animals, respectively; **Figure 7**). After cycloheximide, episodic bouts of LLA appeared in 69% (11/16) of tested animals, 4 of which expressed rhythms that were distinctly PEA-like (**Figure 12D**). LLA coordination across recorded ventral roots is depicted in a polar plot of relative burst timing (**Figure 12E**). Thus, in the absence of protein synthesis, an increased bioavailable source of amino acids enabled the expression of episodic LLA, a distinctive feature of TA-evoked motor rhythms.

DISCUSSION

The neonatal rat spinal cord was shown to contain AADC, the essential synthesis enzyme for TA biosynthesis from their amino acid precursors, as well as their cognate G protein-coupled receptors (TAAR1 and TAAR4) that together define the essential substrates to exert biological actions. Whether and how this intrinsic spinal aminergic modulatory system is recruited is unknown, but exogenously provided TAs clearly promote motor pattern generating circuits. To exert their modulatory actions TAs show dependence on intracellular translocation via Na^+-independent transporters, consistent with prominent intracellularly located TAARs. Unlike the classical monoamines, the TAs can also generate an episodic form of LLA. That episodic motor rhythms are also seen following increases in precursor amino acid availability supports the endogenous TA biosynthesis as a means to promote motor circuit activation.

ANATOMICAL OBSERVATIONS

AADC and tyramine expression in the spinal cord

In both adult and neonate, we regularly observed AADC labeling in cells surrounding the central canal (D cells), in a ventral stream from this region, and in blood vessels. Central canal-related labeling is consistent with previous reports in rat, mouse, and monkey (Jaeger et al., 1983; Nagatsu et al., 1988; Barraud et al., 2010; Li et al., 2014; Wienecke et al., 2014). Labeling in blood vessels has also been reported (Jaeger et al., 1983; Li et al., 2014). AADC immunolabeling was also seen in other gray matter areas and in a midline ventral funiculus white matter bundle (Wienecke et al., 2014). This white matter tract likely represents axon projections of D cells as we observed that DiI applied to this region retrogradely labeled central canal cells. In the neonate, we also observed a ventral collection of $AADC^+$ midline cells emanating from the central canal. These cells may represent a ventral "migratory stream" of active neurogenesis since Wienecke et al. (2014) observed that D cells are $Neun^-$/$doublecortin^+$ in adult rat, and thus likely to be newly generated neurons or neural precursors. Interestingly, motoneurons appeared to be weakly $AADC^+$ though neither *in situ* hybridization nor immunodetection of AADC has been previously reported in rat spinal motoneurons. To support this observation, we mined our earlier microarray expression profiling study on laser capture microdissected

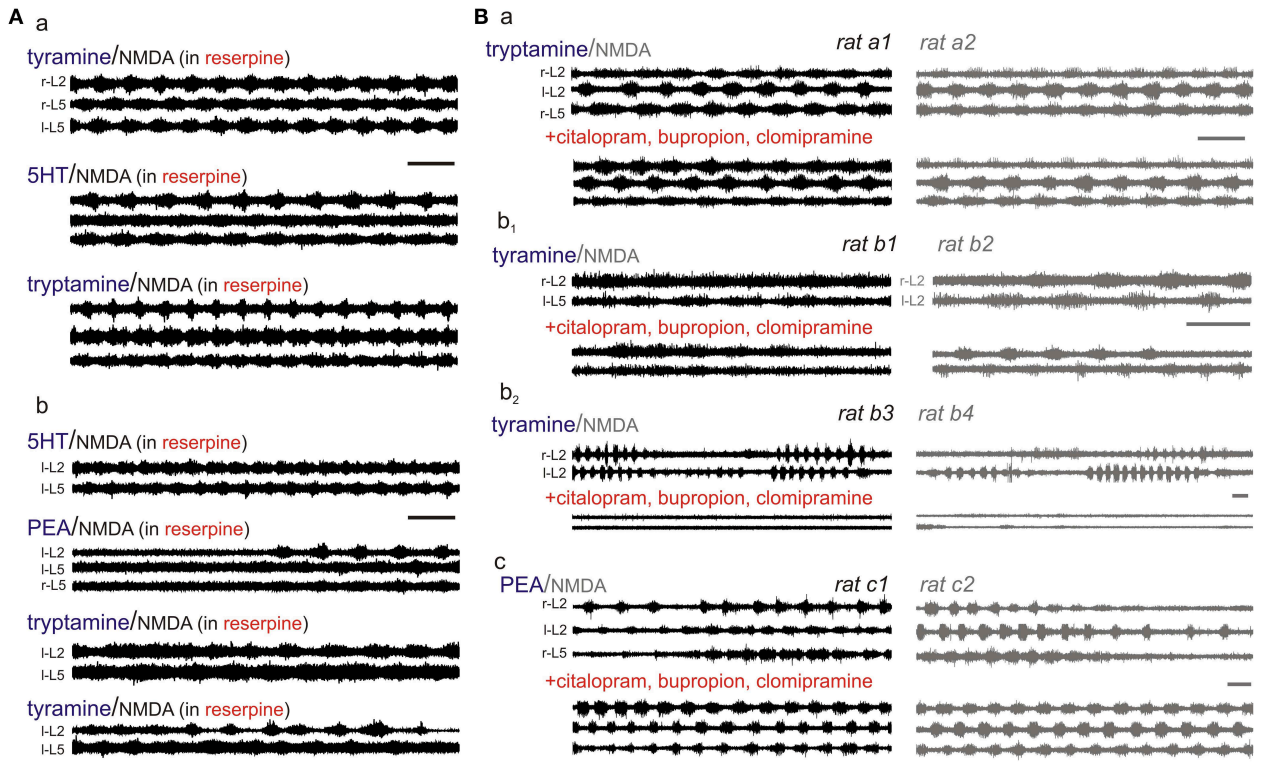

FIGURE 10 | TA-induced LLA is not principally via uptake by high-affinity plasma membrane or vesicular monoamine transporters. (A) The vesicular monoamine transport inhibitor, reserpine (10 μM), did not prevent TA-induced modulatory actions. Shown is evoked LLA in two rats **(a,b) (B)** TA-evoked actions were tested before and after combined application of the transport inhibitors, citalopram (1 μM; a SERT inhibitor), buproprion (1 μM; a DAT inhibitor), and clomipramine (5 μM; a SERT and NET inhibitor). The transport inhibitors were usually without effect as shown for tryptamine **(a)**, but could also depress (**b**; in this case tyramine) or facilitate LLA (**c**; in this case PEA). In all panels, 5-HT and the TAs were always in the presence of NMDA. Scale bars are 10 s in all panels.

medial and lateral motor column motoneurons (Cui et al., 2006). Using the present/absent call in Affymetrix Microarray Suite software, AADC cDNA was detected as present in all samples of motoneurons. In comparison, tyrosine hydroxylase and dopamine-β-hydroxylase cDNA were reported as absent.

Tyramine immunolabeling was also seen in AADC-containing neurons, consistent with AADC-mediated synthesis from tyrosine. It is assumed that tryptamine and PEA would also be synthesized by AADC from their respective amino acid precursors, but could not identify commercially available antibodies with acceptable specificity for these TAs. While the most consistent labeling observed for tyramine and AADC was in central canal midline regions and motoneurons, we noted considerable inter-animal variability. Indeed, the TAs have been described as protean. Metabolic sensitivity to temporal shifts in substrate availability may be a defining feature of the AADC-TA-TAAR modulatory system (Burchett and Hicks, 2006).

Trace amine-associated receptors 1 and 4 in the spinal cord

Previously, *in situ* hybridization, RT-PCR, and LacZ reporter expression studies all observed labeled TAAR1 in the brain (Borowsky et al., 2001; Lindemann et al., 2008) and one report also examined and detected expression in spinal cord (Borowsky et al., 2001). While TAARs are thought to be G_s-coupled (Borowsky et al., 2001), there is also evidence of activation of G_q-coupled signal transduction pathways (Panas et al., 2012). Here, RT-PCR identified expression of several TAARs in the neonatal rat spinal cord, including TAAR1 and TAAR4. We also observed TAAR1 and TAAR4 immunolabeling in neurons near the central canal and in motoneurons. TAAR1 and TAAR4 expression observed in the spinal cord overlapped with the expression of AADC and tyramine. Since tyramine and PEA activate TAAR1 and tryptamine and PEA activate TAAR4 (Borowsky et al., 2001), the TAs have a substrate for biological actions in the spinal cord. Suggestive immunolabeling evidence of a cytoplasmic location of both ligand (tyramine) and receptors (TAAR1 and TAAR4) supports an intracellular activation of signal transduction pathways (Miller, 2011). This is fully consistent with exogenously applied TAs showing dependence on transmembrane transport and taking longer than 5-HT and NA to exert modulatory actions. In summary, the mechanisms for TA synthesis and actions are anatomically coincident, and provide a substrate by which TAs can produce effects on their own.

ELECTROPHYSIOLOGICAL ACTIONS

When applied alone, the monoamines, tryptamine, and tyramine increased motor activity, including via direct excitatory actions on motoneurons. This agrees with a previous study showing that

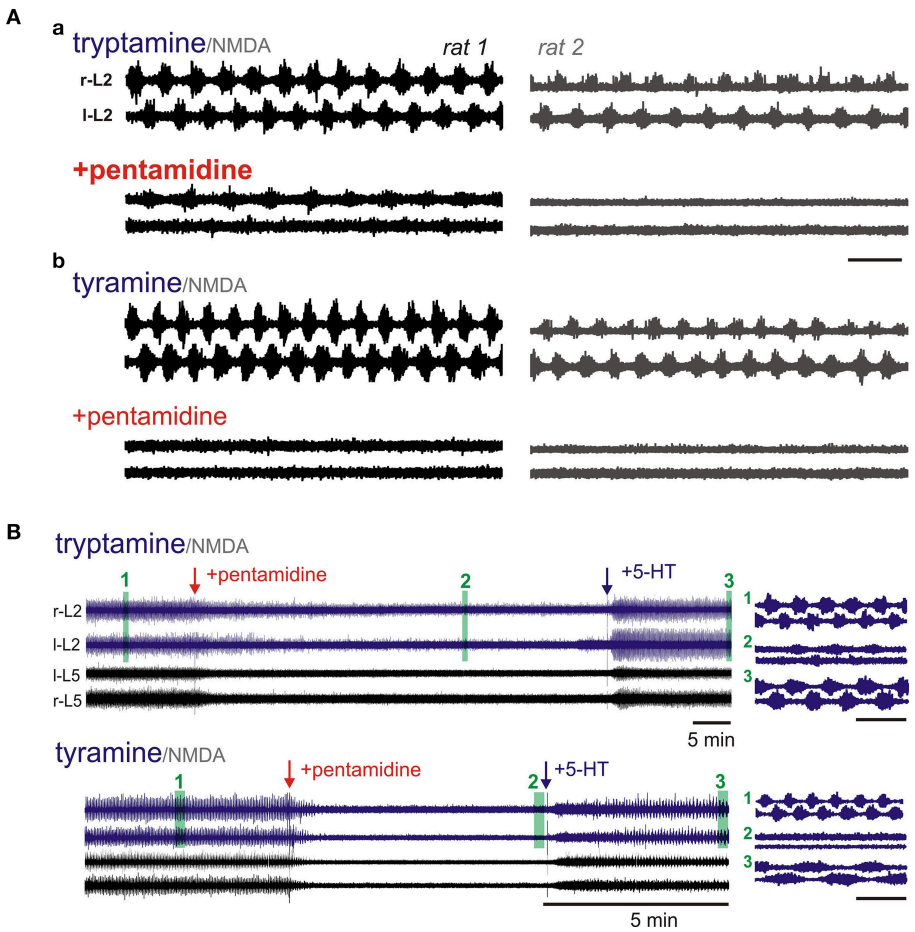

FIGURE 11 | OCT/PMAT transport inhibitors depress or block tryptamine and tyramine-induced LLA. Shown are simultaneous recordings from littermates in the same chamber. **(A_a)** Pentamidine (200 μM) depressed tryptamine-induced LLA. **(A_b)** Following wash, tyramine-induced LLA was also depressed by pentamidine. In both **(A_a,A_b)**, LLA re-emerged with subsequent addition of 5-HT (not shown). **(B)** Time course of pentamidine-induced depression of tyramine and tryptamine-induced LLA in the same animal. LLA re-emerges after application of 5-HT (50 μM). Arrows denote time of pentamidine and 5-HT applications. R-L2 and l-L2 recordings at right present epochs with a shorter time scale for the periods highlighted by numbered green bars. Scale bars are 10 s unless otherwise shown.

tyramine can act directly on motoneurons (Kitazawa et al., 1985). PEA is an agonist at both TAAR1 and TAAR4 receptors (Borowsky et al., 2001; Bunzow et al., 2001); so their expression in motoneurons should also facilitate motor activity. However, no overt actions were observed. One possibility is that there is no membrane transporter for PEA in motoneurons. Another possibility is that TAARs are expressed as heterodimers (e.g., TAAR1/TAAR4) that preferentially interfere with PEA binding (Babusyte et al., 2013). Other possibilities include actions on other receptors (e.g., G_i-coupled α_2-adrenergic) that compete with TAAR1 and TAAR4 mediated actions (Pacifico et al., 2012).

We observed that 5-HT, NA, and DA produced comparable LLA in the presence of NMDA, consistent with reports of sub-locomotor doses of NMDA helping to stabilize and regularize the locomotor rhythm (Sqalli-Houssaini et al., 1993; Cowley and Schmidt, 1994; Kjaerulff et al., 1994; Schmidt et al., 1998). The TAs also produced LLA indistinguishable from that seen with the monoamine transmitters, strongly suggests that the TAs are acting at the level of the locomotor central pattern generator (CPG) and recruiting the same pattern-generating circuits. However, the detailed pattern of activation (which CPG neuron classes are recruited) may not be the same.

The TAs were also generated an episodic form of LLA not seen with the monoamine transmitters, and both continuous and episodic locomotor phenotypes could be observed at different times within an individual animal. Episodic LLA may represent a physiologic pattern recruited by endogenous mechanisms. For example, voluntary wheel running in rats and mice is episodic, occurring in short bouts separated by longer periods (Hanagasioglu and Borbely, 1982; Eikelboom and Mills, 1988; De Bono et al., 2006). Whelan et al. (2000) reported spontaneous episodes of rhythmic ventral root activity in the mouse very similar to those seen here (Whelan et al., 2000).

Episodic LLA patterns may reflect recruitment of additional neurons or intrinsic membrane properties that influence the output of the spinal locomotor CPG. This is explored theoretically

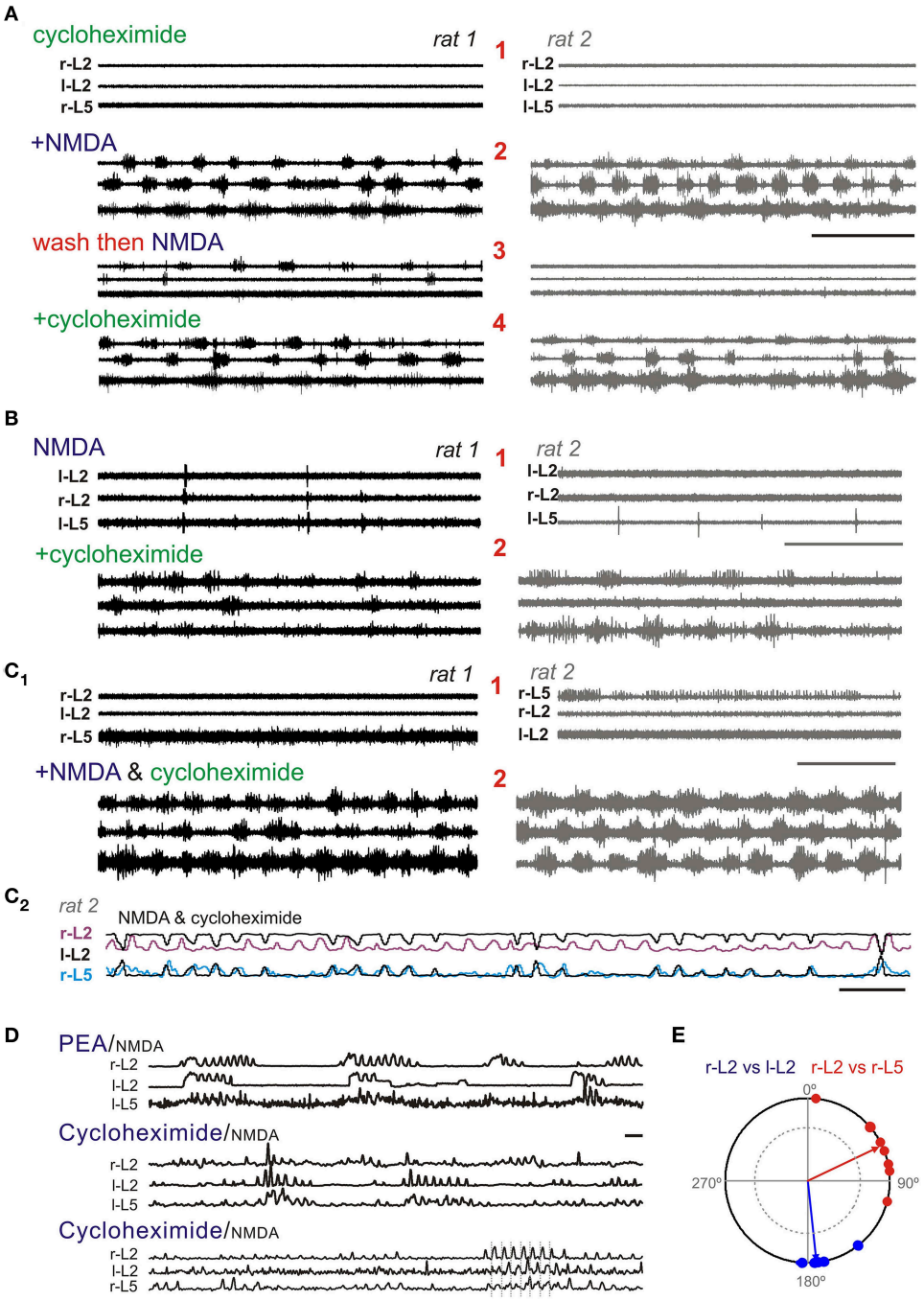

FIGURE 12 | In the presence of NMDA, cycloheximide-induced accumulation of intracellular amino acids leads to the expression or facilitation of LLA. Shown are three separate experiments with pairs of littermate matched spinal cords in the same recording chamber. **(A)** Following pre-incubation of cycloheximide **(1)**, applied NMDA leads to the expression of LLA in both cords **(2)**. Replacement of solution and subsequent application of NMDA **(3)** reinstates some bursting in cord, but a full LLA pattern emerges in both cords only after reapplication of cycloheximide **(4)**. **(B)** NMDA generated some spiking in both cords of this pair **(1)** while addition of cycloheximide led to LLA in both pairs. **(C_1)** In this pair, some spontaneous excitability was seen initially (1) and $NMDA^+$ cycloheximide led to coordinated LLA **(2)**. Note the overall similarity in response in paired spinal cords. **(C_2)** Low-pass filtered recording of additional recorded LLA activity in *rat 2* after cycloheximide and NMDA. l-L2 activity (black) is inverted and adjacent to r-L2 to show anti-phase left-right coordination between flexors, and overlapped with r-L5 activity to show phase coupling with contralateral extensor activity. **(D)** Comparison to PEA evoked rhythm. Longer duration recordings with rectified, low-pass filtered trace compare PEA expression pattern to cycloheximide/NMDA LLA in two separate animals. Scale bar is 10 s in all **(A–D)**. **(E)** Phase relationship of r-L2 burst onset (from 0°) to l-L2 and r-L5 burst onsets (blue and red symbols, respectively). The cycle progresses clockwise from 0° (in-phase) to 180° (out-of-phase) to 360°/0°. Events correspond to bursts in lower record of **(D)** identified with vertical dotted bars. Timing is consistent with LLA. Arrow length represents the concentration (r) about the mean angle (Ø). The inner circle inside the phase diagram denotes the critical r vector calculated from the Rayleigh's Z table using $\alpha = 0.05$ (Zar, 1999).

in **Figure 13A** with the assumption that the TA-induced regular LLA pattern is produced at the CPG level. Episodic modulation of LLA might occur in neurons that project onto the CPG (left illustration) themselves producing a much slower rhythmic synaptic drive onto the CPG. This is consistent with observed waxing and waning of LLA. Obvious candidate neurons are the AADC-expressing D cells surrounding the central canal (described below). Other possible organizations are depicted and explored in **Figure 13A**.

While the function of these D cells in the mammal is still unknown, similarly-located and projecting CSF-contacting neurons in larval zebrafish initiate slow swimming by optogenetic stimulation. Their genetic silencing reduced the frequency of spontaneous locomotion, and they provided the necessary tone for spontaneous forward swimming (Wyart et al., 2009). Based on the similarity in location, it is not unreasonable to consider a comparable role for D cells in the mammal. Aside from CSF-contacting D cells, many other AADC$^+$ neurons were located in adjacent ventromedial locations. This location is consistent with ventromedially located interneurons shown to undergo intrinsic membrane voltage oscillations including in association with neurochemicals that induce LLA (Hochman et al., 1994; Tazerart et al., 2008; Brocard et al., 2013). Thus, D cells, with projections into the ventral funiculus, and previously reported synaptic projections toward motor nuclei (Jaeger et al., 1983) represent a likely source of the episodic modulation of locomotor activity.

TA ACTIONS ARE MECHANISTICALLY DISTINCT FROM THE MONOAMINES TRANSMITTERS

The TAs are structurally similar to the classical monoamines and may act as monoamine receptor agonists. Tryptamine can activate 5-HT$_2$ and 5-HT$_7$ receptors (Boess and Martin, 1994), on which 5-HT induced locomotion is dependent (Madriaga et al., 2004; Liu and Jordan, 2005; Liu et al., 2009), and tryptamine and 5-HT evoke similar locomotor patterns with similar sensitivity to methysergide block. On the other hand, tyramine and PEA-evoked LLA appear to have very low affinity to the monoamine receptors (U'Prichard et al., 1977; Shen et al., 1993; Peddi et al., 2003). Critically, the TAs including tryptamine require 50–100-fold longer incubations periods to activate LLA and this difference is not consistent actions via plasma membrane monoamine receptors. Therefore, tryptamine may require activation of 5HT as well as TAARs to generate LLA.

Slower actions were not due to indirect release of monoamines through their transporters since TA-induced LLA remained following block of VMAT and the Na$^+$-dependent monoamine transporters. Instead, prolonged incubations periods are consistent with observed dependence of tyramine- and tryptamine-evoked LLA on intracellular transport via low affinity Na$^+$-independent membrane transporters. Collectively these observations support TA recruitment of LLA by intrinsic spinal mechanisms independent of descending monoaminergic systems.

Notably, DA also took much longer to initiate LLA. Since DA can be generated in endothelia subsequent to hypoxic stress (Pfeil et al., 2014) and DA is a low affinity agonist at

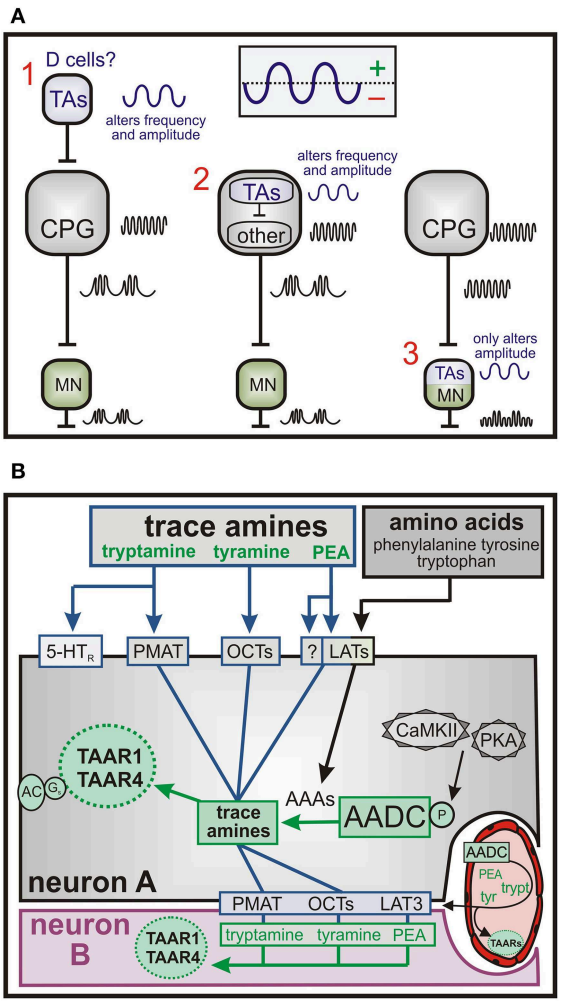

FIGURE 13 | Possible network and cellular pathways for TA-mediated actions. (A) Possible location of TA modulatory actions in the emergence of episodic LLA. **(1)** Episodic bursts upstream of the locomotor central pattern generator (CPG) could include alternating excitation and inhibition. Putative trace aminergic neurons are the AADC$^+$ D cells. Their location is within the spinal region responsible for locomotor rhythmogenesis. Their effect could be to generate a waxing and waning of CPG output to motoneurons (MN). **(2)** Modulatory actions within a subclass of CPG neurons. For example, the synaptic drive provided by the CPG could interact with slow frequency intrinsic voltage oscillations. **(3)** Modulatory actions within motoneurons are not consistent with observed waxing and waning in locomotor CPG frequency. **(B)** Putative transport and intracellular signaling mechanisms for TA actions. The TAs, tyramine and tryptamine, undergo Na$^+$-independent membrane transport via the PCTs and PMAT, respectively. PEA transport could include the LATs, which are also responsible for transport of the precursor aromatic amino acids (AAAs), tyrosine, phenylalanine, and tryptophan. It is also possible for these TAs to be made from their AAA precursors in AADC-containing cells. Once inside, the TAs act on intracellularly-located TAARs to produce Gs-coupled neuromodulatory responses. The TAs could also be generated via AADC in some neurons (here neuron A) or from endothelia then transported into adjacent non-AADC but TAAR-containing neurons to exert neuromodulatory actions. Abbreviations: PMAT, plasma membrane monoamines transporter; OCTs, organic cat ion transporters; LATs, large neutral amino acid transporters; PKA, protein kinase A; cAMKII, calcium/calmodulin dependent protein kinase II; AC, adenylate cyclase; 5-HTR, 5-HT receptor; TA, trace amine; TAAR, trace amine-associated receptor.

TAAR1 in rat (Bunzow et al., 2001), DA-induced LLA may normally associate with vascular release and subsequent intracellular neuronal transport via PMAT.

Dependence on transmembrane transport for PEA-evoked actions was not tested. Biologically, PEA transport is known to occur via a saturable proton-dependent transport process independent of all currently studied OCTs (Fischer et al., 2010). PEA is also a potent cis-transport inhibitor of PMAT (Ho et al., 2011). Some PEA transport may occur by Na^+-independent system L amino acid transporter LAT3 (Babu et al., 2003), which shows widespread expression in the mouse spinal cord (Allen_Spinal_Cord_Atlas, 2009), as well as by simple diffusion (Berry et al., 2013).

As reported for TAAR1 in HEK cells (Bunzow et al., 2001; Miller, 2011), we observed cytoplasmic labeling for TAAR1 and TAAR4, both of which are activated by the TAs (Borowsky et al., 2001). A cytoplasmic location of the ligand and the receptor (e.g., tyramine and TAAR1) would support intracellular activation of signal transduction pathways (Miller, 2011). Such a co-localization would not require release from vesicles and could explain why the TAs do not appear to be found there (Berry, 2004; Burchett and Hicks, 2006).

Putative transport mechanisms for the TA are shown in **Figure 13B**. The TAs, tyramine and tryptamine, can transport via OCTs and PMAT, respectively, whereas PEA may transport via LAT3 but more likely via a currently unidentified transporter. TA biosynthesis can alternatively be generated intracellularly from aromatic amino acid (AAA) precursors in AADC-containing cells to act on intracellularly-located TAARs. This would constitute a form of biochemical integration (Katz and Clemens, 2001). TAs generated via AADC in some neurons (here neuron A) or from endothelia can transport into adjacent non-AADC but TAAR-containing neurons to exert neuromodulatory actions (OCTs and PMAT transport is bidirectional). Last, as AADC and TAARs are present in vasculature, TA modulation of vascular function may lead to secondary actions on neurons (Hardebo et al., 1979; Nagatsu et al., 1988; Broadley, 2010; Anwar et al., 2012).

TA BIOSYNTHESIS AS A SYMPATHETIC AUTONOMIC CELLULAR STRESS RESPONSE

Intrinsic spinal cord AADC activity should enable endogenous synthesis of the TAs from their AAA precursors. However, when the AAAs were bath-applied, no obvious maintained motor rhythms were observed. Since intracellular transport of AAAs may be rapidly sequestered for protein synthesis during this neonatal period of dramatic growth, we blocked protein synthesis with cycloheximide in order to increase biochemically access to AAAs (Beugnet et al., 2003). The effects of cycloheximide were dramatic with LLA developing in all animals. Additional studies are required to directly link increases in AAAs to increases in endogenous TA biosynthesis to induce a neuromodulatory response. However, emergent LLA included TA-like episodic bouts including those with burst structures notably comparable to that seen with PEA.

Access to precursor amino acids for TA biosynthesis may depend heavily stress-induced protein catabolism. As the TAs are known sympathomimetics (Branchek and Blackburn, 2003; Berry, 2004), spinal TAs may function to facilitate motor responses during strong sympathetic nervous system activation. For example, marked PEA increases are seen in human urine after a highly stressful event (Paulos and Tessel, 1982). Just as DA can be generated via AADC in endothelia subsequent to hypoxic stress (Pfeil et al., 2014), intracellularly-synthesized TAs may comprise an integral physiological component of the autonomic stress response.

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REFERENCES

Abbas, A. K. (2013). Evidence for constitutive protein synthesis in hippocampal LTP stabilization. *Neuroscience* 246, 301–311. doi: 10.1016/j.neuroscience.2013.05.011

Allen_Spinal_Cord_Atlas. (2009). *Allen Institute for Brain Science* [Online]. Seattle, WA. Available online at: http://mousespinal.brain-map.org

Ambalavanar, R., and Morris, R. (1989). Fluoro-Gold injected either subcutaneously or intravascularly results in extensive retrograde labelling of CNS neurones having axons terminating outside the blood-brain barrier. *Brain res.* 505, 171–175. doi: 10.1016/0006-8993(89)90133-9

Anwar, M. A., Ford, W. R., Broadley, K. J., and Herbert, A. A. (2012). Vasoconstrictor and vasodilator responses to tryptamine of rat-isolated perfused mesentery: comparison with tyramine and beta-phenylethylamine. *Br. J. Pharmacol.* 165, 2191–2202. doi: 10.1111/j.1476-5381.2011.01706.x

Babu, E., Kanai, Y., Chairoungdua, A., Kim, D. K., Iribe, Y., Tangtrongsup, S., et al. (2003). Identification of a novel system L amino acid transporter structurally distinct from heterodimeric amino acid transporters. *J. Biol. Chem.* 278, 43838–43845. doi: 10.1074/jbc.M305221200

Babusyte, A., Kotthoff, M., Fiedler, J., and Krautwurst, D. (2013). Biogenic amines activate blood leukocytes via trace amine-associated receptors TAAR1 and TAAR2. *J. Leukoc. Biol.* 93, 387–394. doi: 10.1189/jlb.0912433

Barraud, Q., Obeid, I., Aubert, I., Barriere, G., Contamin, H., McGuire, S., et al. (2010). Neuroanatomical study of the A11 diencephalospinal pathway in the non-human primate. *PLoS ONE* 5:e13306. doi: 10.1371/journal.pone.0013306

Barriere, G., Mellen, N., and Cazalets, J. R. (2004). Neuromodulation of the locomotor network by dopamine in the isolated spinal cord of newborn rat. *Eur. J. Neurosci.* 19, 1325–1335. doi: 10.1111/j.1460-9568.2004.03210.x

Berry, M. D. (2004). Mammalian central nervous system trace amines. Pharmacologic amphetamines, physiologic neuromodulators. *J. Neurochem.* 90, 257–271. doi: 10.1111/j.1471-4159.2004.02501.x

Berry, M. D. (2007). The potential of trace amines and their receptors for treating neurological and psychiatric diseases. *Rev. Recent Clin. Trials* 2, 3–19. doi: 10.2174/157488707779318107

Berry, M. D., Shitut, M. R., Almousa, A., Alcorn, J., and Tomberli, B. (2013). Membrane permeability of trace amines: evidence for a regulated, activity-dependent, nonexocytotic, synaptic release. *Synapse* 67, 656–667. doi: 10.1002/syn.21670

Beugnet, A., Tee, A. R., Taylor, P. M., and Proud, C. G. (2003). Regulation of targets of mTOR (mammalian target of rapamycin) signalling by intracellular amino acid availability. *Biochem. J.* 372, 555–566. doi: 10.1042/BJ20021266

Boess, F. G., and Martin, I. L. (1994). Molecular biology of 5-HT receptors. *Neuropharmacology* 33, 275–317. doi: 10.1016/0028-3908(94)90059-0

Borowsky, B., Adham, N., Jones, K. A., Raddatz, R., Artymyshyn, R., Ogozalek, K. L., et al. (2001). Trace amines: identification of a family of mammalian G protein-coupled receptors. *Proc. Natl. Acad. Sci. U.S.A.* 98, 8966–8971. doi: 10.1073/pnas.151105198

Boulton, A. A. (1976). Identification, distribution, metabolism, and function of meta and para tyramine, phenylethylamine and tryptamine in brain. *Adv. Biochem. Psychopharmacol.* 15, 57–67.

Boulton, A. A. (1978). The tyramines: functionally significant biogenic amines or metabolic accidents? *Life Sci.* 23, 659–671. doi: 10.1016/0024-3205(78)90064-4

Boulton, A. A. (1991). Phenylethylaminergic modulation of catecholaminergic neurotransmission. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 15, 139–156. doi: 10.1016/0278-5846(91)90076-D

Boulton, A. A., Juorio, A. V., and Paterson, I. A. (1990). Phenylethylamine in the CNS: effects of monoamine oxidase inhibiting drugs, deuterium substitution and lesions and its role in the neuromodulation of catecholaminergic neurotransmission. *J. Neural Transm. Suppl.* 29, 119–129.

Boulton, A. A., Juorio, A. V., Philips, S. R., and Wu, P. H. (1977). The effects of reserpine and 6-hydroxydopamine on the concentrations of some arylakylamines in rat brain. *Br. J. Pharmacol.* 59, 209–214. doi: 10.1111/j.1476-5381.1977.tb06996.x

Bowman, W. C., Callingham, B. A., and Osuide, G. (1964). EFFECTS OF TYRAMINE ON A SPINAL REFLEX IN THE ANAESTHETISED CHICK. *J. Pharm. Pharmacol.* 16, 505–515. doi: 10.1111/j.2042-7158.1964.tb07505.x

Bradaia, A., Trube, G., Stalder, H., Norcross, R. D., Ozmen, L., Wettstein, J. G., et al. (2009). The selective antagonist EPPTB reveals TAAR1-mediated regulatory mechanisms in dopaminergic neurons of the mesolimbic system. *Proc. Natl. Acad. Sci. U.S.A.* 106, 20081–20086. doi: 10.1073/pnas.0906522106

Branchek, T. A., and Blackburn, T. P. (2003). Trace amine receptors as targets for novel therapeutics: legend, myth and fact. *Curr. Opin. Pharmacol.* 3, 90–97. doi: 10.1016/S1471-4892(02)00028-0

Broadley, K. J. (2010). The vascular effects of trace amines and amphetamines. *Pharmacol. Ther.* 125, 363–375. doi: 10.1016/j.pharmthera.2009.11.005

Brocard, F., Shevtsova, N. A., Bouhadfane, M., Tazerart, S., Heinemann, U., Rybak, I. A., et al. (2013). Activity-dependent changes in extracellular Ca^{2+} and K^{+} reveal pacemakers in the spinal locomotor-related network. *Neuron* 77, 1047–1054. doi: 10.1016/j.neuron.2013.01.026

Bunzow, J. R., Sonders, M. S., Arttamangkul, S., Harrison, L. M., Zhang, G., Quigley, D. I., et al. (2001). Amphetamine, 3,4-methylenedioxymethamphetamine, lysergic acid diethylamide, and metabolites of the catecholamine neurotransmitters are agonists of a rat trace amine receptor. *Mol. Pharmacol.* 60, 1181–1188. doi: 10.1124/mol.60.6.1181

Burchett, S. A., and Hicks, T. P. (2006). The mysterious trace amines: protean neuromodulators of synaptic transmission in mammalian brain. *Prog. Neurobiol.* 79, 223–246. doi: 10.1016/j.pneurobio.2006.07.003

Cazalets, J. R., Borde, M., and Clarac, F. (1995). Localization and organization of the central pattern generator for hindlimb locomotion in newborn rat. *J. Neurosci.* 15, 4943–4951.

Chiellini, G., Frascarelli, S., Ghelardoni, S., Carnicelli, V., Tobias, S. C., Debarber, A., et al. (2007). Cardiac effects of 3-iodothyronamine: a new aminergic system modulating cardiac function. *FASEB J.* 21, 1597–1608. doi: 10.1096/fj.06-7474com

Clarac, F., Pearlstein, E., Pflieger, J. F., and Vinay, L. (2004). The *in vitro* neonatal rat spinal cord preparation: a new insight into mammalian locomotor mechanisms. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 190, 343–357. doi: 10.1007/s00359-004-0499-2

Cowley, K. C., and Schmidt, B. J. (1994). Some limitations of ventral root recordings for monitoring locomotion in the *in vitro* neonatal rat spinal cord preparation. *Neurosci. Lett.* 171, 142–146. doi: 10.1016/0304-3940(94)90625-4

Cowley, K. C., and Schmidt, B. J. (1997). Regional distribution of the locomotor pattern-generating network in the neonatal rat spinal cord. *J. Neurophysiol.* 77, 247–259.

Cui, D., Dougherty, K. J., Machacek, D. W., Sawchuk, M., Hochman, S., and Baro, D. J. (2006). Divergence between motoneurons: gene expression profiling provides a molecular characterization of functionally discrete somatic and autonomic motoneurons. *Physiol. Genomics* 24, 276–289. doi: 10.1152/physiolgenomics.00109.2005

De Bono, J. P., Adlam, D., Paterson, D. J., and Channon, K. M. (2006). Novel quantitative phenotypes of exercise training in mouse models. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 290, R926–R934. doi: 10.1152/ajpregu.00694.2005

Durden, D. A., Philips, S. R., and Boulton, A. A. (1973). Identification and distribution of beta-phenylethylamine in the rat. *Can. J. Biochem.* 51, 995–1002. doi: 10.1139/o73-129

Eikelboom, R., and Mills, R. (1988). A microanalysis of wheel running in male and female rats. *Physiol. Behav.* 43, 625–630. doi: 10.1016/0031-9384(88)90217-X

Engel, K., and Wang, J. (2005). Interaction of organic cations with a newly identified plasma membrane monoamine transporter. *Mol. Pharmacol.* 68, 1397–1407. doi: 10.1124/mol.105.016832

Fischer, W., Neubert, R. H., and Brandsch, M. (2010). Transport of phenylethylamine at intestinal epithelial (Caco-2) cells: mechanism and substrate specificity. *Eur. J. Pharm. Biopharm.* 74, 281–289. doi: 10.1016/j.ejpb.2009.11.014

Garcia-Ramirez, D. L., Calvo, J. R., Hochman, S., and Quevedo, J. N. (2014). Serotonin, dopamine and noradrenaline adjust actions of myelinated afferents via modulation of presynaptic inhibition in the mouse spinal cord. *PLoS ONE* 9:e89999. doi: 10.1371/journal.pone.0089999

Gerin, C., Becquet, D., and Privat, A. (1995). Direct evidence for the link between monoaminergic descending pathways and motor activity. 1. A study with microdialysis probes implanted in the ventral funiculus of the spinal cord. *Brain Res.* 704, 191–201. doi: 10.1016/0006-8993(95)01111-0

Gieseker, E., Williams, K., Sawchuk, M., and Hochman, S. (2004). The trace amine tyramine is found in spinal ventral horn neurons and induces locomotor-like activity in the isolated neonatal rat spinal cord. *Soc. Neurosci. Abstr.* 30.

Gozal, E. A., Sawchuk, M. A., and Hochman, S. (2007). Trace amine immunolabeling and motor patterning in the neonatal rat spinal cord. *Soc. Neurosci. Abstr.* 33.

Gozal, E. A., Williams, K., Sawchuk, M. A., and Hochman, S. (2006). Trace amines recruit motor activity in the isolated neonatal rat spinal cord. *Soc. Neurosci. Abstr.* 32.

Hanagasioglu, M., and Borbely, A. A. (1982). Effect of voluntary locomotor activity on sleep in the rat. *Behav. Brain Res.* 4, 359–368. doi: 10.1016/0166-4328(82)90060-2

Hardebo, J. E., Falck, B., Owman, C., and Rosengren, E. (1979). Studies on the enzymatic blood-brain barrier: quantitative measurements of DOPA decarboxylase in the wall of microvessels as related to the parenchyma in various CNS regions. *Acta Physiol. Scand.* 105, 453–460. doi: 10.1111/j.1748-1716.1979.tb00110.x

Hayes, H. B., Chang, Y. H., and Hochman, S. (2009). An *in vitro* spinal cord-hindlimb preparation for studying behaviorally relevant rat locomotor function. *J. Neurophysiol.* 101, 1114–1122. doi: 10.1152/jn.90523.2008

Ho, H. T., Pan, Y., Cui, Z., Duan, H., Swaan, P. W., and Wang, J. (2011). Molecular analysis and structure-activity relationship modeling of the substrate/inhibitor interaction site of plasma membrane monoamine transporter. *J. Pharmacol. Exp. Ther.* 339, 376–385. doi: 10.1124/jpet.111.184036

Hochman, S., Garraway, S. M., Machacek, D. W., and Shay, B. L. (2001). "5-HT receptors and the neuromodulatory control of spinal cord function," in *Motor Neurobiology of the Spinal Cord*, ed T. C. Cope (Boca Raton: CRC Press), 47–87.

Hochman, S., Gozal, E. A., Hayes, H. B., Anderson, J. T., Deweerth, S. P., and Chang, Y. H. (2012). Enabling techniques for *in vitro* studies on mammalian spinal locomotor mechanisms. *Front. Biosci.* 17, 2158–2180. doi: 10.2741/4043

Hochman, S., Jordan, L. M., and Macdonald, J. F. (1994). N-methyl-D-aspartate receptor-mediated voltage oscillations in neurons surrounding the central canal in slices of rat spinal cord. *J. Neurophysiol.* 72, 565–577.

Jacobs, B. L., and Fornal, C. A. (1993). 5-HT and motor control: a hypothesis. *Trends Neurosci.* 16, 346–352. doi: 10.1016/0166-2236(93)90090-9

Jaeger, C. B., Ruggiero, D. A., Albert, V. R., Joh, T. H., and Reis, D. J. (1984). "Immunocytochemical localization of aromatic-L-amino acid decarboxylase," in *Handbook of Chemical Neuroanatomy*, Vol. 2, *Classical Transmitters in the CNS, Part 1*, eds A. Bjorkland and T. Hokfelt (Amsterdam: Elsevier), 387–408.

Jaeger, C. B., Teitelman, G., Joh, T. H., Albert, V. R., Park, D. H., and Reis, D. J. (1983). Some neurons of the rat central nervous system contain aromatic-L-amino-acid decarboxylase but not monoamines. *Science* 219, 1233–1235. doi: 10.1126/science.6131537

Jonker, J. W., and Schinkel, A. H. (2004). Pharmacological and physiological functions of the polyspecific organic cation transporters: OCT1, 2, and 3 (SLC22A1-3). *J. Pharmacol. Exp. Ther.* 308, 2–9. doi: 10.1124/jpet.103.053298

Jung, N., Lehmann, C., Rubbert, A., Knispel, M., Hartmann, P., Van Lunzen, J., et al. (2008). Relevance of the organic cation transporters 1 and 2 for antiretroviral drug therapy in human immunodeficiency virus infection. *Drug Metab. Dispos.* 36, 1616–1623. doi: 10.1124/dmd.108.020826

Juorio, A. V. (1979). Drug-induced changes in the formation, storage and metabolism of tyramine in the mouse. *Br. J. Pharmacol.* 66, 377–384. doi: 10.1111/j.1476-5381.1979.tb10841.x

Juorio, A. V. (1980). Effects of molindone and fluphenazine on the brain concentration of some phenolic and catecholic amines in the mouse and the rat. *Br. J. Pharmacol.* 70, 475–480. doi: 10.1111/j.1476-5381.1980.tb08726.x

Juorio, A. V. (1988). "Brain beta-phenylethylamine: localization, pathways, and interrelation with catecholamines," in *Progress in Catecholamine Research*, eds A. Dahlstrom, M. Sandler, and R. Belmarker (New York, NY: Alan R. Liss), 433–437.

Karoum, F., Nasrallah, H., Potkin, S., Chuang, L., Moyer-Schwing, J., Phillips, I., et al. (1979). Mass fragmentography of phenylethylamine, m- and p-tyramine and related amines in plasma, cerebrospinal fluid, urine, and brain. *J. Neurochem.* 33, 201–212. doi: 10.1111/j.1471-4159.1979.tb11722.x

Katz, P. S., and Clemens, S. (2001). Biochemical networks in nervous systems: expanding neuronal information capacity beyond voltage signals. *Trends Neurosci.* 24, 18–25. doi: 10.1016/S0166-2236(00)01686-6

Kiehn, O. (2006). Locomotor circuits in the mammalian spinal cord. *Annu. Rev. Neurosci.* 29, 279–306. doi: 10.1146/annurev.neuro.29.051605.112910

Kiehn, O., and Kjaerulff, O. (1996). Spatiotemporal characteristics of 5-HT and dopamine-induced rhythmic hindlimb activity in the *in vitro* neonatal rat. *J. Neurophysiol.* 75, 1472–1482.

Kiehn, O., and Kjaerulff, O. (1998). Distribution of central pattern generators for rhythmic motor outputs in the spinal cord of limbed vertebrates. *Ann. N.Y. Acad. Sci.* 860, 110–129. doi: 10.1111/j.1749-6632.1998.tb09043.x

Kiehn, O., Sillar, K. T., Kjaerulff, O., and McDearmid, J. R. (1999). Effects of noradrenaline on locomotor rhythm-generating networks in the isolated neonatal rat spinal cord. *J. Neurophysiol.* 82, 741–746.

Kitazawa, T., Saito, K., and Ohga, A. (1985). Effects of catecholamines on spinal motoneurones and spinal reflex discharges in the isolated spinal cord of the newborn rat. *Brain Res.* 351, 31–36. doi: 10.1016/0165-3806(85)90228-7

Kjaerulff, O., Barajon, I., and Kiehn, O. (1994). Sulphorhodamine-labelled cells in the neonatal rat spinal cord following chemically induced locomotor activity *in vitro*. *J. Physiol.* 478(Pt 2), 265–273.

Kjaerulff, O., and Kiehn, O. (1996). Distribution of networks generating and coordinating locomotor activity in the neonatal rat spinal cord *in vitro*: a lesion study. *J. Neurosci.* 16, 5777–5794.

Kremer, E., and Lev, T. A. (1997). Localization of the spinal network associated with generation of hindlimb locomotion in the neonatal rat and organization of its transverse coupling system. *J. Neurophysiol.* 77, 1155–1170.

Leo, D., Mus, L., Espinoza, S., Hoener, M. C., Sotnikova, T. D., and Gainetdinov, R. R. (2014). Taar1-mediated modulation of presynaptic dopaminergic neurotransmission: role of D2 dopamine autoreceptors. *Neuropharmacology* 81, 283–291. doi: 10.1016/j.neuropharm.2014.02.007

Li, Y., Li, L., Stephens, M. J., Zenner, D., Murray, K. C., Winship, I. R., et al. (2014). Synthesis, transport, and metabolism of serotonin formed from exogenously applied 5-HTP after spinal cord injury in rats. *J. Neurophysiol.* 111, 145–163. doi: 10.1152/jn.00508.2013

Lindemann, L., Meyer, C. A., Jeanneau, K., Bradaia, A., Ozmen, L., Bluethmann, H., et al. (2008). Trace amine-associated receptor 1 modulates dopaminergic activity. *J. Pharmacol. Exp. Ther.* 324, 948–956. doi: 10.1124/jpet.107.132647

Liu, J., Akay, T., Hedlund, P. B., Pearson, K. G., and Jordan, L. M. (2009). Spinal 5-HT7 receptors are critical for alternating activity during locomotion: *in vitro* neonatal and *in vivo* adult studies using 5-HT7 receptor knockout mice. *J. Neurophysiol.* 102, 337–348. doi: 10.1152/jn.91239.2008

Liu, J., and Jordan, L. M. (2005). Stimulation of the parapyramidal region of the neonatal rat brain stem produces locomotor-like activity involving spinal 5-HT7 and 5-HT2A receptors. *J. Neurophysiol.* 94, 1392–1404. doi: 10.1152/jn.00136.2005

Madriaga, M. A., McPhee, L. C., Chersa, T., Christie, K. J., and Whelan, P. J. (2004). Modulation of locomotor activity by multiple 5-HT and dopaminergic receptor subtypes in the neonatal mouse spinal cord. *J. Neurophysiol.* 92, 1566–1576. doi: 10.1152/jn.01181.2003

Mandela, P., Chandley, M., Xu, Y. Y., Zhu, M. Y., and Ordway, G. A. (2010). Reserpine-induced reduction in norepinephrine transporter function requires catecholamine storage vesicles. *Neurochem. Int.* 56, 760–767. doi: 10.1016/j.neuint.2010.02.011

Mann, R., and Bell, C. (1991). Neuronal metabolism and DOPA decarboxylase immunoreactivity in terminal noradrenergic sympathetic axons of rat. *J. Histochem. Cytochem.* 39, 663–668. doi: 10.1177/39.5.1673138

Martin, L. L., Baker, G. B., and Wood, P. L. (1988). "Tryptamine turnover: effects of drugs," in *Trace Amines: Comparative and Clinical Neurobiology*, eds A. A. Boulton, A. V. Juorio, and R. G. H. Downer (New Jersey, NJ: Humana Press), 95–104, 57–174.

Matsushima, T., and Grillner, S. (1992). Neural mechanisms of intersegmental coordination in lamprey: local excitability changes modify the phase coupling along the spinal cord. *J. Neurophysiol.* 67, 373–388.

Merchenthaler, I. (1991). Neurons with access to the general circulation in the central nervous system of the rat: a retrograde tracing study with fluoro-gold. *Neuroscience* 44, 655–662. doi: 10.1016/0306-4522(91)90085-3

Millan, M. J. (2002). Descending control of pain. *Prog. Neurobiol.* 66, 355–474. doi: 10.1016/S0301-0082(02)00009-6

Miller, G. M. (2011). The emerging role of trace amine-associated receptor 1 in the functional regulation of monoamine transporters and dopaminergic activity. *J. Neurochem.* 116, 164–176. doi: 10.1111/j.1471-4159.2010.07109.x

Murray, K. C., Stephens, M. J., Ballou, E. W., Heckman, C. J., and Bennett, D. J. (2011). Motoneuron excitability and muscle spasms are regulated by 5-HT2B and 5-HT2C receptor activity. *J. Neurophysiol.* 105, 731–748. doi: 10.1152/jn.00774.2010

Nagatsu, I., Sakai, M., Yoshida, M., and Nagatsu, T. (1988). Aromatic L-amino acid decarboxylase-immunoreactive neurons in and around the cerebrospinal fluid-contacting neurons of the central canal do not contain dopamine or serotonin in the mouse and rat spinal cord. *Brain Res.* 475, 91–102. doi: 10.1016/0006-8993(88)90202-8

Nguyen, T. V., and Juorio, A. V. (1989). Binding sites for brain trace amines. *Cell. Mol. Neurobiol.* 9, 297–311. doi: 10.1007/BF00711411

Ono, H., Ito, H., and Fukuda, H. (1991). 2-phenylethylamine and methamphetamine enhance the spinal monosynaptic reflex by releasing noradrenaline from the terminals of descending fibers. *Jpn. J. Pharmacol.* 55, 359–366. doi: 10.1254/jjp.55.359

Pacifico, R., Dewan, A., Cawley, D., Guo, C., and Bozza, T. (2012). An olfactory subsystem that mediates high-sensitivity detection of volatile amines. *Cell Rep.* 2, 76–88. doi: 10.1016/j.celrep.2012.06.006

Panas, M. W., Xie, Z., Panas, H. N., Hoener, M. C., Vallender, E. J., and Miller, G. M. (2012). Trace amine associated receptor 1 signaling in activated lymphocytes. *J. Neuroimmune Pharmacol.* 7, 866–876. doi: 10.1007/s11481-011-9321-4

Paulos, M. A., and Tessel, R. E. (1982). Excretion of beta-phenethylamine is elevated in humans after profound stress. *Science* 215, 1127–1129. doi: 10.1126/science.7063846

Peddi, S., Roth, B. L., Glennon, R. A., and Westkaemper, R. B. (2003). Spiro[9,10-dihydroanthracene]-9,3'-pyrrolidine-a structurally unique tetracyclic 5-HT2A receptor antagonist. *Eur. J. Pharmacol.* 482, 335–337. doi: 10.1016/j.ejphar.2003.09.059

Pfeil, U., Kuncova, J., Bruggmann, D., Paddenberg, R., Rafiq, A., Henrich, M., et al. (2014). Intrinsic vascular dopamine—a key modulator of hypoxia-induced vasodilation in splanchnic vessels. *J. Physiol.* 592(Pt 8), 1745–1756. doi: 10.1113/jphysiol.2013.262626

Philips, S. R., Davis, B. A., Durden, D. A., and Boulton, A. A. (1975). Identification and distribution of m-tyramine in the rat. *Can. J. Biochem.* 53, 65–69. doi: 10.1139/o75-010

Philips, S. R., Durden, D. A., and Boulton, A. A. (1974a). Identification and distribution of p-tyramine in the rat. *Can. J. Biochem.* 52, 366–373. doi: 10.1139/o74-055

Philips, S. R., Durden, D. A., and Boulton, A. A. (1974b). Identification and distribution of tryptamine in the rat. *Can. J. Biochem.* 52, 447–451. doi: 10.1139/o74-068

Premont, R. T., Gainetdinov, R. R., and Caron, M. G. (2001). Following the trace of elusive amines. *Proc. Natl. Acad. Sci. U.S.A.* 98, 9474–9475. doi: 10.1073/pnas.181356198

Reddy, S. V., Maderdrut, J. L., and Yaksh, T. L. (1980). Spinal cord pharmacology of adrenergic agonist-mediated antinociception. *J. Pharmacol. Exp. Ther.* 213, 525–533.

Rekling, J. C., Funk, G. D., Bayliss, D. A., Dong, X. W., and Feldman, J. L. (2000). Synaptic control of motoneuronal excitability. *Physiol. Rev.* 80, 767–852.

Revel, F. G., Moreau, J. L., Gainetdinov, R. R., Bradaia, A., Sotnikova, T. D., Mory, R., et al. (2011). TAAR1 activation modulates monoaminergic neurotransmission, preventing hyperdopaminergic and hypoglutamatergic activity. *Proc. Natl. Acad. Sci. U.S.A.* 108, 8485–8490. doi: 10.1073/pnas.1103029108

Reynolds, I. J., and Aizenman, E. (1992). Pentamidine is an N -methyl-D-aspartate receptor antagonist and is neuroprotective *in vitro*. *J. Neurosci.* 12, 970–975.

Reynolds, I. J., Zeleski, D. M., Rothermund, K. D., Hartnett, K. A., Tidwell, R., and Aizenman, E. (1993). Studies on the effects of several pentamidine analogues on the NMDA receptor. *Eur. J. Pharmacol. Mol. Pharmacol.* 244, 175–179. doi: 10.1016/0922-4106(93)90023-3

Saavedra, J. M. (1989). "á-phenylethylamine, phenylethanoamine, tyramine and octopamine," in *Catecholamines II*, eds U. Trendelenburg and N. Weiner (Berlin: Springer-Verlag), 181–210.

Schmidt, B. J., Hochman, S., and Maclean, J. N. (1998). NMDA receptor-mediated oscillatory properties: potential role in rhythm generation in the mammalian spinal cord. *Ann. N.Y. Acad. Sci.* 860, 189–202. doi: 10.1111/j.1749-6632.1998.tb09049.x

Schmidt, B. J., and Jordan, L. M. (2000). The role of serotonin in reflex modulation and locomotor rhythm production in the mammalian spinal cord. *Brain Res. Bull.* 53, 689–710. doi: 10.1016/S0361-9230(00)00402-0

Schomig, E., Lazar, A., and Grundemann, D. (2006). Extraneuronal monoamine transporter and organic cation transporters 1 and 2: a review of transport efficiency. *Handb. Exp. Pharmacol.* 151–180. doi: 10.1007/3-540-29784-7_8

Shen, Y., Monsma, F. J. Jr., Metcalf, M. A., Jose, P. A., Hamblin, M. W., and Sibley, D. R. (1993). Molecular cloning and expression of a 5-hydroxytryptamine7 serotonin receptor subtype. *J. Biol. Chem.* 268, 18200–18204.

Shreckengost, J., Calvo, J., Quevedo, J., and Hochman, S. (2010). Bicuculline-sensitive primary afferent depolarization remains after greatly restricting synaptic transmission in the mammalian spinal cord. *J. Neurosci.* 30, 5283–5288. doi: 10.1523/JNEUROSCI.3873-09.2010

Spector, S., Melmon, K., Lovenberg, W., and Sjoerdsma, A. (1963). The presence and distribution of tyramine in mammalian tissues. *J. Pharmacol. Exp. Ther.* 140, 229–235.

Sqalli-Houssaini, Y., and Cazalets, J. R. (2000). Noradrenergic control of locomotor networks in the *in vitro* spinal cord of the neonatal rat. *Brain Res.* 852, 100–109. doi: 10.1016/S0006-8993(99)02219-2

Sqalli-Houssaini, Y., Cazalets, J. R., and Clarac, F. (1993). Oscillatory properties of the central pattern generator for locomotion in neonatal rats. *J. Neurophysiol.* 70, 803–813.

Tazerart, S., Vinay, L., and Brocard, F. (2008). The persistent sodium current generates pacemaker activities in the central pattern generator for locomotion and regulates the locomotor rhythm. *J. Neurosci.* 28, 8577–8589. doi: 10.1523/JNEUROSCI.1437-08.2008

Toll, L., Berzetei-Gurske, I. P., Polgar, W. E., Brandt, S. R., Adapa, I. D., Rodriguez, L., et al. (1998). Standard binding and functional assays related to medications development division testing for potential cocaine and opiate narcotic treatment medications. *NIDA Res. Monogr.* 178, 440–466.

Torres, G. E., Gainetdinov, R. R., and Caron, M. G. (2003). Plasma membrane monoamine transporters: structure, regulation and function. *Nat. Rev. Neurosci.* 4, 13–25. doi: 10.1038/nrn1008

U'Prichard, D. C., Greenberg, D. A., and Snyder, S. H. (1977). Binding characteristics of a radiolabeled agonist and antagonist at central nervous system alpha noradrenergic receptors. *Mol. Pharmacol.* 13, 454–473.

Whelan, P., Bonnot, A., and O'Donovan, M. J. (2000). Properties of rhythmic activity generated by the isolated spinal cord of the neonatal mouse. *J. Neurophysiol.* 84, 2821–2833.

Wienecke, J., Ren, L. Q., Hultborn, H., Chen, M., Moller, M., Zhang, Y., et al. (2014). Spinal cord injury enables aromatic l-amino acid decarboxylase cells to synthesize monoamines. *J. Neurosci.* 34, 11984–12000. doi: 10.1523/JNEUROSCI.3838-13.2014

Wolinsky, T. D., Swanson, C. J., Smith, K. E., Zhong, H., Borowsky, B., Seeman, P., et al. (2007). The Trace Amine 1 receptor knockout mouse: an animal model with relevance to schizophrenia. *Genes Brain Behav.* 6, 628–639. doi: 10.1111/j.1601-183X.2006.00292.x

Wyart, C., Del Bene, F., Warp, E., Scott, E. K., Trauner, D., Baier, H., et al. (2009). Optogenetic dissection of a behavioural module in the vertebrate spinal cord. *Nature* 461, 407–410. doi: 10.1038/nature08323

Xie, Z., and Miller, G. M. (2008). Beta-phenylethylamine alters monoamine transporter function via trace amine-associated receptor 1: implication for modulatory roles of trace amines in brain. *J. Pharmacol. Exp. Ther.* 325, 617–628. doi: 10.1124/jpet.107.134247

Xie, Z., Vallender, E. J., Yu, N., Kirstein, S. L., Yang, H., Bahn, M. E., et al. (2008). Cloning, expression, and functional analysis of rhesus monkey trace amine-associated receptor 6: evidence for lack of monoaminergic association. *J. Neurosci. Res.* 86, 3435–3446. doi: 10.1002/jnr.21783

Xie, Z., Westmoreland, S. V., Bahn, M. E., Chen, G. L., Yang, H., Vallender, E. J., et al. (2007). Rhesus monkey trace amine-associated receptor 1 signaling: enhancement by monoamine transporters and attenuation by the D2 autoreceptor *in vitro*. *J. Pharmacol. Exp. Ther.* 321, 116–127. doi: 10.1124/jpet.106.116863

Yamamoto, H., Kamegaya, E., Hagino, Y., Imai, K., Fujikawa, A., Tamura, K., et al. (2007). Genetic deletion of vesicular monoamine transporter-2 (VMAT2) reduces dopamine transporter activity in mesencephalic neurons in primary culture. *Neurochem. Int.* 51, 237–244. doi: 10.1016/j.neuint.2007.06.022

Zar, J. H. (1999). *Biostatistical Analysis: Upper Saddle River*. New Jersey, NJ: Prentice Hall.

Zhu, H., Clemens, S., Sawchuk, M., and Hochman, S. (2007). Expression and distribution of all dopamine receptor subtypes (D(1)-D(5)) in the mouse lumbar spinal cord: a real-time polymerase chain reaction and non-autoradiographic *in situ* hybridization study. *Neuroscience* 149, 885–897. doi: 10.1016/j.neuroscience.2007.07.052

Zimmerman, A. L., Sawchuk, M., and Hochman, S. (2012). Monoaminergic modulation of spinal viscero-sympathetic function in the neonatal mouse thoracic spinal cord. *PLoS ONE* 7:e47213. doi: 10.1371/journal.pone.0047213

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The sacral networks and neural pathways used to elicit lumbar motor rhythm in the rodent spinal cord

Meir Cherniak, Alex Etlin, Ido Strauss, Lili Anglister and Aharon Lev-Tov*

Department of Medical Neurobiology, Institute for Medical Research—Israel-Canada, IMRIC, The Hebrew University Medical School, Jerusalem, Israel

Edited by:
Brian R. Noga, University of Miami, USA

Reviewed by:
Uner Tan, Cukurova University, Turkey
Patrick John Whelan, University of Calgary, Canada
Kristine C. Cowley, University of Manitoba, Canada

***Correspondence:**
Aharon Lev-Tov, Department of Medical Neurobiology, Institute for Medical Research – Israel-Canada, IMRIC, The Hebrew University Medical School, P.O. Box 12272, Jerusalem 9112102, Israel
e-mail: aharonl@ekmd.huji.ac.il

Identification of neural networks and pathways involved in activation and modulation of spinal central pattern generators (CPGs) in the absence of the descending control from the brain is important for further understanding of neural control of movement and for developing innovative therapeutic approaches to improve the mobility of spinal cord injury patients. Activation of the hindlimb innervating segments by sacrocaudal (SC) afferent input and by specific application of neurochemicals to the sacral networks is feasible in the isolated spinal cord preparation of the newborn rat. Here we review our recent studies of sacral relay neurons with lumbar projections and evaluate their role in linking the sacral and thoracolumbar (TL) networks during different motor behaviors. Our major findings show that: (1) heterogeneous groups of dorsal, intermediate and ventral sacral-neurons with ventral and lateral ascending funicular projections mediate the activation of the locomotor CPGs through sacral sensory input; and (2) rhythmic excitation of lumbar flexor motoneurons, produced by bath application of alpha-1 adrenoceptor agonists to the sacral segments is mediated exclusively by ventral clusters of sacral-neurons with lumbar projections through the ventral funiculus.

Keywords: sacrocaudal afferents, calcium imaging, spinal interneurons, ascending pathways, central pattern generators, locomotor, alpha1 adrenoceptors

INTRODUCTION: THE THORACOLUMBAR LIMB-MOVING AND SACROCAUDAL BODY-STABILIZING RHYTHMOGENIC NETWORKS ARE TIGHTLY COUPLED

Spinal neuronal networks known as central pattern generators (CPGs) produce the rhythmic patterned output required for coordinated movements such as swimming and stepping in many species of vertebrates including humans (for reviews see Alford et al., 2003; Kiehn, 2006; Hultborn and Nielsen, 2007; Frigon, 2012). The CPGs are controlled by descending supraspinal commands and can be modulated to produce different patterns and behaviors. However, the CPGs can be also activated and modulated in the absence of descending control from brain by afferent-input and neuroactive compounds. Indeed, recent clinical studies have shown that reactivation of the CPGs in spinal cord injury patients by afferent input is possible and that it improves the motor function and mobility of some of the patients (for review see Hubli and Dietz, 2013). Therefore, it is of particular interest and significance to elucidate the networks and pathways involved in activation and modulation of the CPGs in accessible experimental models. The pattern generating circuitry in rodents activates the limb, trunk, and axial muscles innervating-segments to produce stabilized and coordinated locomotion under different conditions. The rhythmogenic capacity of these networks is preserved *in vitro*, in the isolated spinal cord preparation of newborn rodents (e.g., Kudo and Yamada, 1987; Smith et al., 1988). Therefore, this preparation can be used as an ideal model to study the mechanisms and interactions of the body stabilizing and limb moving networks in the absence of supraspinal control. In previous studies we described a rhythmogenic network in the sacrocaudal (SC) segments of the neonatal rat spinal cord that controls the axial and tail musculature during various movements (Lev-Tov and Delvolvé, 2000; Lev-Tov et al., 2000; Delvolvé et al., 2001; Gabbay et al., 2002). The SC-rhythmogenic network could be activated in surgically detached sacral spinal segments by stimulation of sacrocaudal afferents (SCA; Lev-Tov et al., 2000; Delvolvé et al., 2001), by bath application of noradrenaline (NA) and NMDA (Gabbay and Lev-Tov, 2004) and by the alpha1-adrenoceptor agonist, methoxamine (Gabbay and Lev-Tov, 2004). **Figure 1** shows that the SC rhythmogenic network is co-activated with the hindlimb CPGs in the isolated spinal cord of the neonatal rat in the absence and presence of bath-applied neurochemicals. Coordinated activation of the SC- and locomotor-CPGs could be also demonstrated in response to electrical stimulation of the ventromedial medulla (VMM; Blivis et al., 2007; e.g., Zaporozhets et al., 2004) or SCA in the isolated brainstem-spinal cord preparation (**Figure 1A**, see Blivis et al., 2007; e.g., Gordon et al., 2008).

Most of the studies of rodent rhythmogenesis used various combinations of NMDA and monoamines to produce the rhythm (reviewed in Miles and Sillar, 2011). **Figure 1B** shows that the NMDA/5HT induced rhythm in the sacral spinal segments is nearly abolished when the spinal cord is transected at the lumbosacral junction, while the 0.25–1 Hz lumbar rhythm produced in the presence of methoxamine disappears when the

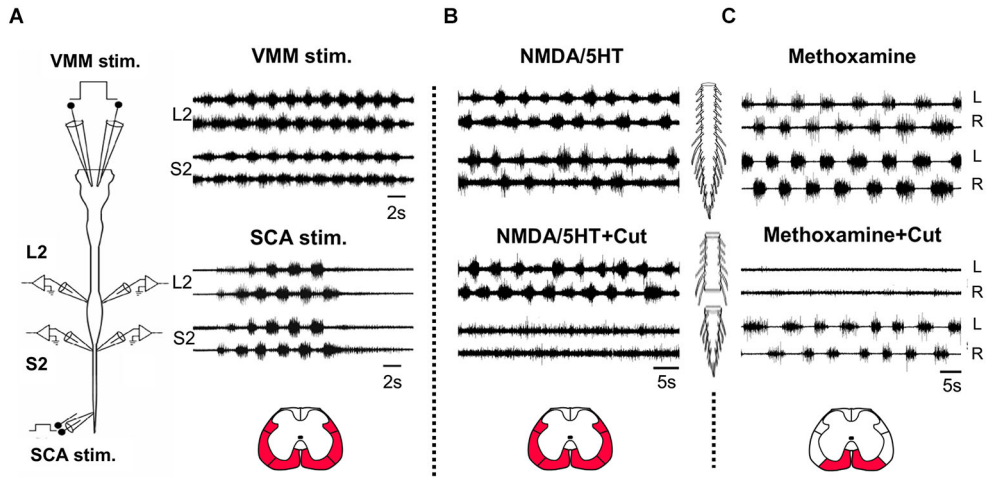

FIGURE 1 | Coordinated activation of the lumbar and sacral rhythmogenic networks. **(A)** Schematic illustration of the isolated brainstem spinal cord preparation of the neonatal rat (left). Stimulation electrodes are bilaterally attached to the ventromedial medulla (VMM) and to sacrocaudal afferents (SCA) entering though S4 or Co1 dorsal roots. Signals are recorded from left and right (L and R) ventral roots of L2 and S2. Right: Recordings of the rhythm produced by VMM (top) and SCA (bottom) stimulation from the left and right L2 and S2 ventral roots. VMM stimulation: 5 ms, 80-pulse 4 Hz train at 1 mA; SCA stimulation: 0.2 ms, 50-pulse 4 Hz train at 25 μA for the SCA. **(B)** The rhythm produced by bath application of 4 μM NMDA and 10 μM 5HT in the isolated spinal cord preparation of the newborn rat and recorded from the left and right L2 and S2 ventral roots, before (NMDA/5HT) and after transection of the cord through the lumbosacral junction (NMDA+5HT+Cut). **(C)** The rhythm produced by bath application of methoxamine (100 μM) in the isolated spinal cord preparation of the newborn rat and recorded from the left and right L2 and S2 ventral roots, before (Methoxamine) and after (Methoxamine+Cut) transection of the cord through the lumbosacral junction. The red regions in the cross sections illustrated below denote the major white matter funiculi at the lumbosacral junction, linking the sacral and lumbar networks to produce coordinated rhythmic activity under the experimental conditions specified in (**A**), (**B**) and (**C**), respectively.

sacral segments are disconnected from the lumbar spinal cord (**Figure 1C**). These findings suggest that the lumbar-CPGs drive the sacral-CPGs in the presence of NMDA/5HT and that the sacral-CPGs drive the rostral-lumbar segments to produce rhythmic bursting in the presence of methoxamine (see also Gabbay and Lev-Tov, 2004). The coupling between the thoracolumbar (TL) and SC-CPGs during the rhythm induced by sensory and brainstem stimulation was found to be in the rostro-caudal direction (Bonnot et al., 2002; Lev-Tov and O'Donovan, 2009; Etlin et al., 2010; Lev-Tov et al., 2010; Strauss, 2011). On the other hand, caudo-rostral coupling between spinal networks is also well known in many vertebrates (see changes in the direction of propagation when switching from forward to backwards swimming in lampreys, Islam et al., 2006) including the neonatal rat spinal cord preparation during neurochemically induced motor rhythms (Falgairolle and Cazalets, 2007).

Our interest in the SC-network stems from two main reasons. First, it provides basic knowledge regarding stabilization of the body axis and the use of the tail musculature during rhythmic limb movements. Second, SC-interneurons may serve as important relays onto the locomotor generators during a nocifensive/escape type of behavior triggered by stimulation of SC dermatomes (Smith et al., 1988; Lev-Tov and Delvolvé, 2000; Lev-Tov et al., 2000; Whelan et al., 2000; Delvolvé et al., 2001; Bonnot et al., 2002; Strauss and Lev-Tov, 2003). Moreover, the ability to activate the locomotor CPGs in the absence of descending control from the brain via sacral relays has a potential clinical significance for future treatments of spinal cord injury patients (see below).

More specifically, the capacity to produce the locomotor rhythm by SCA depends largely on synaptic activation of sacral relay neurons with lumbar projections (Strauss and Lev-Tov, 2003; Blivis et al., 2007; Etlin et al., 2010, 2013; Lev-Tov et al., 2010). The ability of sacral CPGs to produce an alternating left-right lumbar-flexor bursting in response to sacral application of methoxamine, also depends on activation of sacral relay neurons with rostral lumbar projections (Gabbay and Lev-Tov, 2004; as in **Figure 1C**). Here we briefly review our studies of the mediating relay neurons and the pathways involved in linking the sacral and lumbar networks under these conditions and discuss possible mechanisms and implications of our findings.

I- ACTIVATION OF LOCOMOTOR CPGs BY SACROCAUDAL AFFERENT INPUT

MEDIATION OF SCA-INDUCED LOCOMOTOR ACTIVITY BY SACRAL RELAY-NEURONS WITH LUMBAR PROJECTIONS

Afferent input is a potent activator of the locomotor CPGs in the absence of the descending control from the brain in humans and experimental mammalian models (Grillner and Rossignol, 1978; Grillner and Zangger, 1979; Duysens and Pearson, 1980; Pearson and Rossignol, 1991; Prochazka et al., 1997; Dietz and Duysens, 2000; Dietz et al., 2002; Pearson, 2004; for reviews see Edgerton et al., 2008; Dietz, 2009). We showed that mechanical and radiant heat stimulation of SC dermatomes (Lev-Tov et al., 2000; and Blivis et al., 2007, respectively; e.g., Mandadi and Whelan, 2009) as well as electrical stimulation of SCA induces coordinated activation of the

locomotor and SC-CPGs (Lev-Tov et al., 2000; Strauss and Lev-Tov, 2003; Klein and Tresch, 2010; for the mouse see Whelan et al., 2000).

The SCA-induced locomotor rhythm in the newborn rat spinal cord, could be blocked by bathing the sacral segments in low-calcium high-magnesium Kreb's saline, by selective sacral application of the non-NMDA receptor blocker CNQX (Strauss and Lev-Tov, 2003) or the mu-opioid agonist DAMGO (Blivis et al., 2007). Using surgical and pharmacological manipulations of the spinal cord, we found that the sacral relay neurons, activated by SCA to produce the locomotor rhythm, project to the lumbar segments through the ventral (VF), ventrolateral/lateral (VLF/LF) and dorsolateral (DLF) white matter funiculi (Strauss and Lev-Tov, 2003; Etlin et al., 2010; Lev-Tov et al., 2010). These studies further showed that the capacity of SCA input to produce the locomotor rhythm depends not only on activation of sacral relay-neurons with direct projections to the lumbar CPGs, but also on serial recruitment of multi-funicular sacral-propriospinal neurons interposed between the second order neurons and the hindlimb-CPGs.

SEGMENTAL AND SPATIAL DISTRIBUTION OF SACRAL RELAY NEURONS WITH LUMBAR PROJECTIONS

Confocal imaging of sacral relay neurons with lumbar projections, back-labeled through cut VF, VLF/LF and DLF axon bundles at the lumbosacral junction, was used to map their segmental and spatial distribution, and lumbar projection patterns (Etlin et al., 2010; Lev-Tov et al., 2010). **Figure 2** shows low-power projected confocal images of sacral relay-neurons in whole-mount transparent preparations of the spinal cord, labeled through the cut VF (**Figure 2A**) and the combined VLF/LF ("VLF", **Figure 2B**) axons at the left lumbosacral junction (see, respective scheme above each micrograph; also Etlin et al., 2010). These confocal micrographs demonstrate that most of the lumbar VF projections are crossed, and the majority of the lumbar VLF/LF projections are un-crossed (see also the schemes in A and B: crossed projections in blue, uncrossed projections in white). The DLF lumbar projections (not shown) were uncrossed. The back-labeled DLF-neurons were revealed mainly within the dorsal horn laminae, while most of the VF- and VLF/LF-neurons were in laminae VII, VIII and IX and the deep dorsal horn laminae (Etlin et al., 2010).

SACROCAUDAL PRIMARY AFFERENTS AND INTRASPINAL NEURONS INNERVATE SACRAL RELAY-NEURONS WITH LUMBAR PROJECTIONS

After mapping the distribution of sacral relay-neurons and their lumbar projection patterns, we examined whether these neurons are contacted by SCA, and whether these contacts are glutamatergic as predicted by Strauss and Lev-Tov (2003). Using back-labeling of sacral VF-neurons, anterograde labeling of SCA and immunostaining for vesicular glutamate transporters 1 and 2, we found that many VF-neurons are innervated by primary afferents immunoreactive for VGluT1, some VF-neurons were innervated by VGluT2^{+} primary afferents, and large proportions of the VF-neurons received VGluT2^{+} contacts from intraspinal neurons (Etlin et al., 2013). These findings suggest (e.g., Alvarez et al., 2004; Liu et al., 2010; Scherrer et al., 2010; Rogoz et al., 2012) that the sacral VF-neurons can be activated directly by glutamatergic proprioceptive and nociceptive primary afferents and indirectly by glutamatergic-interneurons (Etlin et al., 2013).

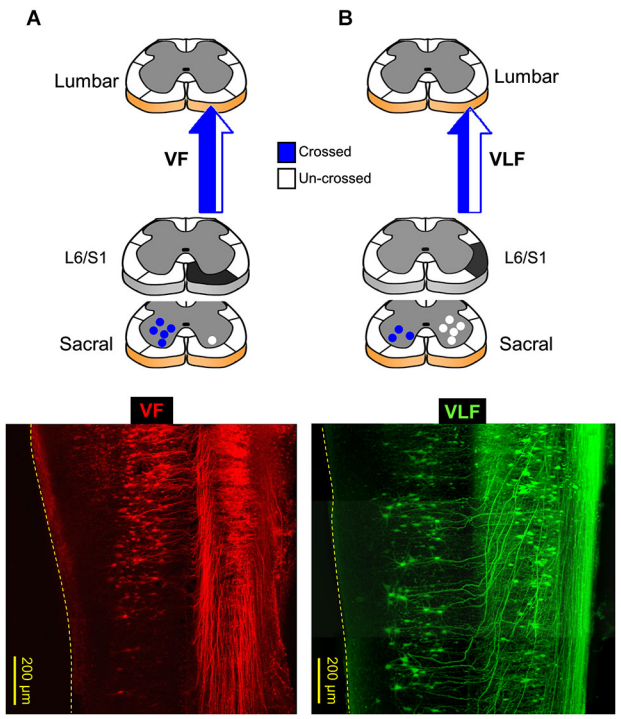

FIGURE 2 | Lumbar projections of sacral relay neurons through the ventral and ventrolateral white matter funiculi (VF and VLF; A and B, respectively). Bottom panels are confocal projected images of two transparent whole-mount spinal cord preparations in which sacral neurons with lumbar projections were back-labeled through the VF (**A**, red) and VLF (**B**, green). On top are illustrations of lumbar projections through the VF and VLF. **(A)** The lumbar projections of sacral neurons, labeled through the VF at the lumbosacral junction (L6/S1, black), are mainly crossed (blue). **(B)** More than 60% of the lumbar projections of sacral neurons, labeled through the VLF at the lumbosacral junction (L6/S1, black), are un-crossed (white). Note that the crossed lumbar projections enter the VLF after traveling along the VF (VLF micrograph; e.g., Etlin et al., 2010). The projections through the VLF are the combined projections through VLF/LF as originally described in Etlin et al. (2010).

SACRAL VF-NEURONS SERVE AS AN IMPORTANT LINK BETWEEN SCA AND THE HINDLIMB-CPGs

Etlin et al. (2010) have shown that bilateral interruption of the VF at the lumbosacral junction caused more severe interference to the lumbar rhythm produced by SCA stimulation than lesion of any other pair of white matter funiculi. They also showed that following multifunicular lesions that spared only one of the funiculi, the most regular and robust lumbar rhythm was obtained when the VF was left bilaterally intact (Etlin et al., 2010). Therefore, sacral relay-neurons projecting to the lumbar segments through the VF (VF-neurons) have been suggested to contribute more substantially than other sacral relay-neurons to activation of the lumbar-CPGs (Strauss and Lev-Tov, 2003; Etlin et al., 2010; Lev-Tov et al., 2010). These VF-neurons are innervated by glutamatergic SCA and intraspinal neurons (see above). The question raised next is whether VF-neurons are activated by

SCA-stimulation, and if so, how their activity is related to the concomitant motor-output.

Figure 3 demonstrates calcium imaging of back-filled VF-neurons and concurrent electrophysiological recordings of the motor-output produced in the rostral-lumbar and sacral segments of the isolated spinal cord during SCA stimulation (**Figure 3A**). Recordings were obtained from the ventral surface of the cord after prolonged back-loading with the calcium sensor (Calcium green dextran) through cut bundles of right VF-axons at the lumbosacral junction. The fluorescence changes ($\Delta F/F$) produced by calcium transients during the stimulus trains applied to SCA at two different intensities are shown in **Figures 3B,C**. The rhythmic $\Delta F/F$ of the imaged left VF-neuron during the higher-intensity stimulus train, exhibits a characteristic rhythmic pattern, in phase with the left ventral root bursting (**Figure 3C**). Our experiments revealed that more than 50% of the back-labeled VF-neurons could be activated by SCA stimulation. Forty-one percent of the activated VF-neurons exhibited tonic pattern, 18% showed pure rhythmic pattern, 34.5% had a tonic pattern with superimposed oscillations, and 6.2% fired irregularly during the stimulus trains. Analysis of the 161 crossed-projecting VF-neurons that exhibited oscillatory drive (with and without background tonic activity), revealed that ∼70% of them were in phase with the ipsilateral-motor output and ∼30% were in phase with the contralateral motor output (Etlin et al., 2013). We have suggested that VF-neurons with ipsilateral phase-preference are crossed-inhibitory and those with contralateral phase-preference are crossed-excitatory commissural neurons with lumbar projections (Etlin et al., 2013). This suggestion is based on the special pattern produced by sacral networks (alternating left-right rhythm and in-phase flexor-extensor activation at a given side of the sacral cord), and on studies of the phase preference of Vo commissural neurons (Lanuza et al., 2004) and lamina VIII GABAergic neurons (Wu et al., 2011).

Another interesting finding involves the relation between rhythmic bursting of the sacral CPG, the activity pattern of VF-neurons, and lumbar motor-output. **Figure 3D** demonstrates that application of the NMDA-receptor blocker APV to the sacral segments reduces the activity of the sacral-CPGs without blocking the concomitant lumbar-rhythm. Under these conditions, the tonic drive of the imaged VF-neurons is not altered (compare Sacral APV to APV wash) while the oscillatory drive of the neurons is completely blocked in ∼60% of the cells with the combined rhythmic/tonic pattern and of the cells with the rhythmic pattern, and is markedly attenuated in the remaining ∼40% of these cells (Etlin et al., 2013). When APV is washed out, the sacral-CPGs are vigorously activated; a strong rhythmic drive develops in the imaged neuron and the concomitant lumbar motor-output is strengthened significantly. All optical and electrophysiological activities are abolished in the presence of the non-NMDA receptor antagonist CNQX (Sacral CNQX), and reappear after washing the CNQX (CNQX wash). Collectively these findings suggest the following: (1) the ability of SCA stimulation to activate the VF-neurons and the CPGs depends on non-NMDA receptor-mediated synaptic transmission in the sacral segments; (2) activation of the lumbar CPGs by SCA stimulation can be obtained when the activity of the sacral CPGs is reduced or nearly blocked (see also Strauss and Lev-Tov, 2003), and when the drive of the recruited VF-neurons is purely tonic; and (3) the activities of VF-neurons and the locomotor CPGs are maximized when the SC CPGs are activated. Thus, sacral VF-neurons are suggested to be a significant link between SCA and the locomotor CPGs.

II. CAUDO-ROSTRAL COUPLING BETWEEN SACRAL AND ROSTRAL LUMBAR NETWORKS DURING ALPHA1-ADRENOCEPTOR AGONIST ACTIVATION OF THE SACRAL CPGs

The spinal cord receives extensive noradrenergic innervation (e.g., Gabbay and Lev-Tov, 2004). Noradrenaline is known to initiate locomotor activity and modulate locomotor-like activity in a number of adult mammals (For review see Miles and Sillar, 2011). In the isolated spinal cord of neonatal rodents, NA produces a very slow rhythm with various irregularities (Kiehn et al., 1999; Cazalets and Bertrand, 2000; Sqalli-Houssaini and Cazalets, 2000). When applied to the sacral segments of the neonatal rat spinal cord, NA produces a short lasting (1–2 min) "fast" alternating left-right rhythm in the sacral and lumbar segments before transforming into a very slow non-locomotor rhythm (Gabbay and Lev-Tov, 2004). The "fast" NA rhythm was found to be blocked by alpha1 and not by alpha2 adrenoceptor antagonists (Gabbay et al., 2002). A "fast" (0.25–1 Hz) and robust alternating left-right rhythm could be produced and maintained in the isolated spinal cord of neonatal rats, in the presence of the alpha1-adrenoceptor agonist methoxamine (see **Figures 1**, **4**). Our findings, that the methoxamine rhythm persists in the sacral segments and is blocked in the lumbar segments after transecting the spinal cord at the lumbosacral junction (**Figure 1C**, and Gabbay and Lev-Tov, 2004), suggest that the rhythm originates in the sacral segments of the spinal cord. The experiment shown in **Figures 4B,C** demonstrates that the 0.25–1 Hz methoxamine rhythm appears in both sacral and lumbar segments of the spinal cord only when methoxamine is added to the sacral but not to the TL spinal segments. Thus, there must be a potent coupling between the sacral CPGs and the rostral lumbar spinal segments.

What is the nature of the lumbar bursting produced by sacrally applied methoxamine? Gabbay and Lev-Tov (2004) showed that addition of methoxamine to the sacral segments produces rhythmic bursts in lumbar flexor but not in extensor motoneurons. They provided evidence suggesting that the sacral-CPGs do not activate the lumbar-CPGs in the presence of sacral methoxamine, but rather activate lumbar flexor-motoneurons and lumbar commissural-neurons (see Figure 9 in Gabbay and Lev-Tov, 2004, for the hypothetical organization of the circuitry). To study the link between the sacral-CPGs and the rostral lumbar segments under these conditions, we tried to determine the minimal anatomical configuration that is required to produce the 0.25–1 Hz rhythm in rostral lumbar motoneurons by sacrally applied methoxamine. Our most recent data (see abstracts by Cherniak et al., 2011; Roisman et al., 2014), revealed that: (1) the ability of methoxamine to produce the 0.25–1 Hz lumbar rhythm depends on intact connectivity between the TL and the first two sacral spinal segments (S1–S2); (2) the lumbar rhythm persists after removal of the dorsal aspect of the

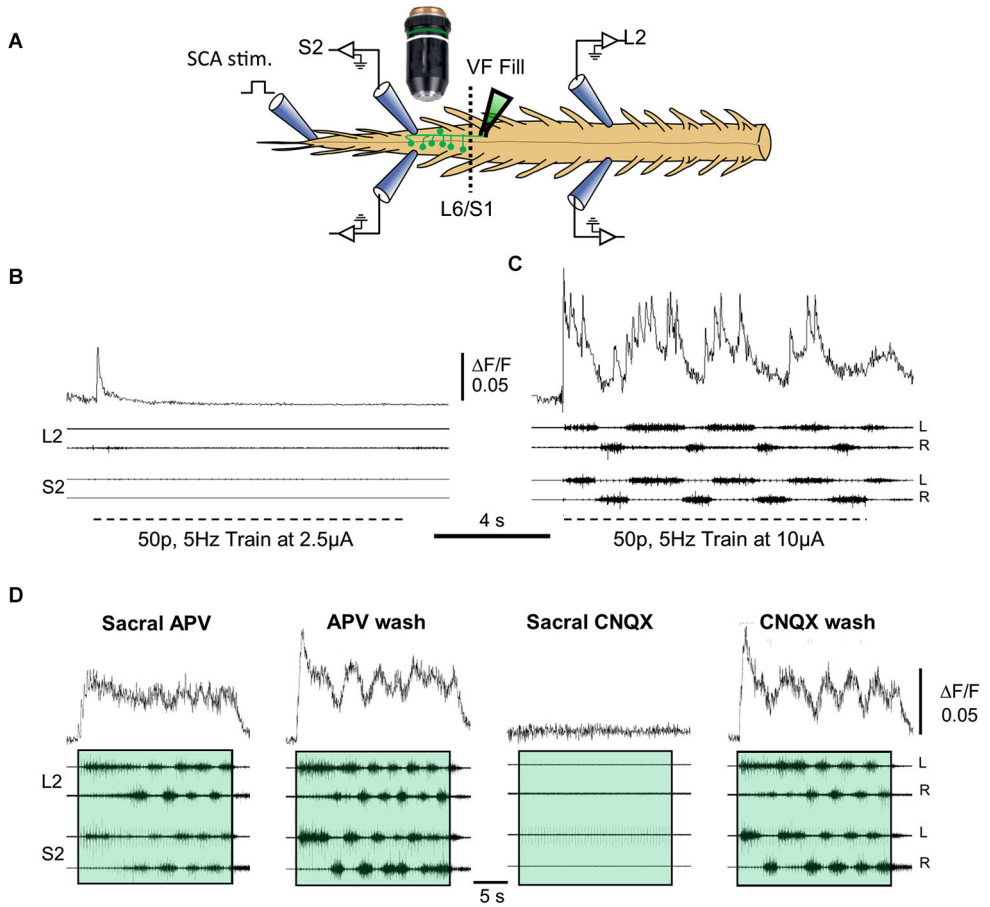

FIGURE 3 | Correlation between the activity of VF neurons and the motor output during SCA stimulation. **(A)** Illustration of the experimental set up used for simultaneous imaging of VF neurons and electrophysiological recordings of the motor output during the rhythm produced by SCA stimulation. Sacral relay neurons are loaded with calcium green dextran through the cut VF at the lumbosacral junction (L6/S1) in the isolated spinal cord preparation of the neonatal rat (VF fill). The preparation is mounted with ventral side up (in this example) in a dual chamber experimental bath (dashed line at the L6/S1 denotes the Vaseline barrier separating the two chambers). The motor output produced by stimulation of Co1 or S4 dorsal roots (SCA stimulation) is recorded from the left and right L2 and S2 ventral roots. Calcium transients produced in fluorescently labeled sacral VF-neurons are imaged simultaneously using epifluorescence microscopy with attached 14 bit CCD camera (for further details see Etlin et al., 2013). The difference image (ΔF/F) of imaged VF neurons and the simultaneous L2 and S2 ventral root recordings during SCA stimulation under different conditions are superimposed in **(B–D)**. The relations between the optical and electrophysiological signals are analyzed using the cross Wavelet and cross Wavelet coherence methods (Mor and Lev-Tov, 2007; Etlin et al., 2013). **(B,C)** Top traces are the activity patterns imaged from a left S2 VF-neuron (ΔF/F) back-labeled from the right VF with Calcium green dextran during 0.1 ms, 50-pulse, 5 Hz stimulus trains applied to the Co1 dorsal root at 2.5 and 10 μA (**B** and **C**, respectively). Below are the corresponding motor outputs (four bottom traces) recorded from the left and right ventral roots of S2 and L2, simultaneously with the optical signals. **(D)** The activity imaged from a VF-neuron (ΔF/F, upper records) and the motor output recorded from the left and right S2 and L2 ventral roots (four bottom records), during stimulation of the Co1 dorsal root, in the presence of 20 μM of sacrally applied APV (Sacral APV) and 35 min after APV wash (APV wash). Sacral addition of 10 μM CNQX under these conditions (Sacral CNQX) blocked the activity. The block is alleviated 60 min after CNQX wash (CNQX wash). Trains are confined within colored transparent rectangles. The oscillatory drive of the cells is markedly reduced by APV and is abolished by CNQX. For further details, see text and Etlin et al. (2013). Fifty-pulse 2.5 Hz stimulus trains were applied at 10 μA to produce the rhythm. **(B–D)** Modified from Etlin et al. (2013).

sacral cord down to the central canal, but not below it; and (3) the methoxamine-induced lumbar rhythm depends on ventral sacral-neurons projecting rostrally only through ventral funiculi. Following the latter finding, we imaged the activity of ventral clusters of back-labeled VF-neurons in the presence of methoxamine (**Figure 4D**) and studied their relation to the concomitant motor-output produced in the sacral- and lumbar-segments. The activity pattern of these ventral clusters of sacral VF-neurons in the presence of methoxamine was rhythmic in most cases with phase preference mainly to the ipsilateral motor output. **Figure 4E** shows the gradual appearance of rhythmic calcium transients in a left VF-neuron back-loaded with calcium green dextran and rhythmic bursting in sacral- and lumbar-segments in the presence of methoxamine. Regular optical and electrophysiological oscillatory signals developed after 10 min exposure to methoxamine. The ΔF/F of the left VF neuron imaged in

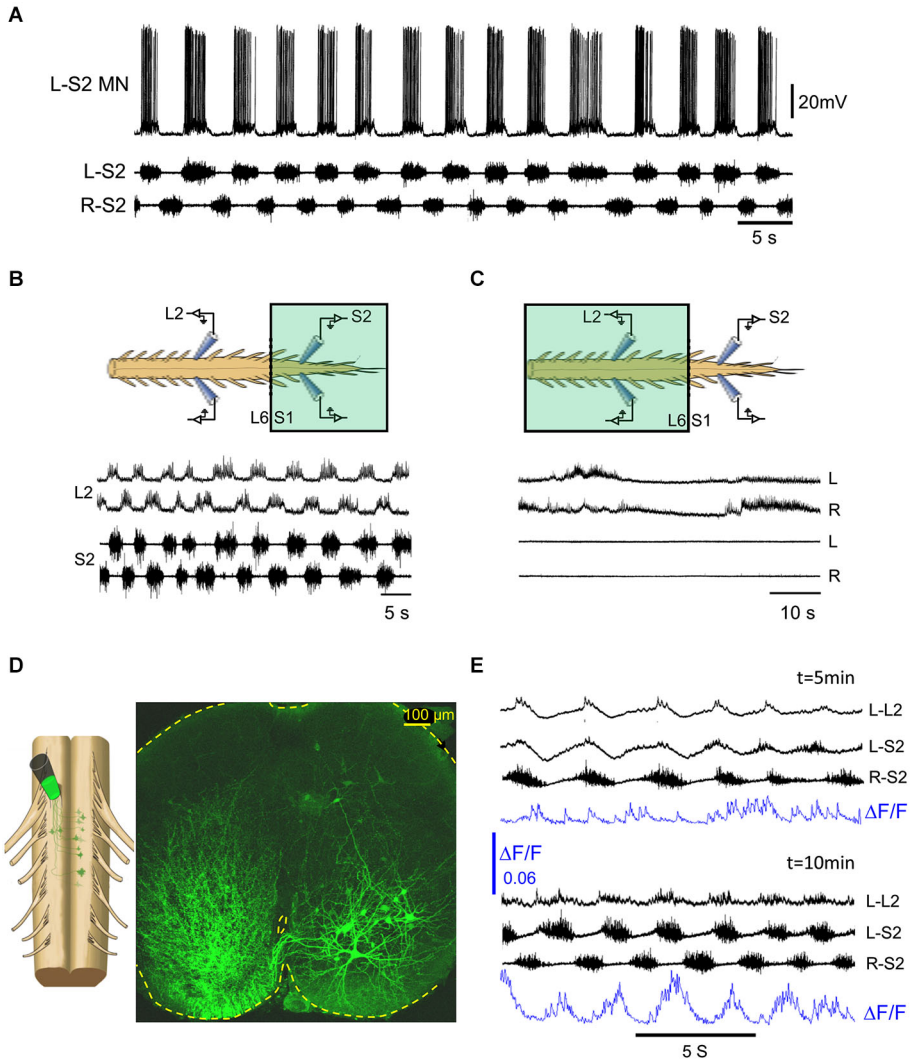

FIGURE 4 | The methoxamine-induced 0.25–1 Hz rhythm in the sacral spinal segments is relayed to the rostral lumbar network by sub-clusters of ventrally located sacral VF neurons.
(A) Intracellular recordings from a left S2 motoneuron (L-S2 MN) are superimposed with concurrent recordings from the left and right S2 ventral roots in the presence of bath-applied methoxamine (100 µM) to the surgically detached sacral segments of the spinal cord. **(B)** Recordings from the left and right L2 and S2 ventral roots showing alternating left-right bursting when methoxamine is applied to the sacral compartment (colored rectangle) of a dual chamber experimental bath. **(C)** Recordings from the left and right L2 and S2 ventral roots in the experiment described in **(B)** after washing the sacrally applied methoxamine and adding methoxamine to the TL compartment (colored rectangle) of the experimental bath. The sacral rhythmic activity is blocked, while a slow rhythm appears in the lumbar segments. **(D)** Confocal micrograph of a 70 µm cross section through the S2 spinal segment shows left sacral neurons back-loaded with fluorescein-dextran through the right VF at the lumbosacral junction (dye back-loading is illustrated on the left). **(E)** Imaging the activity developed in a ventral S2 VF-neuron back-loaded with Calcium green dextran and viewed from the ventral aspect of the isolated spinal cord (blue, ΔF/F) and the concurrently recorded motor output from the left L2 and the left and right S2 ventral roots, 5 and 10 min after addition of methoxamine to the experimental bath. The 0.1 Hz–10 Khz ventral root recordings were not high-pass filtered to reveal the early subthreshold activity in L–L2.

this experiment is in phase with the ipsilateral motor output (L-S2).

We suggest that sub-clusters of ventrally located sacral-neurons mediate the generation of the rhythmic bursting in lumbar flexor-motoneurons by sacrally applied methoxamine. Studies of trans-synaptic labeling of sacral VF- and other sacral-interneurons, using GFP-encoded retrograde virus injection to hindlimb muscles (e.g., Hadas et al., 2014), suggest oligo-synaptic connectivity between sacral VF-neurons and rostral lumbar motoneurons. Additional experiments are needed to further clarify this issue and determine the functionality of this connectivity.

CONCLUSIONS AND SUGGESTIONS

In this manuscript, we focused on the mediating role of sacral-neurons in linking the sacral and lumbar networks during

rhythmic motor activity produced by SCA input and by selective activation of sacral-neurons by alpha1 adrenoceptor agonists. While SCA stimulation activates successfully both the sacral and lumbar CPGs, sacrally applied methoxamine activates the sacral CPGs and thereby produces alternating left-right bursting in rostral lumbar motoneurons. The lumbar rhythmic bursting produced by SCA stimulation is mediated mainly by relay-neurons with direct and indirect multifunicular lumbar-projections, while the methoxamine-induced rhythm is mediated by sacral-neurons that project rostrally only through the VF. We provided evidence that the drive produced by the activated VF relay neurons and the pathways associated with them, may turn-on the locomotor pattern generators when the sacral afferents are stimulated, and stimulate lumbar flexor motoneurons in the presence of methoxamine. Thus, sub-populations of sacral VF-neurons are suggested to project to flexor motoneurons that are driven by the more excitable and dominant rostral lumbar oscillators (e.g., Cazalets et al., 1995; Kjaerulff and Kiehn, 1996; Cowley and Schmidt, 1997; Kremer and Lev-Tov, 1997; Bonnot et al., 2002; for review see Kiehn, 2006), and to the locomotor CPGs (Etlin et al., 2010, 2013). Modulation of the activity of these two sets of projections enables separate and/or simultaneous control on the frequency and power of the rhythmic lumbar output under different conditions (see Finkel et al., 2014), and thereby plays a significant role in shaping the final motor-output.

Further studies are required to clarify the role of short- and long-ascending propriospinal pathways from the sacral to the lumbar cord (Bras et al., 1988; Grottel et al., 1998; Dutton et al., 2006), and of lumbar collaterals of spinothalamic, spinocerebellar or spinoreticular pathways (Leah et al., 1988; Edgley and Grant, 1991; Yamada et al., 1991; Katter et al., 1996; Matsushita, 1999; Garifoli et al., 2006) in activation of the locomotor CPGs, and the exact mechanisms by which inter-enlargements coupling is achieved. It is also important to verify whether it is possible to extrapolate our findings in newborn rodent to the adult spinal cord, and to evaluate the potential clinical significance of the ability to activate and modulate the CPG action via sacral relay neurons in the absence of descending supraspinal control.

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REFERENCES

Alford, S., Schwartz, E., and Viana di Prisco, G. (2003). The pharmacology of vertebrate spinal central pattern generators. *Neuroscientist* 9, 217–228. doi: 10.1177/1073858403009003014

Alvarez, F. J., Villalba, R. M., Zerda, R., and Schneider, S. P. (2004). Vesicular glutamate transporters in the spinal cord, with special reference to sensory primary afferent synapses. *J. Comp. Neurol.* 472, 257–280. doi: 10.1002/cne.20012

Blivis, D., Mentis, G. Z., O'Donovan, M. J., and Lev-Tov, A. (2007). Differential effects of opioids on sacrocaudal afferent pathways and central pattern generators in the neonatal rat spinal cord. *J. Neurophysiol.* 97, 2875–2886. doi: 10.1152/jn.01313.2006

Bonnot, A., Whelan, P. J., Mentis, G. Z., and O'Donovan, M. J. (2002). Spatiotemporal pattern of motoneuron activation in the rostral lumbar and the sacral segments during locomotor-like activity in the neonatal mouse spinal cord. *J. Neurosci.* 22:RC203.

Bras, H., Cavallari, P., and Jankowska, E. (1988). Demonstration of initial axon collaterals of cells of origin of the ventral spinocerebellar tract in the cat. *J. Comp. Neurol.* 273, 584–592. doi: 10.1002/cne.902730412

Cazalets, J. R., and Bertrand, S. (2000). Coupling between lumbar and sacral motor networks in the neonatal rat spinal cord. *Eur. J. Neurosci.* 12, 2993–3002. doi: 10.1046/j.1460-9568.2000.00169.x

Cazalets, J. R., Borde, M., and Clarac, F. (1995). Localization and organization of the central pattern generator for hindlimb locomotion in newborn rat. *J. Neurosci.* 15, 4943–4951.

Cherniak, M., Etlin, A., Federman, N., Srauss, I., and Lev-Tov, A. (2011). Ventral horn clusters of sacral neurons are activated by α1 adrenoceptor agonists to produce "fast" rhythmic bursts in sacral and rostral lumbar motoneurons. *J. Mol. Neurosci.* 45(Suppl. 1), S24.

Cowley, K. C., and Schmidt, B. J. (1997). Regional distribution of the locomotor pattern-generating network in the neonatal rat spinal cord. *J. Neurophysiol.* 77, 247–259.

Delvolvé, I., Gabbay, H., and Lev-Tov, A. (2001). The motor output and behavior produced by rhythmogenic sacrocaudal networks in spinal cords of neonatal rats. *J. Neurophysiol.* 85, 2100–2110.

Dietz, V. (2009). Body weight supported gait training: from laboratory to clinical setting. *Brain Res. Bull.* 78, I–VI. doi: 10.1016/S0361-9230(08)00410-3

Dietz, V., and Duysens, J. (2000). Significance of load receptor input during locomotion: a review. *Gait Posture* 11, 102–110. doi: 10.1016/S0966-6362(99)00052-1

Dietz, V., Müller, R., and Colombo, G. (2002). Locomotor activity in spinal man: significance of afferent input from joint and load receptors. *Brain* 125, 2626–2634. doi: 10.1093/brain/awf273

Dutton, R. C., Carstens, M. I., Antognini, J. F., and Carstens, E. (2006). Long ascending propriospinal projections from lumbosacral to upper cervical spinal cord in the rat. *Brain Res.* 1119, 76–85. doi: 10.1016/j.brainres.2006.08.063

Duysens, J., and Pearson, K. G. (1980). Inhibition of flexor burst generation by loading ankle extensor muscles in walking cats. *Brain Res.* 187, 321–332. doi: 10.1016/0006-8993(80)90206-1

Edgerton, V. R., Courtine, G., Gerasimenko, Y. P., Lavrov, I., Ichiyama, R. M., Fong, A. J., et al. (2008). Training locomotor networks. *Brain Res. Rev.* 57, 241–254. doi: 10.1016/j.brainresrev.2007.09.002

Edgley, S. A., and Grant, G. M. (1991). Inputs to spinocerebellar tract neurons located in stilling's nucleus in the sacral segments of the rat spinal cord. *J. Comp. Neurol.* 305, 130–138. doi: 10.1002/cne.903050112

Etlin, A., Blivis, D., Ben-Zwi, M., and Lev-Tov, A. (2010). Long and short multifunicular projections of sacral neurons are activated by sensory input to produce locomotor activity in the absence of supraspinal control. *J. Neurosci.* 30, 10324–10336. doi: 10.1523/JNEUROSCI.1208-10.2010

Etlin, A., Finkel, E., Mor, Y., O'Donovan, M. J., Anglister, L., and Lev-Tov, A. (2013). Characterization of sacral interneurons that mediate activation of locomotor pattern generators by sacrocaudal afferent input. *J. Neurosci.* 33, 734–747. doi: 10.1523/JNEUROSCI.4390-12.2013

Falgairolle, M., and Cazalets, J. R. (2007). Metachronal coupling between spinal neuronal networks during locomotor activity in newborn rat. *J. Physiol. Lond.* 580, 87–102. doi: 10.1113/jphysiol.2006.115709

Finkel, E., Etlin, A., Cherniak, M., Mor, Y., Lev-Tov, A., and Anglister, L. (2014). Neuroanatomical basis for cholinergic modulation of locomotor networks by sacral relay neurons with ascending lumbar projections. *J. Comp. Neurol.* 522, 3437–3455. doi: 10.1002/cne.23613

Frigon, A. (2012). Central pattern generators of the mammalian spinal cord. *Neuroscientist* 18, 56–69. doi: 10.1177/1073858410396101

Gabbay, H., Delvolvé, I., and Lev-Tov, A. (2002). Pattern generation in caudal-lumbar and sacrococcygeal segments of the neonatal rat spinal cord. *J. Neurophysiol.* 88, 732–739.

Gabbay, H., and Lev-Tov, A. (2004). Alpha-1 adrenoceptor agonists generate a "fast" NMDA receptor-independent motor rhythm in the neonatal rat spinal cord. *J. Neurophysiol.* 92, 997–1010. doi: 10.1152/jn.00205.2004

Garifoli, A., Maci, T., Perciavalle, V., and Perciavalle, V. (2006). Organization of bilateral spinal projections to the lateral reticular nucleus of the rat. *Arch. Ital. Biol.* 144, 145–157.

Gordon, I. T., Dunbar, M. J., Vanneste, K. J., and and Whelan, P. J. (2008). Interaction between developing spinal locomotor networks in the

neonatal mouse. *J. Neurophysiol.* 100, 117–128. doi: 10.1152/jn.00829.2007

Grillner, S., and Rossignol, S. (1978). On the initiation of the swing phase of locomotion in chronic spinal cats. *Brain Res.* 146, 269–277. doi: 10.1016/0006-8993(78)90973-3

Grillner, S., and Zangger, P. (1979). On the central generation of locomotion in the low spinal cat. *Exp. Brain Res.* 34, 241–261. doi: 10.1007/BF00235671

Grottel, K., Krutki, P., and Mrówczyński, W. (1998). Triple projections of neurones located in S1 and S2 segments of the cat spinal cord to the C6 segment, the cerebellum and the reticular formation. *Exp. Physiol.* 83, 737–746.

Hadas, Y., Etlin, A., Falk, H., Avraham, O., Kobiler, O., Panet, A., et al. (2014). A 'tool box' for deciphering neuronal circuits in the developing chick spinal cord. *Nucleic Acids Res.* doi: 10.1093/nar/gku750. [Epub ahead of print].

Hubli, M., and Dietz, V. (2013). The physiological basis of neurorehabilitation—locomotor training after spinal cord injury. *J. Neuroeng. Rehabil.* 10:5. doi: 10.1186/1743-0003-10-5

Hultborn, H., and Nielsen, J. B. (2007). Spinal control of locomotion–from cat to man. *Acta Physiol. (Oxf)* 189, 111–121. doi: 10.1111/j.1748-1716.2006.01651.x

Islam, S. S., Zelenin, P. V., Orlovsky, G. N., Grillner, S., and Deliagina, T. G. (2006). Pattern of motor coordination underlying backward swimming in the lamprey. *J. Neurophysiol.* 96, 451–460. doi: 10.1152/jn.01277.2005

Katter, J. T., Dado, R. J., Kostarczyk, E., and Giesler, G. J. Jr. (1996). Spinothalamic and spinohypothalamic tract neurons in the sacral spinal cord of rats. I. Locations of antidromically identified axons in the cervical cord and diencephalon. *J. Neurophysiol.* 75, 2581–2605.

Kiehn, O. (2006). Locomotor circuits in the mammalian spinal cord. *Annu. Rev. Neurosci.* 29, 279–306. doi: 10.1146/annurev.neuro.29.051605.112910

Kiehn, O., Sillar, K. T., Kjaerulff, O., and McDearmid, J. R. (1999). Effects of noradrenaline on locomotor rhythm-generating networks in the isolated neonatal rat spinal cord. *J. Neurophysiol.* 82, 741–746.

Kjaerulff, O., and Kiehn, O. (1996). Distribution of networks generating and coordinating locomotor activity in the neonatal rat spinal cord in vitro: a lesion study. *J. Neurosci.* 16, 5777–5794.

Klein, D. A., and Tresch, M. C. (2010). Specificity of intramuscular activation during rhythms produced by spinal patterning systems in the in vitro neonatal rat with hindlimb attached preparation. *J. Neurophysiol.* 104, 2158–2168. doi: 10.1152/jn.00477.2010

Kremer, E., and Lev-Tov, A. (1997). Localization of the spinal network associated with generation of hindlimb locomotion in the neonatal rat and organization of its transverse coupling system. *J. Neurophysiol.* 77, 1155–1170.

Kudo, N., and Yamada, T. (1987). N-methyl-D,L-aspartate-induced locomotor activity in a spinal cord-hindlimb muscles preparation of the newborn rat studied in vitro. *Neurosci. Lett.* 75, 43–48. doi: 10.1016/0304-3940(87)90072-3

Lanuza, G. M., Gosgnach, S., Pierani, A., Jessell, T. M., and Goulding, M. (2004). Genetic identification of spinal interneurons that coordinate left-right locomotor activity necessary for walking movements. *Neuron* 42, 375–386. doi: 10.1016/S0896-6273(04)00249-1

Leah, J., Menétrey, D., and de Pommery, J. (1988). Neuropeptides in long ascending spinal tract cells in the rat: evidence for parallel processing of ascending information. *Neuroscience* 24, 195–207. doi: 10.1016/0306-4522(88)90323-5

Lev-Tov, A., and Delvolvé, I. (2000). Pattern generation in non-limb moving segments of the mammalian spinal cord. *Brain Res. Bull.* 53, 671–675. doi: 10.1016/S0361-9230(00)00400-7

Lev-Tov, A., Delvolvé, I., and Kremer, E. (2000). Sacrocaudal afferents induce rhythmic efferent bursting in isolated spinal cords of neonatal rats. *J. Neurophysiol.* 83, 888–894.

Lev-Tov, A., Etlin, A., and Blivis, D. (2010). Sensory-induced activation of pattern generators in the absence of supraspinal control. *Ann. N Y Acad. Sci.* 1198, 54–62. doi: 10.1111/j.1749-6632.2009.05424.x

Lev-Tov, A., and O'Donovan, M. (2009). "Spinal cord: neonatal circuits," in *Encyclopedia of Neuroscience*, ed L. R. Squire, Editor-in-Chief (Oxford: Academic Press), 61–65.

Liu, Y., Abdel Samad, O., Zhang, L., Duan, B., Tong, Q., Lopes, C., et al. (2010). VGLUT2-dependent glutamate release from nociceptors is required to sense pain and suppress itch. *Neuron* 68, 543–556. doi: 10.1016/j.neuron.2010.09.008

Mandadi, S., and Whelan, P. J. (2009). A new method to study sensory modulation of locomotor networks by activation of thermosensitive cutaneous afferents using a hindlimb attached spinal cord preparation. *J. Neurosci. Methods* 182, 255–259. doi: 10.1016/j.jneumeth.2009.06.011

Matsushita, M. (1999). Projections from lowest lumbar and sacral caudal segments to the cerebellar nuclei in the rat, studied by anterograde axonal tracing. *J. Comp. Neurol.* 404, 21–32. doi: 10.1002/(SICI)1096-9861(19990201)404:1<21::AID-CNE2>3.0.CO;2-7

Miles, G. B., and Sillar, K. T. (2011). Neuromodulation of vertebrate locomotor control networks. *Physiology (Bethesda)* 26, 393–411. doi: 10.1152/physiol.00013.2011

Mor, Y., and Lev-Tov, A. (2007). Analysis of rhythmic patterns produced by spinal neural networks. *J. Neurophysiol.* 98, 2807–2817. doi: 10.1152/jn.00740.2007

Pearson, K. G. (2004). Generating the walking gait: role of sensory feedback. *Prog. Brain Res.* 143, 123–129. doi: 10.1016/S0079-6123(03)43012-4

Pearson, K. G., and Rossignol, S. (1991). Fictive motor patterns in chronic spinal cats. *J. Neurophysiol.* 66, 1874–1887.

Prochazka, A., Gillard, D., and Bennett, D. J. (1997). Positive force feedback control of muscles. *J. Neurophysiol.* 77, 3226–3236.

Rogoz, K., Lagerström, M. C., Dufour, S., and Kullander, K. (2012). VGLUT2-dependent glutamatergic transmission in primary afferents is required for intact nociception in both acute and persistent pain modalities. *Pain* 153, 1525–1536. doi: 10.1016/j.pain.2012.04.017

Roisman, R., Cherniak, M., and Lev-Tov, A. (2014). Caudo-rostral coupling between alpha-1 adrenoceptor activated cluster of sacral interneurons and rostral lumbar motoneurons. *J. Mol. Neurosci.* 53(Suppl. 1), S107–S108.

Scherrer, G., Low, S. A., Wang, X., Zhang, J., Yamanaka, H., Urban, R., et al. (2010). VGLUT2 expression in primary afferent neurons is essential for normal acute pain and injury-induced heat hypersensitivity. *Proc. Natl. Acad. Sci. U S A* 107, 22296–22301. doi: 10.1073/pnas.1013413108

Smith, J. C., Feldman, J. L., and Schmidt, B. J. (1988). Neural mechanisms generating locomotion studied in mammalian brain stem-spinal cord in vitro. *FASEB J.* 2, 2283–2288.

Sqalli-Houssaini, Y., and Cazalets, J. R. (2000). Noradrenergic control of locomotor networks in the in vitro spinal cord of the neonatal rat. *Brain Res.* 852, 100–109. doi: 10.1016/S0006-8993(99)02219-2

Strauss, I. (2011). *Coupling Between Rhythmogenic Networks in the Isolated Spinal Cord of the Neonatal Rat.* PhD thesis, Jerusalem: Hebrew University.

Strauss, I., and Lev-Tov, A. (2003). Neural pathways between sacrocaudal afferents and lumbar pattern generators in neonatal rats. *J. Neurophysiol.* 89, 773–784. doi: 10.1152/jn.00716.2002

Whelan, P., Bonnot, A., and O'Donovan, M. J. (2000). Properties of rhythmic activity generated by the isolated spinal cord of the neonatal mouse. *J. Neurophysiol.* 84, 2821–2833.

Wu, L., Sonner, P. M., Titus, D. J., Wiesner, E. P., Alvarez, F. J., and Ziskind-Conhaim, L. (2011). Properties of a distinct subpopulation of GABAergic commissural interneurons that are part of the locomotor circuitry in the neonatal spinal cord. *J. Neurosci.* 31, 4821–4833. doi: 10.1523/JNEUROSCI.4764-10.2011

Yamada, J., Shirao, K., Kitamura, T., and Sato, H. (1991). Trajectory of spinocerebellar fibers passing through the inferior and superior cerebellar peduncles in the rat spinal cord: a study using horseradish peroxidase with pedunculotomy. *J. Comp. Neurol.* 304, 147–160. doi: 10.1002/cne.903040111

Zaporozhets, E., Cowley, K. C., and Schmidt, B. J. (2004). A reliable technique for the induction of locomotor-like activity in the in vitro neonatal rat spinal cord using brainstem electrical stimulation. *J. Neurosci. Methods* 139, 33–41. doi: 10.1016/j.jneumeth.2004.04.009

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Changes in functional properties and 5-HT modulation above and below a spinal transection in lamprey

Matthew I. Becker and David Parker*

Department of Physiology, Development and Neuroscience, University of Cambridge, Cambridge, UK

Edited by:
Brian R. Noga, University of Miami, USA

Reviewed by:
Hans Hultborn, University of Copenhagen, Denmark
María Celina Rodicio, University of Santiago de Compostela, Spain

****Correspondence:***
David Parker, Department of Physiology, Development and Neuroscience, University of Cambridge, Anatomy Building, Downing Site, Cambridge CB2 3DY, UK
e-mail: Djp27@cam.ac.uk

In addition to the disruption of neural function below spinal cord injuries (SCI), there also can be changes in neuronal properties above and below the lesion site. The relevance of these changes is generally unclear, but they must be understood if we are to provide rational interventions. Pharmacological approaches to improving locomotor function have been studied extensively, but it is still unclear what constitutes an optimal approach. Here, we have used the lamprey to compare the modulatory effects of 5-HT and lesion-induced changes in cellular and synaptic properties in unlesioned and lesioned animals. While analyses typically focus on the sub-lesion spinal cord, we have also examined effects above the lesion to see if there are changes here that could potentially contribute to the functional recovery. Cellular and synaptic properties differed in unlesioned and lesioned spinal cords and above and below the lesion site. The cellular and synaptic modulatory effects of 5-HT also differed in lesioned and unlesioned animals, again in region-specific ways above and below the lesion site. A role for 5-HT in promoting recovery was suggested by the potential for improvement in locomotor activity when 5-HT was applied to poorly recovered animals, and by the consistent failure of animals to recover when they were incubated in PCPA to deplete 5-HT. However, PCPA did not affect swimming in animals that had already recovered, suggesting a difference in 5-HT effects after lesioning. These results show changes in 5-HT modulation and cellular and synaptic properties after recovery from a spinal cord transection. Importantly, effects are not confined to the sub-lesion spinal cord but also occur above the lesion site. This suggests that the changes may not simply reflect compensatory responses to the loss of descending inputs, but reflect the need for co-ordinated changes above and below the lesion site. The changes in modulatory effects should be considered in pharmacological approaches to functional recovery, as assumptions based on effects in the unlesioned spinal cord may not be justified.

Keywords: spinal cord, neuromodulation, spinal cord injury, lamprey, 5-HT

INTRODUCTION

Spinal cord injury is associated with a loss of sensory and motor function below a lesion site due to damage to ascending and descending tracts and local circuitry, as well as disturbances of autonomic function. There is currently no effective intervention to overcome the effects of SPI (Verma et al., 2008). A major focus is on promoting the regeneration of axons across lesion sites. This occurs spontaneously in lower vertebrates (Tanaka and Ferretti, 2009), where it is assumed to account for the functional recovery that can occur in these systems. However, in addition to loss of descending and ascending inputs across the lesion site, there are also various functional changes below the lesion site in animal models and in the human spinal cord (Cohen et al., 1988; McClellan, 1994; Bennett et al., 2001; Edgerton et al., 2001; Pearson, 2001; Rossignol et al., 2001; Wolpaw and Tennissen, 2001; Li and Bennett, 2003; Grasso et al., 2004; Li et al., 2004; Harkema, 2008; Cooke and Parker, 2009; Boulenguez et al., 2010; Rossignol and Frigon, 2011; Roy et al., 2012; Vajn et al., 2014). The role of these changes is generally unclear: they could facilitate regeneration strategies by compensating for the reduction of descending inputs (e.g., by changing functional properties to allow a reduced number of descending inputs to evoke the same functional effect as in the unlesioned cord); or they could complicate these approaches by requiring regenerated inputs to interact appropriately with varying sub-lesion networks (Nahar et al., 2012).

Pharmacological approaches to restoring function after spinal injury have been attempted in experimental and clinical settings (Rossignol et al., 2001; Tillakaratne et al., 2002; Barbeau and Norman, 2003; Parker, 2005). There is a vast literature on drug effects but still little insight into what constitutes an

Abbreviations: 5-HT, 5-hydroxytryptamine; CV, coefficient of variation; RMP, resting membrane potential; PCPA, p-Chlorophenylalanine; GABA, gamma-aminobutyric acid; EMG, electromyogram; sAHP, slow afterhyperpolarisation; SiIN, small ipsilateral inhibitory interneuron; LIN, lateral interneuron; ScIN, small crossing inhibitory interneuron; CCIN, crossed caudal interneuron.

optimal pharmacological approach. This is complicated by the diverse neuron and state-specific effects of modulators, novel effects caused by interactions between modulatory systems (see Katz and Edwards, 1999), and the general difficulty of linking cellular effects to network outputs and behavior. Among the various transmitter systems, 5-HT has arguably been studied most extensively. 5-HT has significant effects on locomotion and sensory processing in the unlesioned spinal cord (Schmidt and Jordan, 2000), and there is evidence that constitutive activation of 5-HT_2 receptors (Murray et al., 2010; D'Amico et al., 2013) or the application of 5-HT receptor agonists (see Gimenez y Ribotta et al., 1998; Hains et al., 2001; Hochman et al., 2001; Antri et al., 2003; see Gackière and Vinay (2014) for a recent review) can improve locomotor function after injury. However, the mechanisms underlying any improvements, which would ideally be targeted to improve functional recovery, are unclear.

The larval and juvenile adult lamprey has been used as a model system for studying the recovery of locomotor function after spinal cord lesions. These studies have largely focused on regeneration, which occurs to a comparable extent in both developmental stages (Cohen et al., 1988; Lurie and Selzer, 1991; McClellan, 1994; Oliphint et al., 2010), but changes in the anatomy and functional properties of spinal cord neurons have also been examined (see Yin et al., 1987). Our previous analyses have shown that cellular and synaptic properties of larval motor neurons and spinal interneurons are altered below the lesion site (Cooke and Parker, 2009). Proprioceptive feedback is also potentiated after lesioning, and the modulation of proprioceptive inputs by GABA and somatostatin is altered (Hoffman and Parker, 2011; Svensson et al., 2013).

The lamprey offers a simpler system in which to examine injury-induced changes, and ultimately try and establish their influence on recovery. Here we have examined potential lesion-induced differences in modulation by comparing the effects of 5-HT in unlesioned animals and in lesioned animals. We have extended our analyses to examine effects in young adults. While recovery in adult lampreys has been studied (Cohen et al., 1989), most studies have focused on larval animals. Given that insight is needed into how the mature nervous system responds to injury, we felt that extending our analysis to, albeit juvenile, adult animals was a useful addition. We have also examined effects below and above the lesion site. Changes below the lesion site are routinely examined, but above lesion effects have received relatively little attention (see Grasso et al., 2004). While changes below the lesion site could be an attempt to compensate for the loss of descending inputs, changes above the lesion site may also result from the loss of ascending inputs, or the need to adjust supra-lesion activity to the changes that occur below the lesion site. While in many cases preliminary, the results suggest differences in functional properties and their modulation by 5-HT after lesioning that occur in region-specific ways above and below the lesion site.

MATERIALS AND METHODS

Juvenile adult lampreys (*Petromyzon marinus*) between 100–130 mm were obtained from commercial suppliers (Acme Lamprey Company, Maine, USA). All experiments were performed under license and conformed to the requirements of the UK Home Office Animals (Scientific Procedures) Act 1986. For spinal cord transections animals were anesthetized by immersion in MS-222 (300 mg/ml, pH adjusted to 7.4) and a ∼5 mm dorsal incision was made approximately 1 cm below the last gill to expose the spinal cord. The spinal cord was transected with iridectomy scissors and the incision site sealed with tissue glue (Vetbond). Following transection animals were kept at 20°C for 8–10 weeks (unlesioned animals were also kept at this temperature): at this time most animals had recovered locomotor function (McClellan, 1994) and the incision site had healed completely.

Video and electromyogram (EMG) recordings were made from unlesioned animals and animals 8–10 weeks after lesioning. EMG recordings were made by inserting bipolar Teflon insulated electrodes (0.075 mm diameter) into the muscle under MS-222 anesthesia. The electrodes were inserted approximately 0.5 cm rostral and caudal to the lesion site. After recovery from anesthesia muscle activity was recorded as the animals swam in a plastic aquarium (29 × 23.5 × 5.5 cm). Swimming activity was initiated by lightly pinching the tail or head of the animal using serrated forceps: stimulation was given 30 s after the end of the previous swimming episode. A qualitative assessment of the functional recovery was made from video recordings based on the scale derived by (Ayers (1989); see Cooke and Parker, 2009; Hoffman and Parker, 2011). EMG and extracellular activity was recorded using an A-M Systems 1700 Differential AC amplifier. All data acquisition and analysis was done using a computer equipped with an analog to digital interface (Digidata 1322A, Molecular Devices) and pClamp9 software (Molecular Devices). From the EMG of each animal a quantitative analysis of swimming was performed by measuring the episode length (duration of EMG activity), the cycle period, the coefficient of variation of bursting, and the intersegmental phase lag. Lampreys undergo a change in body size during development. However, the spinal cord represents ∼80% of the total body length in each of the life cycle stages (Ruiz et al., 2004). A linear function can fit the relationship of segment to body length, where each of the assumed 100 spinal segments along the spinal cord increases proportionally with body length (Ruiz et al., 2004):

$$y = 0.0066x + 0.058$$

y is the segment length and x the total body length (Ruiz et al., 2004). Animals were measured from the tip of the oral hood to the end of the tail. To determine intersegmental phase lag (Φ), the following equation was used (Boyd and McClellan, 2002):

$$\phi = [d/T]/N$$

d is the interval between rostral and caudal bursts in the same cycle on the same side; T is the cycle time, which is inversely proportional to the bursting frequency; N is the number of intervening segments which is divided by y.

The modulation of swimming was assessed by applying 5-HT (10–500 μM) to the aquarium water: plateau effects on the

episode length and coefficient of variation (CV; standard deviation of the cycle duration divided by the mean cycle duration), aspects where significant effects of 5-HT were seen, occurred at a concentration of 200–400 μM (see **Figures 8A,B**; results presented in **Figure 9** show only the effects of 500 μM 5-HT). While we do not know the final concentration of 5-HT in the spinal cord, we are confident that this application route allows access to the CNS as there were changes in the swimming behavior of lesioned and unlesioned animals. To examine the role of 5-HT in locomotor function and recovery we incubated animals in p-Chlorophenylalanine (PCPA; 0.006 g in 200 ml of water equivalent to 150 μM), which depletes 5-HT (Hashimoto and Fukuda, 1991; Airhart et al., 2012). Unlesioned animals were also examined after 72 h in PCPA: in this case there were marked effects on behavior that again suggest access to the CNS through this administration route. Lesioned animals were incubated for 6 weeks (PCPA was changed every 3 days), and then placed in normal aquarium water for at least two weeks to avoid acute effects of PCPA (in unlesioned animals swimming behavior had recovered within 3–5 days after removal from PCPA).

For intracellular and extracellular recordings animals were anesthetized with MS-222 and the spinal cord and notochord were removed from the trunk region (i.e., between the last gill and the start of the dorsal fin) in oxygenated lamprey Ringer at 4°C (Ringer contents: 138 mM NaCl, 2.1 mM KCl; 1.8 mM $CaCl_2$; 2.6 mM $MgCl_2$; 4.0 mM D-(+)-glucose; 2.0 mM HEPES; 0.5 mM L-glutamine, bubbled with O_2 and adjusted to pH 7.4 with 1 M NaOH). The spinal cord was isolated from the notochord and pinned to a Sylgard lined chamber and superfused with lamprey Ringer at 10°C. Intracellular recordings were made from cells above and below the lesion site using an Axoclamp 2B amplifier (Molecular Devices). Motor neurons (identified by spikes in a ventral root following an evoked action potential in the cell body) and unidentified cells were examined in unlesioned animals and in lesioned animals above and below the lesion site. Given the relative size and number of different types of neurons, unidentified cells are likely to be motor neurons: as there were no obvious differences in motor neurons and unidentified cells they were grouped for analysis (identified motor neurons constituted at least 80% of the sample size in each analysis). Recordings were not made from different classes of interneurons as this analysis is not trivial even in this "simpler" system (Parker, 2006, 2010) and we initially need to identify the changes that invite targeted cell and synapse-specific analyses. While the analysis essentially treats the locomotor network as a functional unit, using motor neurons allowed us to assay cellular properties, while also inferring something about premotor inputs from spontaneous synaptic activity. Cells were typically sought 2–3 segments rostral or caudal to the lesion site. The resting membrane potential (RMP) was examined after the cell had stabilized (∼2 min); the input resistance was measured by injecting 100 ms hyperpolarising current pulses (−0.5 to −2.5 nA) into cells under discontinuous current clamp (DCC; sampling frequency between 2–3 KHz); excitability was examined by injecting 100 ms depolarizing current pulses (0.5–2.5 nA) into the cells under DCC.

Spontaneous spinal cord activity was recorded extracellularly by placing a glass suction electrode on the surface of the spinal cord to cover the cell body area, and intracellularly by recording spontaneous synaptic activity from a cell in thirty 1 s sweeps. Cord and intracellular activity was rectified and integrated in Clampfit to quantify the summed activity. As the cellular activity was rectified it reflected the total changes in synaptic input from premotor neurons and associated changes in resting potential and thus provided a measure of the total subthreshold activity of the cell. To examine EPSP and IPSP properties specifically (albeit from unidentified presynaptic sources) spontaneous PSPs were instead measured over a 40 s period in each cell using the event detection feature in Clampfit.

The properties of specific presynaptic inputs were examined by making paired intracellular recordings from postsynaptic motor neurons and Müller reticulospinal axons. These inputs were also used to examine the modulatory effects of 5-HT, but for this analysis cord stimulation-evoked responses were also used when reticulospinal axons that connected to the recorded postsynaptic cell could not be found. Cord-stimulation responses were evoked by stimulating the medial column that runs between the cell body layer (the region where locomotor network interneurons and motor neurons are located) and the midline of the spinal cord extracellularly using a glass suction electrode for 1 ms at 0.1 Hz for 50 s. The stimulation strength was adjusted to evoke what appeared to be a unitary EPSP, determined by the short rise time, longer decay time, and absence of multiple peaks (however, with any extracellular stimulation the potential exists for multiple inputs being evoked onto the postsynaptic cell). This was done in control and in the presence of 5-HT: the overlaid PSPs were averaged and the peak amplitude was measured. Putative monosynaptic reticulospinal-evoked inputs were examined by intracellular stimulation of reticulospinal axons in the medial column above the lesion site several segments rostral to the postsynaptic cell at 20 Hz for 1 s, followed by low frequency recovery test pulses (250 ms, 550 ms, 2 s, and 4 s after the end of the train). The amplitude of the initial EPSP and of EPSPs over the spike train was measured from the baseline preceding each EPSP to the peak of the EPSP. The trains were evoked at 20 s intervals. As there is no activity-dependent plasticity of the input at this frequency, the initial EPSP in the spike trains provided a measure of single low-frequency-evoked EPSPs. Putative monosynaptic connections were determined by the presence of reliable EPSPs that occurred with a consistent short latency (typically <2–3 ms) in response to presynaptic stimulation at 20 Hz (note that functionally weak connections (e.g., regenerated axons) could fail the criteria for monosynapticity even though the connections were monosynaptic). Inputs were considered to be putatively polysynaptic if the inputs were unreliable (i.e., presynaptic spikes failed to evoke a PSP; see **Figure 5C**), had a relatively long latency to the postsynaptic response (10 ms or greater; see **Figure 5D**), and had multiple peaks on the postsynaptic depolarization (see **Figures 5D,E**). The unequivocal identification of monosynaptic or polysynaptic inputs is not trivial (Berry and Pentreath, 1976), and the use of the term putative relates to the likelihood that an input is mono- or polysynaptic.

Drugs were purchased from Sigma-Aldrich. Drugs were applied to the isolated spinal cord by superfusion using a peristaltic pump. The data presented here are taken from two batches of animals examined over a two year period, and does not include data from previous analyses. Statistical analyses were performed in Graphpad Prism using Wilcoxon matched pairs test for paired comparisons or a Kruskal-Wallis test with a *post hoc* Dunns test for multiple comparisons. The graphs show mean responses ± SEM.

RESULTS

EFFECTS OF 5-HT IN UNLESIONED AND LESIONED ANIMALS

We examined if there were changes in modulatory effects after spinal cord lesions by comparing the effects of 5-HT in the isolated spinal cord of lesioned and unlesioned animals. As we have used juvenile adults rather than larvae for the first time in our analyses, we have also compared cellular and synaptic properties in lesioned and unlesioned spinal cords (see Cooke and Parker, 2009 for analyses in larvae).

There were no significant differences in unlesioned and lesioned animals (either above or below the lesion site) in the resting potential (RMP; **Figure 1A**), input resistance (**Figure 1B**), I-V relationship (**Figure 1B**), the slow afterhyperpolarisation (sAHP) amplitude (**Figure 1C**), or the excitability (**Figure 1D**; see **Table 1**).

In cells from unlesioned animals, 1 μM 5-HT non-significantly hyperpolarised the RMP (−1.16 ± 0.5 mV, $n = 6$; **Figure 2A**), an effect that did not differ significantly to that in lesioned animals above (−0.95 ± 0.5 mV, $n = 19$) or below the lesion site (−1.3 ± 0.6 mV, $n = 22$; $p > 0.05$; data not shown). With 10 μM 5-HT (Harris-Warrick and Cohen, 1985) there was again a consistent, but non-significant, hyperpolarization of the membrane potential in unlesioned animals ($n = 15$ of 16; −0.61 ± 0.34 mV; **Figure 2A**). However, below the lesion site it depolarized the membrane potential in the majority of cells ($n = 16$ of 30 cells), but in the population as a whole there was still a mean hyperpolarization of −0.54 ± 0.39 mV, while above the lesion site it depolarized the membrane potential in 9 of 24 cells, which in this case gave a mean depolarization of 0.19 ± 0.76 mV (**Figure 2A**). While the mean RMP changes by 10 μM 5-HT were not significantly different (**Figure 2B**), there was a significant increase in the proportion of cells that depolarized above and below the lesion site compared to unlesioned animals ($p < 0.05$, Chi square). The RMP effect thus changed from a highly consistent hyperpolarization in unlesioned animals to a more variable effect where there was a mix of depolarization and hyperpolarization.

There was no significant effects of 1 μM 5-HT on the input resistance, I-V relationship, excitability, or sAHP amplitude in unlesioned animals or lesioned animals above or below the lesion site (data not shown). For 10 μM 5-HT there was again no significant effect on the excitability or I-V relationship in cells from unlesioned animals or lesioned animals above or below the lesion site ($p > 0.05$; data not shown), but it did significantly reduce the sAHP amplitude

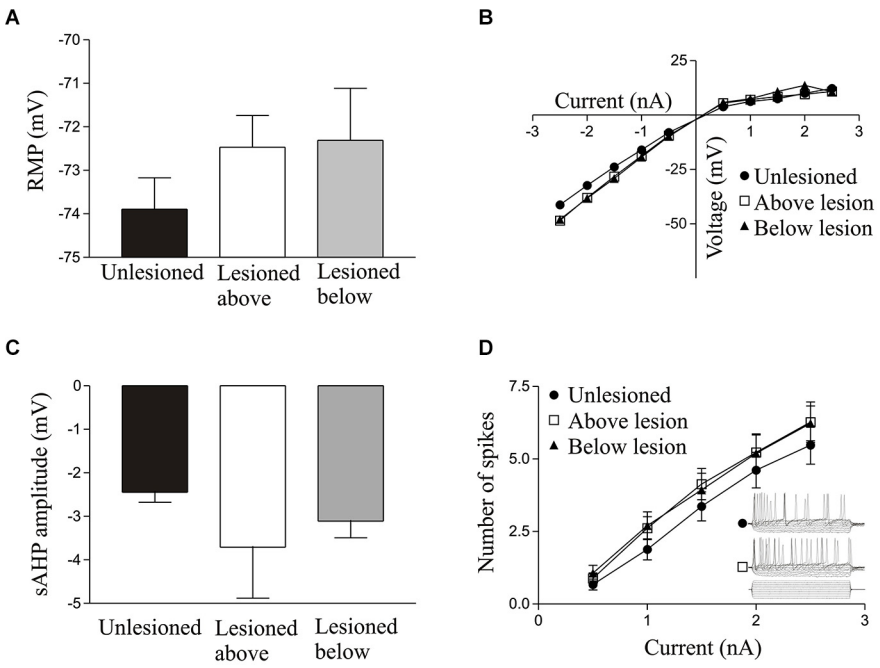

FIGURE 1 | Graphs showing values in unlesioned and lesioned animals for the resting membrane potential (A), I-V relationship **(B)**, slow afterhyperpolarisation (sAHP) amplitude **(C)**, excitability **(D)**; the inset shows sample 100 ms traces from cells in an unlesioned animal and a cell above the lesion site).

Table 1 | Comparison of cellular properties in unlesioned and lesioned animals.

	RMP	Input resistance	sAHP	Cord integrated	Cell integrated
Unlesioned	−73.9 ± 0.7 mV (*n* = 74)	16.2 ± 1.7 MΩ (*n* = 59)	−2.4 ± 0.2 mV (*n* = 59)	8.6 ± 0.3 (*n* = 42)	257 ± 22 (*n* = 42)
Above lesion	−72.5 ± 0.7 mV (*n* = 42)	19.4 ± 1.7 MΩ (*n* = 32)	−3.7 ± 1.2 mV (*n* = 32)	15.6 ± 1.9*# (*n* = 42)	349 ± 38* (*n* = 42)
Below lesion	−72.3 ± 1.2 mV (*n* = 81)	19.4 ± 2.3 MΩ (*n* = 66)	−3.1 ± 0.04 mV (*n* = 66)	11 ± 0.7* (*n* = 66)	340 ± 25* (*n* = 66)

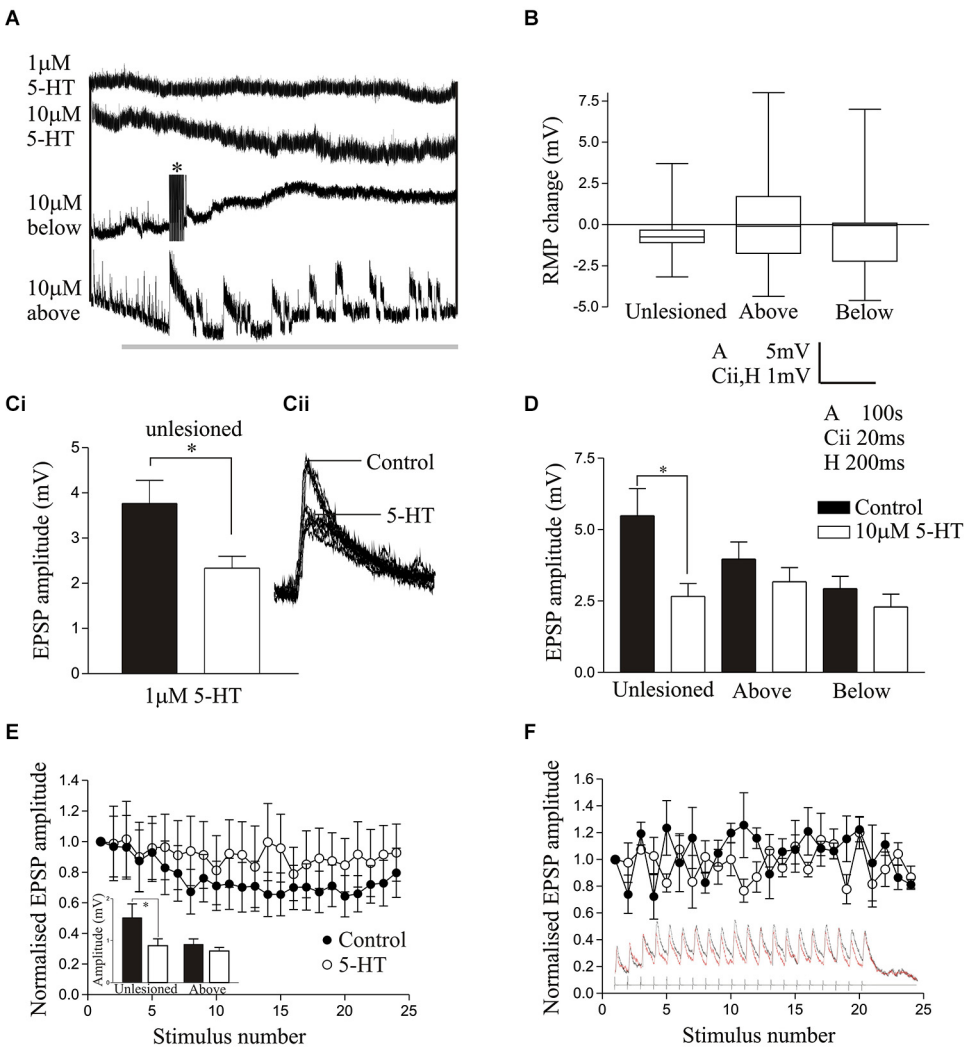

FIGURE 2 | (A) The effects of 1 μM 5-HT and 10 μM 5-HT on the resting membrane potential of spinal cord neurons from unlesioned animals and lesioned animals above and below the lesion site. The asterix shows truncated spikes evoked by 5-HT. **(B)** Graph comparing the change in RMP by 10 μM 5-HT in unlesioned, above lesion and below lesion cells. **(Ci)** Graph and traces **(Cii)** showing the effect of 1 μM 5-HT on the cord stimulation-evoked EPSP amplitude. On this and other graphs * indicates statistically significant differences. **(D)** The effect of 10 μM 5-HT on the amplitude of cord-evoked EPSPs. Note that while there was a significant reduction in unlesioned animals, there was no significant effect above or below the lesion site. **(E)** The reticulospinal input over a spike train in an unlesioned animal in control and in 10 μM 5-HT. The inset graph shows the effect of 10 μM 5-HT on the initial EPSP in spike trains from an unlesioned and above lesion experiment. **(F)** Graph showing the lack of effect of 10 μM 5-HT on the activity-dependent plasticity of a reticulospinal input to a cell above the lesion site. The inset shows the synaptic input over the spike train.

in each case (unlesioned (n = 11), above lesion (n = 10), lesioned below (n = 25), the reduction not differing significantly in the different conditions ($p > 0.05$; data not shown).

Cord-stimulation-evoked EPSPs were significantly reduced in spinal neurons by 1 μM 5-HT in unlesioned animals (**Figures 2Ci,ii**; $p < 0.05$, n = 6), and in lesioned animals above (n = 19) and below the lesion site (n = 20), the magnitude of

the EPSP reduction not differing significantly between conditions ($p > 0.05$; data not shown). Similarly, 10 μM 5-HT also significantly reduced the amplitude of cord stimulation-evoked EPSPs in unlesioned animals ($n = 11$; $p < 0.05$; **Figure 2D**). However, it had no overall significant effect on the EPSP amplitude above ($n = 21$) or below ($n = 26$) the lesion site ($p > 0.05$; **Figure 2D**), suggesting a weakening of 5-HT synaptic effects after lesioning. The effects of 10 μM 5-HT were also examined on reticulospinal-evoked EPSPs in spinal neurons in unlesioned animals ($n = 11$) and in lesioned animals above the lesion site ($n = 3$): it is generally harder to find connections in lesioned animals, especially below the lesion site, which may be a reflection of the sparseness of regenerated inputs (Oliphint et al., 2010). The sample of above lesion connections is too small for a statistical analysis and only shows potential trends. There was a significant reduction of the initial EPSP amplitude in the spike train by 10 μM 5-HT, but as with cord stimulation-evoked EPSP there was no change in the EPSP amplitude above the lesion site (inset **Figure 2E**). 5-HT non-significantly reduced depression in unlesioned animals (**Figure 2E**), possibly due to the reduction of the initial EPSP amplitude, but there was no 5-HT change in the properties of the EPSP in reticulospinal axons above the lesion site (**Figure 2F**).

Reticulospinal-evoked EPSPs examined in the isolated spinal cord were used to examine lesion-induced differences in the basic properties of evoked synaptic inputs. There was no significant difference in the initial EPSP amplitude in the spike train in unlesioned or lesioned animals above or below the lesion site (**Table 2**; **Figures 3A,Bi**). The EPSP rise time did not differ, but the half width was significantly greater above and below the lesion site than in unlesioned animals (**Figure 3Bii**; **Table 2**). This could reflect potentiation of the NMDA component of the EPSP, which correlates with the EPSP half-width (Dale and Grillner, 1986). The slow synaptic depolarization over spike trains seen in lesioned larval animals (Cooke and Parker, 2009) was present (**Figure 3A**). This effect was significantly greater in lesioned than in unlesioned animals, but it did not differ significantly above and below the lesion site (**Figures 3A,Biii**; **Table 2**). The mechanisms underlying this effect are unknown: it is not blocked by high calcium Ringer (data not shown), suggesting either a monosynaptic or a strong polysynaptic effect (see Berry and Pentreath, 1976). It was probably not due to the increased EPSP half-width, as there was no significant correlation between the slow depolarization amplitude and the half-width above or below the lesion (**Figure 3C**; $r^2 = 0.21$ above, $r^2 = 0.06$ below, $p > 0.05$). In unlesioned animals and lesioned animals below the lesion site when all connections were averaged the EPSP depressed across the spike train (**Figure 3D**). However, above the lesion site the input significantly facilitated ($p < 0.05$; **Figures 3A,D**). This facilitation from an unchanged initial EPSP amplitude will make connections above the lesion functionally stronger, and suggests a functional difference above and below the lesion site.

The integrated synaptic activity (see Section Methods) was significantly increased in cells above and below the lesion site compared to cells from unlesioned animals (**Figures 4A,B**; **Table 1**). The integrated spontaneous activity

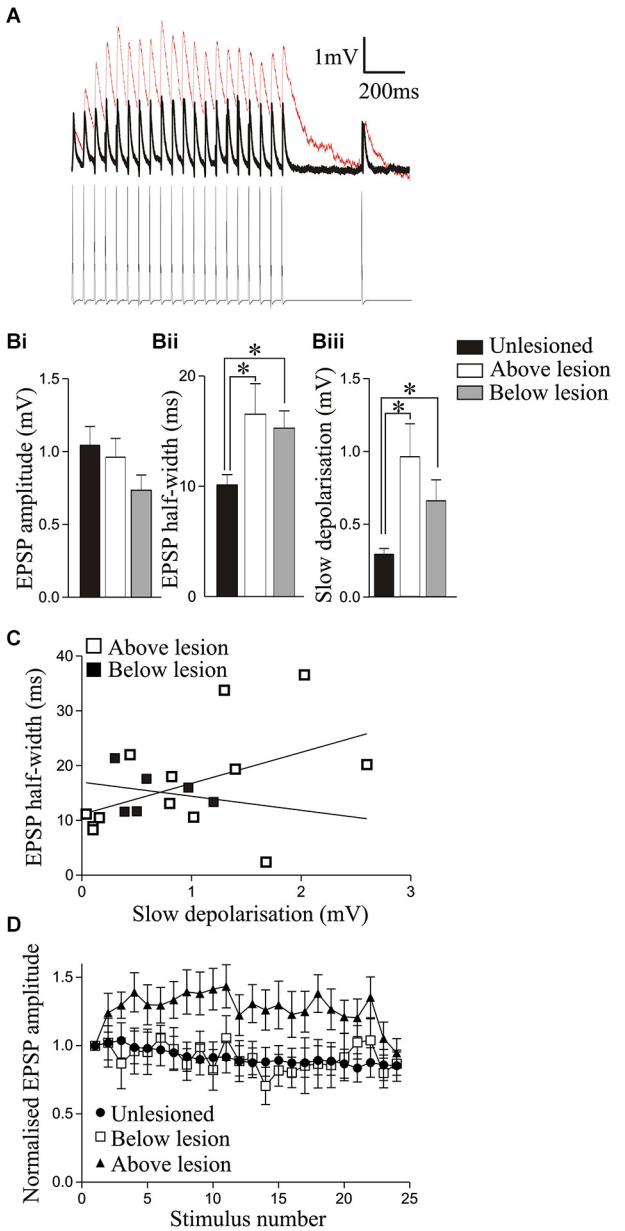

FIGURE 3 | Analysis of synaptic inputs from reticulospinal axons in unlesioned and lesioned animals above and below the lesion site. **(A)** Traces showing the reticulospinal axon-evoked input in a cell recorded above the lesion site (red line) and in an unlesioned cord (black line). Note that the individual EPSPs in the lesioned cord sit on a slow depolarization. **(Bi)** The amplitude of the initial reticulospinal EPSP in spike trains did not differ significantly in unlesioned, above lesion, or below lesion cells, but the half-width **(Bii)** and the amplitude of the slow depolarization (measured from the pre-stimulation baseline to the baseline preceding the 20th EPSP); **(Biii)** were significantly greater above and below the lesion site compared to cells from unlesioned animals. **(C)** Graph showing the lack of correlation between the EPSP half-width and the slow synaptic depolarization amplitude above and below the lesion site. **(D)** Changes in the activity-dependent plasticity of reticulospinal inputs. Note that while inputs from unlesioned animals and below the lesion site depressed, the input above the lesion site facilitated. The *x* axis shows the stimuli number. The

(*Continued*)

FIGURE 3 | Continued
first 20 are successive stimuli all at 20 Hz; from 21–24 they are 250 ms, 550 ms, 2 s, and 4 s after the end of the 20 Hz train (see Section Methods).

recorded extracellularly from the surface of the cord was also significantly increased above and below the lesion site compared to unlesioned animals. In this case activity above the lesion site was also significantly greater than activity below (**Figures 4A,B**; **Table 1**), which again suggests a functional difference on either side of the lesion site. There was no significant difference in the effect of 1 μM 5-HT on integrated synaptic or extracellular activity ($p > 0.05$; **Figure 4C**). However, there was a significant increase in integrated synaptic activity in 10 μM 5-HT below ($p < 0.05$, $n = 22$), but not above ($n = 15$), the lesion site compared to unlesioned animals ($n = 9$; **Figure 4D**), and a significant decrease in the integrated extracellular activity below compared to both unlesioned and above lesion values ($p < 0.05$; **Figure 4D**).

As the integrated synaptic activity reflects the total spontaneous synaptic input, discrete spontaneous EPSPs and IPSPs were measured to examine this activity further. There were no significant differences in the amplitude or number of spontaneous EPSPs in cells from unlesioned animals or lesioned animals above and below the lesion site (**Figures 5A,B**; **Table 3**). However, the number and amplitude of IPSPs was significantly greater below the lesion site than in unlesioned animals, and the number of IPSPs was also significantly greater below than above the lesion (**Figures 5A,B**; **Table 3**). This relative increase in inhibition could contribute to the reduced integrated cord activity below the lesion site (**Figure 4B**), and the change in integrated spontaneous synaptic activity (**Figures 4A,B**). The increase in inhibition was supported by the increase in the proportion of putative polysynaptic IPSPs (see Section Methods) seen in paired recordings from reticulospinal axons and motor neurons below the lesion site (**Figure 5C**; $n = 1$ of 17 in unlesioned animals,

Table 2 | Comparison of reticulospinal-evoked ESPP amplitudes in unlesioned and lesioned animals.

	RS EPSP amplitude	RS EPSP rise time	RS EPSP half-width	Slow depolarization	Train
Unlesioned	1.12 ± 0.11 mV (*n* = 47)	2.8 ± 0.7 ms (*n* = 17)	10.1 ± 0.9 ms (*n* = 17)	0.29 ± 0.04 mV (*n* = 24)	0.87 ± 0.1 (*n* = 24)
Above lesion	0.96 ± 0.13 mV (*n* = 13)	2.5 ± 0.7 ms (*n* = 13)	15.5 ± 9.9 ms (n = 13)*	0.96 ± 1.8 mV (*n* = 13)*	0.92 ± 0.2 (*n* = 13)
Below lesion	0.73 ± 0.1 mV (*n* = 6)	2.9 ± 0.1.1 ms (*n* = 6)	15.3 ± 3.8 ms (*n* = 6)*	0.76 ± 0.8 mV (*n* = 6)*	1.2 ± 0.12 (*n* = 6)*

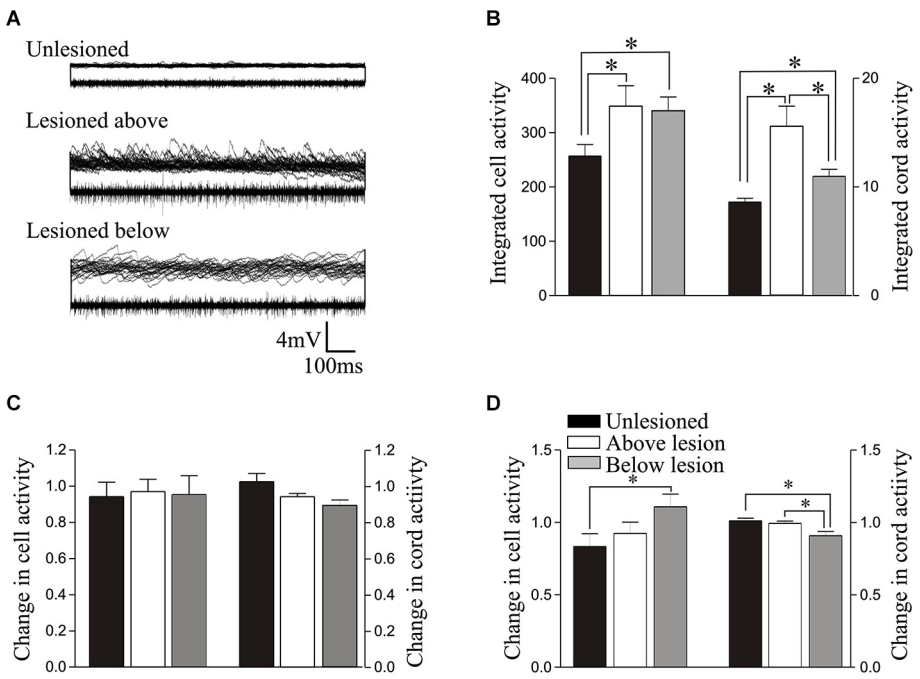

FIGURE 4 | (A) Overlaid traces showing spontaneous synaptic activity recorded intracellularly (top of each of the paired trace) and activity recorded extracellularly from the surface of the spinal cord (bottom of each of the paired trace) from an unlesioned spinal cord, and from a lesioned spinal cord above and below the lesion site. **(B)** Graph showing the rectified and integrated cell and cord activity. **(C)** Graph showing the normalized change in integrated cord and cell activity by 1 μM 5-HT. **(D)** Comparison of the normalized changes in integrated cell and cord activity by 10 μM 5-HT. The key on D relates to all graphs on this figure.

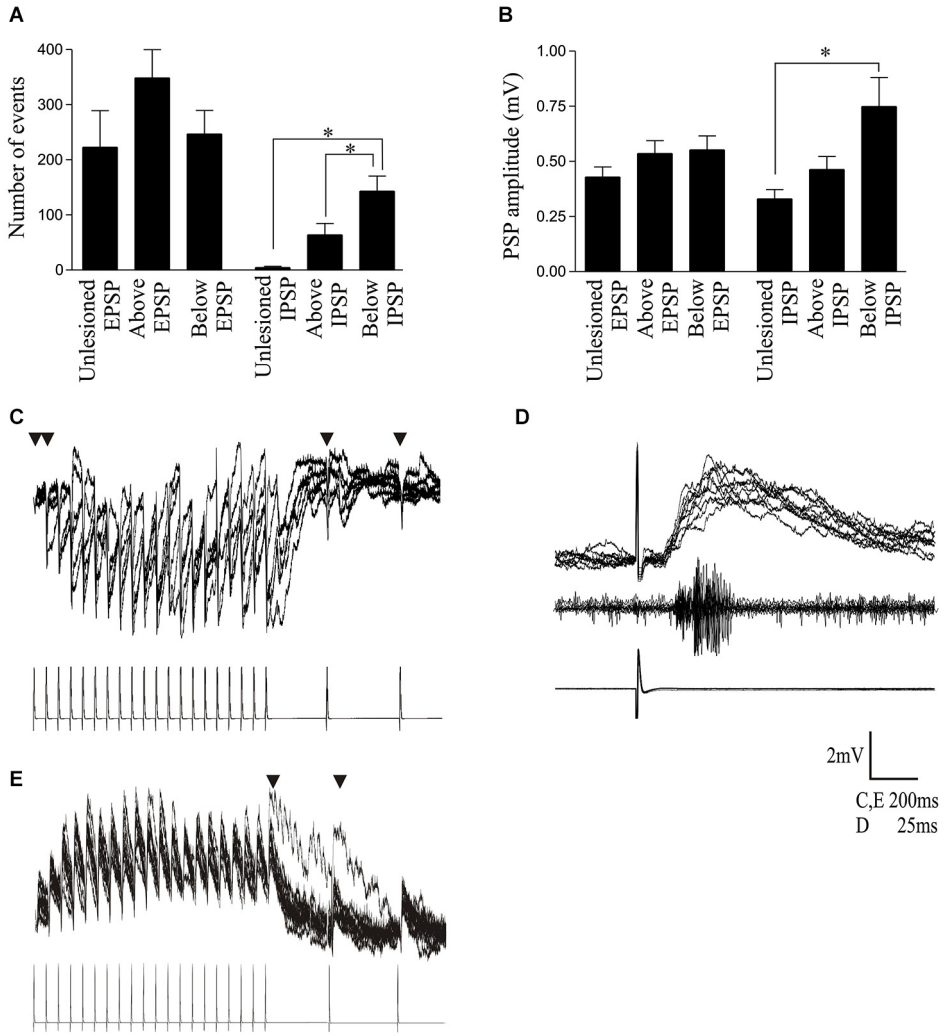

FIGURE 5 | (A) Graph showing the number of spontaneous EPSPs and IPSPs in cells from unlesioned animals and above and below the lesion site. **(B)** Graph showing the spontaneous EPSP and IPSP amplitude. **(C)** Traces showing a polysynaptic IPSP evoked in a cell below the lesion site in response to 20 Hz stimulation of a reticulospinal axon. The inverted triangle indicates where presynaptic spikes failed to evoke a PSP on at least 50% of presynaptic stimulation trials. **(D)** Example of a polysynaptic EPSP evoked in a cell above the lesion site. Note the latency (∼10 ms) before the response began and the multiple peaks on the depolarization. The middle trace shows a burst of activity recorded from the surface of the spinal cord approximately 5 segments below the cell, which also suggests activation of several cells by the single presynaptic spike. **(E)** Evidence of polysynaptic EPSP evoked by stimulating a reticulospinal axon that evoked a monosynaptic EPSP in a postsynaptic spinal cord neuron above the lesion site at 20 Hz. The inverted triangle indicates where polysynaptic EPSPs were evoked.

Table 3 | Characterization of spontaneous PSPs in unlesioned and lesioned animals.

	Spontaneous EPSP Amp	Spontaneous EPSP No.	Spontaneous IPSP Amp	Spontaneous IPSP No.
Unlesioned	0.46 ± 0.03 mV (*n* = 34)	215 ± 38 (*n* = 34)	0.32 ± 0.04 mV (*n* = 33)	7 ± 18 (*n* = 33)
Above lesion	0.53 ± 0.06 mV (*n* = 19)	348 ± 51 (*n* = 19)	0.46 ± 0.12 mV (*n* = 18)	68 ± 21 (*n* = 18)
Below lesion	0.51 ± 0.03 mV (*n* = 24)	229 ± 40 (*n* = 24)	0.67 ± 0.04 mV (*n* = 23)*	145 ± 28 (*n* = 23)*#

n = 3 of 6 lesioned animals, $p < 0.05$, Chi square). While the properties of spontaneous PSPs above the lesion site did not differ to unlesioned animals, there was additional evidence for an increase in excitatory connectivity: putative polysynaptic EPSPs were more common above the lesion site than in unlesioned animals (*n* = 1 of 47 in unlesioned compared to 7 of 13 in lesioned animals, $p < 0.05$, Chi square; see **Figures 5D,E**). This suggests a strengthening of evoked feedforward excitatory connections despite the lack of change of spontaneous inputs (see Goel and Buonomano, 2013).

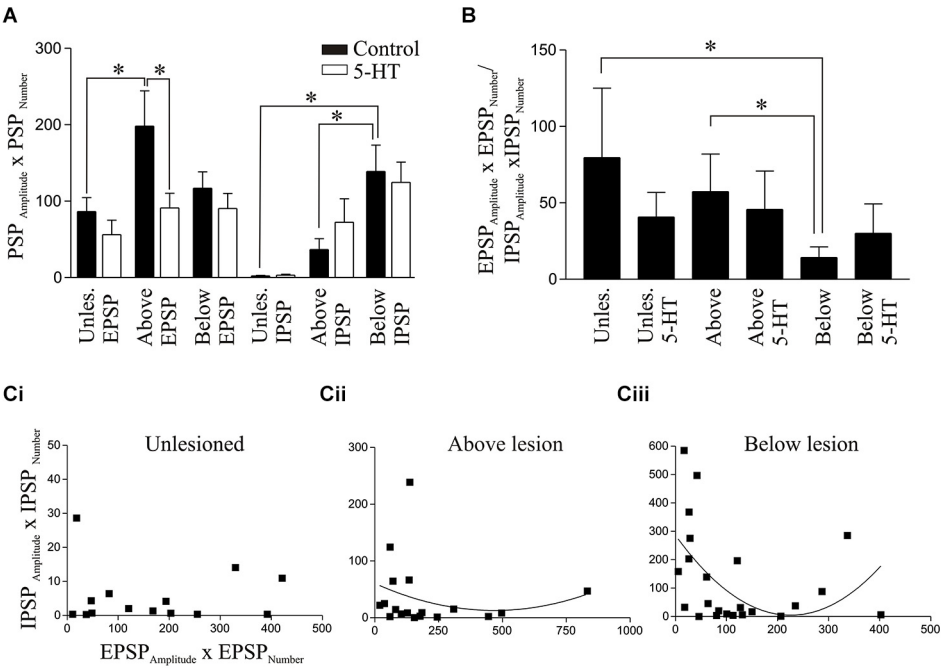

FIGURE 6 | Analysis of spontaneous PSPs. (A) Graphs showing the product of the PSP amplitude and frequency for EPSPs and IPSPs in unlesioned and lesioned animals in control and in 1 μM 5-HT. **(B)** The ratio of the $EPSP_{Amplitude} \times EPSP_{Number}$ and $IPSP_{Amplitude} \times IPSP_{Number}$ in unlesioned animals and above and below the lesion site in control and in 1μM 5-HT. Note that the values were calculated for each cell and summed, and not from taking the averaged values for EPSPs and IPSPs from **(A)**. Correlation of the $EPSP_{Amplitude} \times EPSP_{Number}$ and $IPSP_{Amplitude} \times IPSP_{Number}$ in cells from unlesioned **(Ci)**, above lesion **(Cii)**, and below lesion spinal cords **(Ciii)**.

An estimate of the summed spontaneous excitatory or inhibitory drive for each cell was quantified from the product of the PSP amplitude and number ($PSP_{Amplitude} \times PSP_{Number}$). The IPSP value for spinal cord injured animals was significantly greater below than those in both the unlesioned and above lesion values, while the EPSP value was significantly greater above than in unlesioned animals (**Figure 6A**). When the EPSP:IPSP ratio ($EPSP_{Amplitude} \times EPSP_{Number}$ /$IPSP_{Amplitude} \times IPSP_{Number}$) was calculated for each cell there was a significant reduction below the lesion site compared to unlesioned and above lesion cases, presumably reflecting the increase in inhibition (**Figure 6B**). This relationship was examined further by correlating the $IPSP_{Amplitude} \times IPSP_{Number}$ against the $EPSP_{Amlitude} \times EPSP_{Number}$. This analysis showed little relationship in unlesioned animals where there was a similar inhibitory value over a range of excitatory values (**Figure 6Ci**). Below the lesion site the $IPSP_{Amplitude} \times IPSP_{Number}$ was larger with smaller and larger excitatory values, possibly reflecting the increased feedforward activation of inhibitory interneurons (see **Figure 5C**): at intermediate levels of excitation IPSP values were low, giving a parabolic relationship. This was only significant below the lesion (below $r^2 = 0.25$, $p < 0.05$; above lesion $r^2 = 0.04$, $p > 0.05$; (**Figures 6Ci–iii**), and suggests a reorganization of the spinal cord circuitry that reflects a need for greater inhibition below the lesion site as excitation is increased.

There was no significant effect of 1 μM 5-HT on the spontaneous EPSP or IPSP amplitude in cells from unlesioned animals or cells above or below the lesion site ($p > 0.05$, $n = 6$; **Figure 7A**). However, it significantly reduced the number of EPSPs and the $EPSP_{Amplitude} \times EPSP_{Number}$ above the lesion site ($p < 0.05$; **Figures 6A**, **7B**), suggesting a lesion-induced change in 5-HT sensitivity at this site. The analysis of 5-HT effects on spontaneous PSPs was complicated by two features: firstly there was marked variability in initial values, especially for the number of PSPs (range from <10 to >600); and secondly, 5-HT could evoke oscillations of the membrane potential that could be associated with phases of increased and decreased spontaneous inputs (see **Figure 2A**). These oscillations are difficult to control for here: effects were measured 10 min after 5-HT application and varying this to select a region where there was or was not an oscillation would bias the analysis. The variability of initial values could, however, be addressed by normalizing the control values and analyzing the change in 5-HT (**Figures 7C,D**). In this case there were again no significant differences, but the greater variability after lesioning can be seen from the increased ranges when the data is shown on box-plots. 10 μM 5-HT also failed to significantly affect the spontaneous IPSP or EPSP amplitude or number, but it significantly reduced the $EPSP_{Amplitude} \times EPSP_{Number}$ in unlesioned animals (**Figure 7Ei**). Variability was again a feature, especially

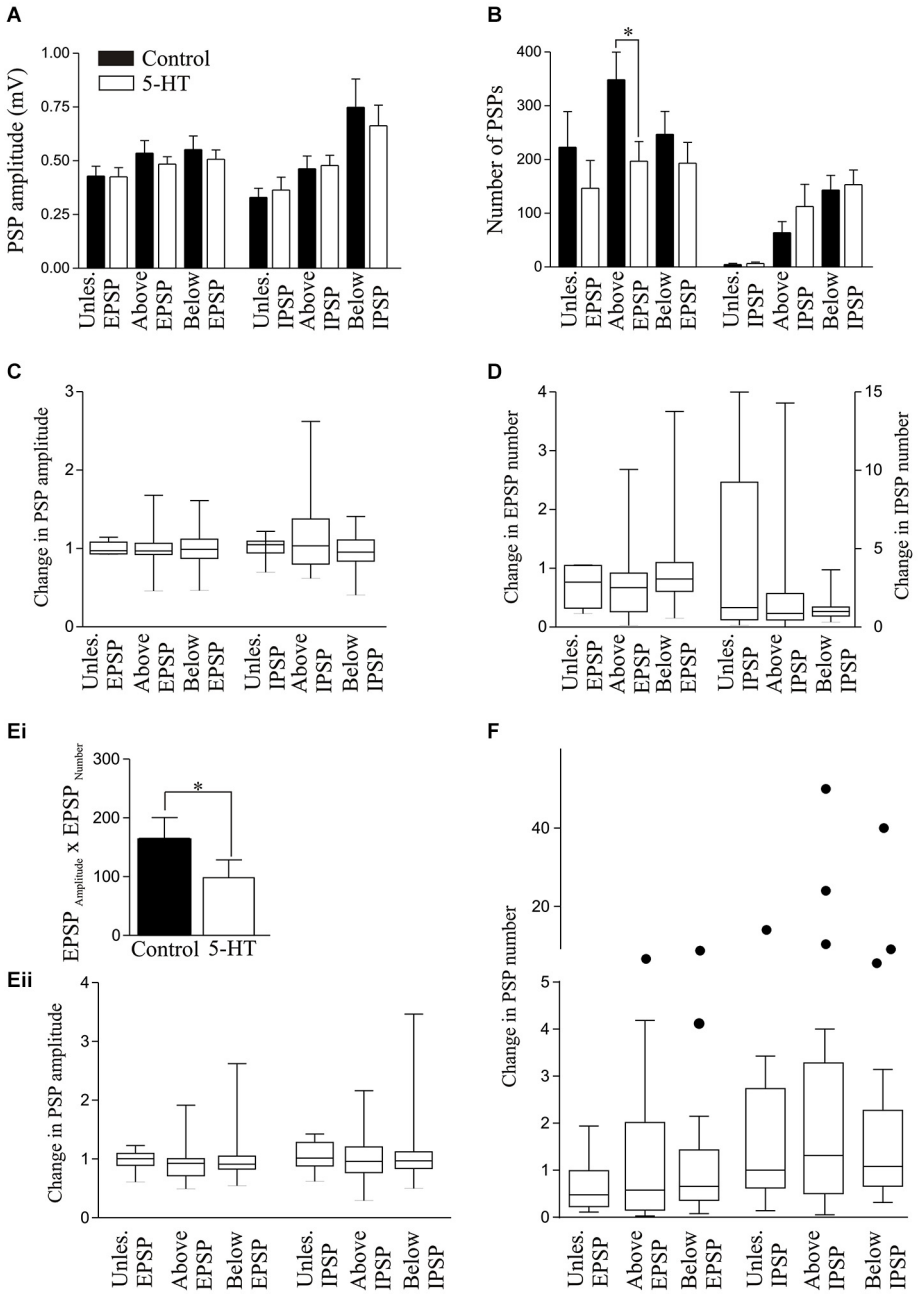

FIGURE 7 | Effect of 1 μM 5-HT on the spontaneous PSP amplitude (A) and number (B). **(C)** Graph showing the normalized change in PSP amplitude in 5-HT. **(D)** Graph showing the normalized change in PSP number in 5-HT. **(Ei)** Graph showing the significant reduction of the $EPSP_{Amplitude} \times EPSP_{Number}$ in unlesioned animals by 10 μM 5-HT. **(Eii)** The effects of 5-HT on the normalized change in PSP amplitudes by 5-HT. **(F)** Graph showing the normalized change in the spontaneous PSP number in control and 10 μM 5-HT. The circles represent Tukey outliers, and highlight the increased variability in numbers after lesioning.

for the number of events. However, no significant effects were revealed by normalizing the change in 5-HT to the pre-5-HT value (**Figures 7Eii,F**).

In summary, these results show significant differences in cellular and synaptic properties and their modulation by 5HT in the isolated lesioned spinal cords, and that these changes differ above and below the lesion site.

5-HT EFFECTS ON SWIMMING

The changes in the modulatory effects of 5-HT in lesioned animals obviously need to be understood in the context of their role in functional recovery. This is difficult to address as it requires direct links between cellular/synaptic effects and behavior, something that is far from trivial even in this simpler system (see Parker, 2006, 2010). To provide a basis for these analyses we

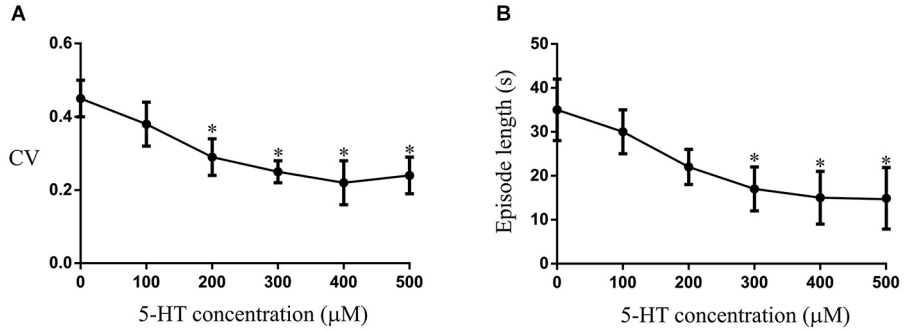

FIGURE 8 | The effects of different concentrations of 5-HT applied to the aquarium water on the CV(A) and episode length (B). The asterix shows that significant effects occurred at 200–300 μM, with a plateau effect occurring at 300 μM.

need to know the behavioral effects of 5-HT. This was examined in intact swimming lampreys. In unlesioned animals (n = 10) significant effects of 5-HT on the CV and episode length occurred at concentrations of between 200–300 μM (**Figures 8A,B**). The effects of 500 μM 5-HT are presented in **Figure 9**. 5-HT had variable non-significant effects on the swimming episode length ($p > 0.05$; **Figure 9Ai**; all values on this figure are 500 μM). Similar effects were seen for the cycle period. Overall there was no significant change ($p > 0.05$), but control values varied and in animals with shorter cycle periods (<400 ms) the cycle period was increased (**Figure 9Aii**). There was no significant effect on the phase lag ($p > 0.05$; **Figure 9Aiii**), but the variability of swimming was reduced by 5-HT, shown by the significant reduction of the coefficient of variation (CV; $p < 0.05$; **Figure 9Aiv**).

In lesioned animals (n = 20), the swimming episode length was significantly reduced by 5-HT at higher concentrations (400–500 μM; $p < 0.05$; **Figure 9Bi**), although the effect was again greater in animals that showed longer swimming episodes in control. In these animals, there was no significant overall or apparent state-dependent effect on the cycle period (**Figure 9Bii**) or the phase lag (**Figure 9Biii**; $p > 0.05$), but the CV was again significantly reduced ($p < 0.05$; **Figure 9Biv**).

While the n number is low (n = 3), 5-HT could improve swimming in poorly recovered animals (swimming score of 2). In these animals activity was absent below the lesion site but was evoked by 5-HT, and the activity above the lesion site became more regular (**Figure 9C**). These effects resulted in an increase in the swimming score from 2 to 3/4, and so swimming was still far from fully recovered.

The role of 5-HT in promoting recovery was examined using PCPA to deplete 5-HT (Hashimoto and Fukuda, 1991; Airhart et al., 2012). PCPA did not cause any acute effects (tested from 1–24 h after incubation; data not shown), but incubating unlesioned animals in PCPA for 72 h markedly disrupted locomotor activity to an extent that locomotor parameters could not be measured for a quantitative analysis. This suggests a necessary role for 5-HT in normal swimming (**Figures 9Di,ii**). On removal from PCPA the animals regained good locomotor function within 3–5 days, to an extent that the episode length, cycle period, intersegmental phase lag, and CV did not differ significantly to non-exposed control animals (data not shown). Incubating lesioned animals in PCPA for 6 weeks resulted in poor recovery in 5 of 5 animals (all assessed as stage 2 or 3; **Figure 9E**). A matched group of control lesioned animals that were not incubated in PCPA all recovered good function (stage 5 or 6; $p < 0.05$ Chi square). The failure of PCPA incubated animals to recover was not due to the acute effect of PCPA as animals were tested after being removed from PCPA for at least 2 weeks. However, in the five animals that recovered in the absence of PCPA, incubation in PCPA for 5 days 10 weeks after lesioning resulted in no disruption of locomotor activity (**Figure 9F**). This contrasts the effect in unlesioned animals and suggests that while 5-HT is necessary for swimming in unlesioned animals and for recovery after lesioning, it is not needed to maintain recovery.

RELATIONSHIP TO DEGREE OF RECOVERY

We have previously found significant differences in larval animals separated into those that recovered well or poorly (see Cooke and Parker, 2009; Hoffman and Parker, 2011). We thus separated lesioned animals into those that recovered well or poorly by taking the upper (stage 5 or 6, n = 39) and lower levels of the swimming score (stage 2 or 3, n = 10).

There was no significant difference in RMP, input resistance, or the sAHP amplitude above or below the lesion site in the isolated spinal cord from animals that recovered well or poorly (data not shown). Excitability did not differ to that in unlesioned animals for either those that recovered well (n = 20) or poorly (n = 10), but above the lesion site excitability was significantly reduced with current steps from 1–2.5 nA in poorly recovered animals (**Figure 10Ai**). A similar effect occurred below the lesion site, but here excitability in response to 0.5–2 nA current steps was significantly lower in poorly recovered animals (n = 14) than those that recovered well (n = 33; **Figure 10Aii**).

There was a significant increase in spontaneous EPSP and IPSP amplitudes above the lesion site in the isolated spinal cord from poorly recovered animals (n = 6) compared to those that recovered well (n = 10; **Figure 10Bi**), while the number of EPSPs was significantly less in poorly recovered animals (**Figure 10Bii**). The spontaneous EPSP amplitude was also significantly greater

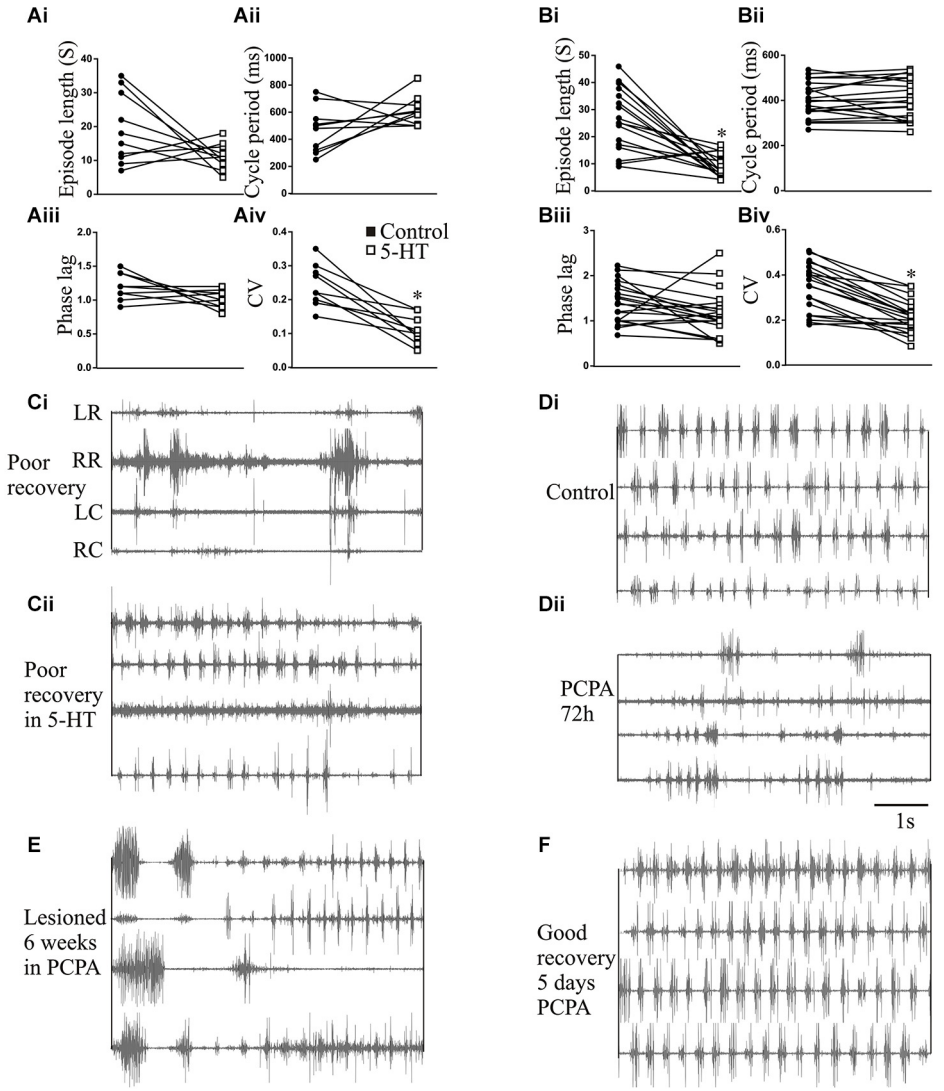

FIGURE 9 | The effects of 5-HT and 5-HT depletion on swimming. Graphs showing the effects of 5-HT (500 μM) applied to the test chamber water on the episode length **(Ai)**, cycle period **(Aii)**, intersegmental phase lag **(Aiii)**, and burst coefficient of variation (CV; **Aiv**) in unlesioned animals, and the effects of 5-HT (500 μM) on the episode length **(Bi)**, cycle period **(Bii)**, intersegmental phase lag **(Biii)**, and burst coefficient of variation (CV; **Biv**) in lesioned animals. **(Ci)** EMG activity recorded in a poorly recovered animal and **(Cii)** the swimming in the same animal after 5-HT (500 μM) application. Electrode placement relative to the lesion site is shown to the left of the trace: LR, left rostral; RR, right rostral; LC, left caudal; RC, right caudal. **(Di)** EMG activity recorded in an unlesioned animal in control and **(Dii)** 72 h after incubation in PCPA to deplete 5-HT. **(E)** Traces showing disrupted swimming activity recorded 9 weeks after lesioning in an animal incubated in PCPA for the first six weeks after lesioning. **(F)** Lack of effect of 5 days incubation in PCPA in an animal that had recovered 10 weeks after a spinal cord lesion.

below the lesion site in poorly recovered animals (n = 6) compared to those that recovered well (n = 17). The $EPSP_{Amplitude} \times EPSP_{Number}$ was significantly greater above the lesion site in animals that recovered well than in those that recovered poorly (**Figure 10C**), an effect that was consistent with an increase in the integrated synaptic activity above the lesion site in animals that recovered well (data not shown): no other values differed significantly.

For 5-HT (10 μM) effects, above the lesion site the only significant difference was that the sAHP was only significantly reduced in animals that recovered well (**Figure 10Di**). Below the lesion site the 5-HT effect on the RMP was significantly different. In unlesioned animals 10 μM 5-HT non-significantly hyperpolarised the membrane potential (n = 15 of 16, mean effect −0.61 ± 0.34 mV; **Figure 2A**). In good recovery there were variable effects on the RMP (n = 3 depolarize, n = 4 hyperpolarize, n = 5 unchanged) that gave a mean change of 0.31 ± 0.74 mV (n = 12), but in cells from poorly recovered there was a hyperpolarization in every cell that was significantly different to cells from animals that recovered well (−2.7 ± 0.86 mV; $p < 0.05$, n = 10; **Figure 10Dii**).

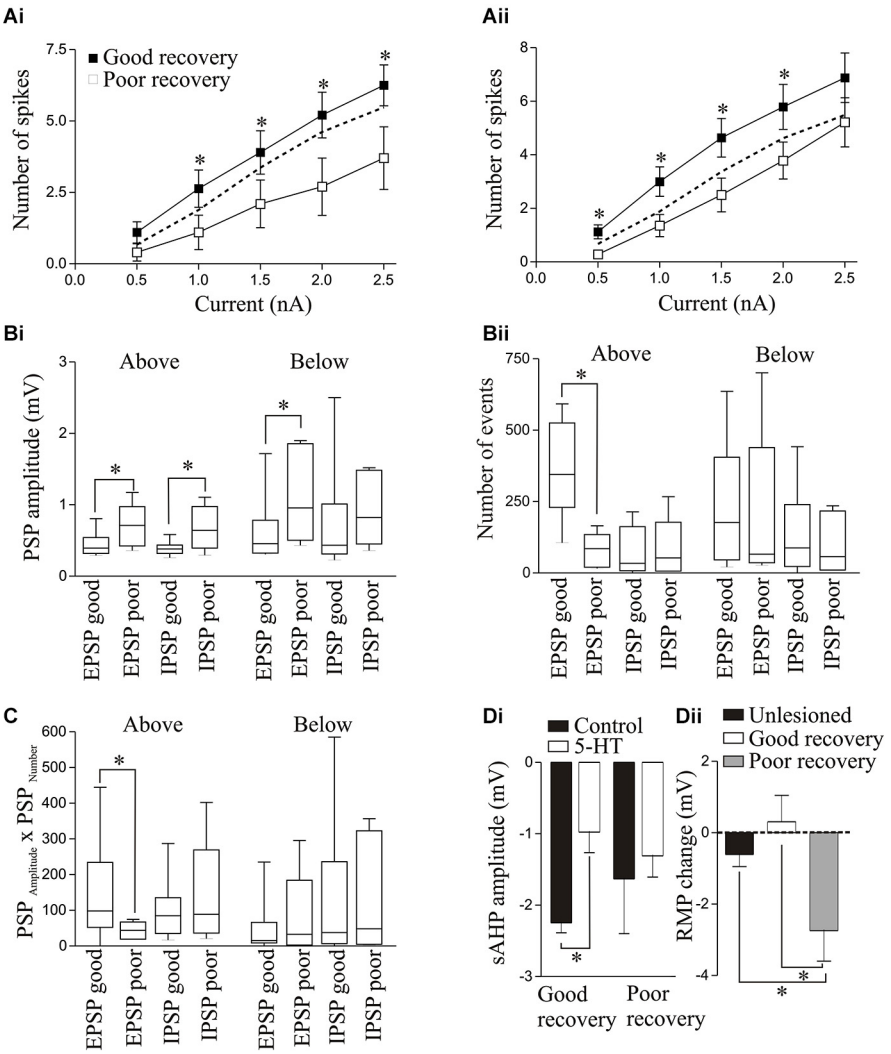

FIGURE 10 | Excitability in response to 100ms current pulse steps in animals that recovered well or poorly above (Ai) and below (Aii) the lesion site. The dashed line shows the unlesioned response. The spontaneous PSP amplitude **(Bi)** and number **(Bii)**, and the $PSP_{Amplitude} \times PSP_{Number}$ **(C)** above and below the lesion site in animals that recovered well or poorly. **(Di)** Graph showing the significant reduction in the sAHP amplitude by 5-HT (10 μM) T (10HT (above the lesion site in animals that recovered well but not in those that recovered poorly. **(Dii)** Graph showing the change in resting membrane potential (RMP) by 5-HT below the lesion site in unlesioned animals and animals that recovered well or poorly.

DISCUSSION

While several of the effects shown here are preliminary and require further analysis, the results show that there are changes in cellular and synaptic properties in the isolated lesioned compared to the isolated unlesioned spinal cord; that these changes differ above and below the lesion site; and that the modulatory effects of 5-HT are altered by lesioning, with effects again differing above and below the lesion site.

There were several changes in functional properties in lesioned animals. Spontaneous synaptic and cord activity was increased above and below the lesion site compared to unlesioned animals. The reticulospinal axon-evoked EPSP half-width and the slow synaptic depolarization during trains of action potentials were also greater above and below the lesion site compared to unlesioned animals. However, the activity-dependent plasticity of reticulospinal inputs differed above and below the lesion site: depression occurred below that matched that seen in unlesioned animals, but above the lesion site the input facilitated which will make these connections functionally stronger. Facilitation is typically associated with a lower release probability and smaller initial EPSP amplitude (Zucker and Regehr, 2002). The size of the active zone can correlate with the release probability (see Holderith et al., 2012 and references therein), and the active zone is reduced at regenerated larval lamprey Müller reticulospinal axons (Oliphint et al., 2010). However, there was no reduction of the initial EPSP amplitude which would be expected if the release probability was reduced. A reduction of release probability to allow facilitation without a reduction of the initial EPSP amplitude is possible if the number of available vesicles is increased (Bevan and Parker,

2004), but this possibility requires further analysis, as do the general properties of synapses made by regenerated axons. For example, while the mean amplitude of monosynaptic EPSPs does not differ in unlesioned and lesioned animals, regenerated Müller axons make fewer synaptic contacts below the lesion site (Oliphint et al., 2010). The generation of an EPSP of comparable amplitude to that in unlesioned animals from a smaller number of synaptic contacts should require some change in the release properties or postsynaptic effects of the individual contacts.

The $EPSP_{Amplitude} \times EPSP_{Number}$ was greater above the lesion site than in unlesioned animals, suggesting potentiation of the excitatory drive. This could relate to the increased incidence of putative polysynaptic EPSPs above the lesion site: this is consistent with greater feedforward excitation, and could reflect potentiation of connections onto or between the excitatory interneurons (EIN), changes in EIN excitability (Parker, 2003), or strengthening of crossing excitatory connections (ScIN or CCIN; Buchanan, 2001; Parker, 2003). Conversely, putative polysynaptic IPSPs, the spontaneous IPSP amplitude and number, and the $IPSP_{Amplitude} \times IPSP_{Number}$ were greater below the lesion site. This suggests changes in inhibitory premotor interneuron excitability or synaptic strengths (this could either occur ipsilaterally (SiIN or LIN; Buchanan, 2001; Parker, 2003) or contralaterally (ScIN or CCIN; Buchanan, 2001; Parker, 2006)) or a change in the excitatory drive to these cells. The latter effect is supported by the parabolic relationship between the $EPSP_{Amplitude} \times EPSP_{Number}$ and $IPSP_{Amplitude} \times IPSP_{Number}$. These region specific changes in inhibition and excitation now suggest specific network synaptic interactions that can be targeted for analysis above and below the lesion site.

Here we have used juvenile adults for the first time in our analyses rather than larvae (Cooke and Parker, 2009; Hoffman and Parker, 2011). Several effects differed here to those in larvae. This could reflect developmental influences (Parker and Gilbey, 2007; Cooke et al., 2012), not a surprising conclusion but one that merits consideration to avoid the mixing of developmental stages. In contrast to larvae (Cooke and Parker, 2009), there were no significant changes in cellular properties (resting potential, input resistance, sAHP amplitude, or excitability). This suggests a potential developmental switch from changes in cellular to changes in synaptic properties. Here inhibition was also greater below the lesion site, but in larvae inhibition was only increased in poorly recovered animals. However, as noted above this increased inhibition could reflect an increase in feedforward excitation.

We have also examined changes above the lesion site for the first time. That there are changes here argues against lesion-induced differences simply being a consequence of the removal of descending regulatory inputs (which has been considered to explain spasticity below lesion sites; Dietz, 2002) or a compensatory response to the removal of descending excitation (Cooke and Parker, 2009). The above lesion effects may instead reflect the need for changes at multiple sites that act together to regulate the integrated activity of the supra and sub-lesion spinal cord to generate an efficient motor output. Increased activity above the lesion site may also be needed to drive activity below, either neuronally through increased excitation of regenerated propriospinal axons, or mechanically by strong supra-lesional movements that propagate below the lesion site to be relayed to the spinal cord by potentiated proprioceptive inputs (Hoffman and Parker, 2011). Grasso et al. (2004) provided evidence of mechanical effects after injury to the human spinal cord (e.g., using arm and body movements to assist leg movement), and Shah et al. (2013) showed that forelimb training increased hindlimb function in rats, suggesting that mechanical propagation of this sort is not simply a peculiarity of the lamprey. In contrast to many species, ascending inputs also regenerate in lamprey (Armstrong et al., 2003), and the reduction or loss of these ascending inputs could provide a signal to cells above the lesion site that drive the supra-lesion changes in functional properties.

LESION-INDUCED CHANGES IN THE EFFECTS OF 5-HT

A principal focus of the analysis was to compare the effects of 5-HT in lesioned and unlesioned animals. The potential role for neuromodulators, especially 5-HT, in restoring function after SPI has been a major focus of research (see Rossignol et al., 2001). However, little is known if or how injury influences modulatory effects. Changes in modulation could occur directly through changes in the properties of transmitter receptors or second messenger pathways, indirectly through altered interactions between modulatory systems as a result of the loss or reduction of one or more transmitter systems, or through state-dependent effects caused by the lesion-induced changes in cellular and synaptic properties. The different effects of 5-HT in unlesioned and lesioned animals shown here add to the evidence of changes in modulatory effects after spinal cord lesions (Svensson et al., 2013). In unlesioned animals, 5-HT had effects that should generally reduced excitation, shown by the consistent, albeit non-significant, hyperpolarization of the membrane potential and the reduction of evoked EPSP amplitudes and spontaneous EPSPs, but in lesioned animals both of these effects were absent. The differences in 5-HT effects after lesioning suggest that pharmacological approaches to functional recovery should not be assumed from analyses in unlesioned spinal cords. There were also differences in 5-HT effects above and below the lesion site. As systemic drug application will act at both sites it may be beneficial to target potential regional effects. This would be possible if effects are mediated by different receptor subtypes.

The behavioral analyses of 5-HT effects on swimming provide preliminary evidence of a role for 5-HT in recovery. While there are several caveats to the analysis of swimming, including unknown drug concentrations in the CNS and uncertainty of their sites of action, a consistent result in these experiments was that putative 5-HT depletion using PCPA led to a failure of recovery (note however that we have not measured 5-HT levels after this treatment). Exogenous and endogenous 5-HT slows the frequency of network activity in unlesioned animals (Harris-Warrick and Cohen, 1985; Christenson et al., 1989; Kemnitz et al., 1995; Martin, 2002). 5-HT originates from three sources in lamprey: descending inputs from rhombencephalic neurons in

the brainstem that run in the lateral tract; an intrinsic ventromedial spinal cord plexus; and fibers entering via the dorsal root ganglion (Cohen et al., 2005). Knowledge of the pharmacology of 5-HT receptors in lamprey lags that in mammals, where 5-HT_{1A} and 5-$HT_{2A,C}$ receptors have been implicated in either improved functional recovery or pathogenesis after spinal lesions (e.g., Gimenez y Ribotta et al., 1998; Giroux et al., 1999; Hains et al., 2001; Hochman et al., 2001; Antri et al., 2003; Murray et al., 2010; Kong et al., 2011; see Gackière and Vinay (2014) for a recent review). Currently we know that the 5-HT-mediated reduction of the post-spike sAHP and fictive locomotor frequency in unlesioned mature adult animals seem to be mediated by a 5-HT_{1A} or 5-HT_2 – like receptor, as the effects were mimicked by agonists of both of these receptors and blocked by the spiperone (a 5-HT_{1A} or 5-HT_2 antagonist), but they were not blocked by specific 5-HT_2 antagonists: 5-HT_3 and 5-HT_4 agonists and antagonists were without effect (Wikström et al., 1995). As in mammals (Giroux et al., 1999; Otoshi et al., 2009), in the larval lamprey 5-HT immunoreactivity was significantly reduced below the lesion in the lateral tract and ventromedial plexus 10 weeks after lesioning, the time point examined here (Cohen et al., 2005). At 10 weeks there was an increase in 5-HT immunoreactivity in the ventromedial plexus immediately rostral to the lesion site, possibly due to sprouting of spared fibers (Cohen et al., 2005). These changes in 5-HT levels and regeneration did not correlate with the degree of functional recovery (Cohen et al., 1999; Christenson et al., 1989). While the pharmacology of 5-HT effects in the lesioned spinal cord is unknown, 5-HT_{1A} receptor levels are increased 1–3 weeks after lesioning immediately above and 1–7 weeks immediately below the lesion site (Cornide-Petronio et al., 2014; note that the lesions in the Cohen et al., 2005 study were more caudal lesions to those used here, and the Cornide-Petronio et al., 2014 lesions more rostral). The relevance of this transient receptor up regulation, which also occurs in the cat (Giroux et al., 1999), is currently unknown, but it may relate to the need for 5-HT during the recovery period suggested by the PCPA experiments.

Assuming no volume transmission across the lesion, there is an obvious potential for differences in 5-HT levels on either side of a lesion site. In lamprey this could lead to faster activity below, where 5-HT levels are reduced, and slower 5-HT-modulated activity above the lesion site (Harris-Warrick and Cohen, 1985). The differences in activity either side of the lesion site would require an intersegmental co-ordinating signal that ensures the activity is properly integrated: failure to do this could result in poor locomotor recovery (Cohen et al., 1999; Christenson et al., 1989). In this context it could be speculated that the reduction of 5-HT effects in lesioned animals could help to reduce potential 5-HT-driven disparities in frequency by reducing the overall influence of 5-HT. The functional changes in basic cellular and synaptic properties may also contribute to this effect. Accepting the assumption that the swimming frequency reflects the degree of excitatory drive (Brodin et al., 1985), the greater inhibition below the lesion site would reduce the frequency but the increased excitation above would increase it: this could help to offset the differences in frequency caused by differences in 5-HT levels. This could be tested by manipulating excitability in unlesioned and lesioned animals either side of the lesion site. While this can easily be done for fictive locomotion, the differences in 5-HT effects on phase lag in the intact and fictive conditions (Harris-Warrick and Cohen, 1985; Kemnitz et al., 1995) raise the issue of how well fictive activity represents normal function (see also discussion of fictive activity in Ayers et al., 1983; Wang and Jung, 2002; Parker and Srivastava, 2013). Also, the discussion above only considers differences in 5-HT: a wide range of transmitters are released from descending neurons whose levels could differ either side of the lesion (Brodin et al., 1988). Differences in the effects of these individual transmitters will also have to be considered, as well as interactions between them.

A major issue is to place the differences in 5-HT effects in lesioned animals into a functional context. While this lack of understanding is an obvious weakness, similar uncertainty exists over the effects of 5-HT in unlesioned animals despite several studies that examine these effects. 5-HT has numerous cellular and synaptic effects in unlesioned animals: it reduces somatosensory and reticulospinal-evoked EPSP amplitudes (Buchanan and Grillner, 1991; El Manira et al., 1997), and has varied cell and synapse-specific effects on locomotor network neurons (see Parker, 2006 for details). How these effects influence the fictive or actual locomotor output in unlesioned animals is unknown. This understanding relies on knowing the segmental and intersegmental network organization: while this has been repeatedly claimed to be characterized there are still significant gaps in our understanding of even the basic network architecture (see Parker, 2006, 2010). The diverse cellular and synaptic effects of 5-HT would then need to be considered. It is claimed that the effects of 5-HT on fictive activity are explained by a reduction of the slow calcium-dependent (K_{Ca}) sAHP following an action potential or via activation of NMDA channels in the crossing (CC) inhibitory interneurons, which delays switching of activity between the two sides and thus increases the cycle period (e.g., Matsushima and Grillner, 1992). However, this argument is flawed: relating effects solely to the CC interneurons begs the question as 5-HT affects the sAHP in other cells than the CC interneurons in ways that could increase the frequency; cells typically generate a small number of spikes which limits the possibility of sAHP summation; and blocking the sAHP with apamin has no effect on fictive locomotion over most of the frequency range (it only slows activity with lower NMDA concentrations, and this seems to be associated with disruption of the activity; see Buchanan, 2001). Understanding the role of the changes seen after lesioning in any system will require a consideration of how the varied functional effects act within well-defined locomotor networks.

CONCLUSIONS

Pharmacological, electrical stimulation, and training effects modulate distinct functions in experimental and clinical situations (Courtine et al., 2009; Harkema et al., 2011; van den Brand et al., 2012). Knowing the changes in the lesioned spinal cord, how they relate to the degree of recovery, and how they respond to manipulations should allow these approaches to be targeted to maximize beneficial effects. Promoting regeneration remains the

dominant approach to recovery from SPI, but the changes that occur in the lesioned spinal cord, either directed or non-directed (Beauparlant et al., 2013), could alter the response of networks to restored inputs (Bradbury and McMahon, 2006; Nahar et al., 2012), making a consideration of these changes necessary even if regeneration is assumed to be the dominant factor. If recovery reflects the integrated effects of functional changes above, below, and across the lesion site, as seems likely, perturbation of any component will only show necessity, not sufficiency in recovery, and thus all factors need to be considered. While we have identified various changes after lesioning in the lamprey model in this and previous studies, we currently do not know how these effects influence recovery. In addition to understanding the mechanisms underlying these effects, we now need to understand how these effects act in a defined, re-organized locomotor network and from this how they influence behavioral recovery.

REFERENCES

Airhart, M. J., Lee, D. H., Wilson, T. D., Miller, B. E., Miller, M. N., Skalko, R. G., et al. (2012). Adverse effects of serotonin depletion in developing zebrafish. *Neurotoxicol. Teratol.* 34, 152–160. doi: 10.1016/j.ntt.2011.08.008

Antri, M., Mouffle, C., Orsal, D., and Barthe, J. Y. (2003). 5-HT1A receptors are involved in short- and long-term processes responsible for 5-HT-induced locomotor function recovery in chronic spinal rat. *Eur. J. Neurosci.* 18, 1963–1972. doi: 10.1046/j.1460-9568.2003.02916.x

Armstrong, J., Zhang, L., and McClellan, A. (2003). Axonal regeneration of descending and ascending spinal projection neurons in spinal cord-transected larval lamprey. *Exp. Neurol.* 180, 156–166. doi: 10.1016/s0014-4886(02)00057-2

Ayers, J. (1989). "Recovery of oscillator function following spinal regeneration in the sea lamprey," in *Cellular and Neuronal Oscillators*, ed J. Jacklet (New York: Marcel Dekker), 349–383.

Ayers, J., Carpenter, G. A., Currie, S., and Kinch, J. (1983). Which behavior does the lamprey central motor program mediate? *Science* 221, 1312–1314. doi: 10.1126/science.6137060

Barbeau, H., and Norman, K. (2003). The effect of noradrenergic drugs on the recovery of walking after spinal cord injury. *Spinal Cord* 41, 137–143. doi: 10.1038/sj.sc.3101374

Beauparlant, J., van den Brand, R., Barraud, Q., Friedli, L., Musienko, P., Dietz, V., et al. (2013). Undirected compensatory plasticity contributes to neuronal dysfunction after severe spinal cord injury. *Brain* 136, 3347–3361. doi: 10.1093/brain/awt204

Bennett, D. J., Li, Y., Harvey, P. J., and Gorassini, M. (2001). Evidence for plateau potentials in tail motoneurons of awake chronic spinal rats with spasticity. *J. Neurophysiol.* 86, 1972–1982.

Berry, M. S., and Pentreath, V. W. (1976). Criteria for distinguishing between monosynaptic and polysynaptic transmission. *Brain Res.* 105, 1–20. doi: 10.1016/0006-8993(76)90919-7

Bevan, S., and Parker, D. (2004). Metaplastic facilitation and ultrastructural changes in synaptic properties are associated with long-term modulation of the lamprey locomotor network. *J. Neurosci.* 24, 9458–9468. doi: 10.1523/jneurosci.3391-04.2004

Boulenguez, P., Liabeuf, S., Bos, R., Bras, H., Jean-Xavier, C., Brocard, C., et al. (2010). Down-regulation of the potassium-chloride cotransporter KCC2 contributes to spasticity after spinal cord injury. *Nat. Med.* 16, 302–308. doi: 10.1038/nm.2107

Boyd, M. R., and McClellan, A. D. (2002). Changes in locomotor activity parameters with variations in cycle time in larval lamprey. *J. Exp. Biol.* 205, 3707–3716.

Bradbury, E. J., and McMahon, S. B. (2006). Spinal cord repair strategies: why do they work? *Nat. Rev. Neurosci.* 7, 644–653. doi: 10.1038/nrn1964

Brodin, L., Grillner, S., Dubuc, R., Ohta, Y., Kasicki, S., and Hökfelt, T. (1988). Reticulospinal neurons in lamprey: transmitters, synaptic interactions and their role during locomotion. *Arch. Ital. Biol.* 126, 317–345.

Brodin, L., Grillner, S., and Rovainen, C. (1985). N-methyl-D-aspartate (NMDA), kainate and quisqualate receptors and the generation of fictive locomotion in the lamprey spinal cord. *Brain Res.* 325, 302–306. doi: 10.1016/0006-8993(85)90328-2

Buchanan, J. T. (2001). Contributions of identifiable neurons and neuron classes to lamprey vertebrate neurobiology. *Prog. Neurobiol.* 63, 441–466. doi: 10.1016/s0301-0082(00)00050-2

Buchanan, J. T., and Grillner, S. (1991). 5-Hydroxytryptamine depresses reticulospinal excitatory postsynaptic potentials in motoneurons of the lamprey. *Neurosci. Lett.* 122, 41–44.

Christenson, J., Franck, J., and Grillner, S. (1989). Increase in endogenous 5-hydroxytryptamine levels modulates the central network underlying locomotion in the lamprey spinal cord. *Neurosci. Lett.* 100, 188–192. doi: 10.1016/0304-3940(89)90682-4

Cohen, A., Abdelnabi, M., Guan, L., Ottinger, M., and Chakrabarti, L. (2005). Changes in distribution of serotonin induced by spinal injury in larval lampreys: evidence from immunohistochemistry and HPLC. *J. Neurotrauma* 22, 172–188. doi: 10.1089/neu.2005.22.172

Cohen, A. H., Baker, M. T., and Dobrov, T. A. (1989). Evidence for functional regeneration in the adult lamprey spinal cord following transection. *Brain Res.* 496, 368–372. doi: 10.1016/0006-8993(89)91090-1

Cohen, A., Kiemel, T., Pate, V., Blinder, J., and Guan, L. (1999). Temperature can alter the function outcome of spinal cord regeneration in larval lampreys. *Neuroscience* 90, 957–965. doi: 10.1016/s0306-4522(98)00502-8

Cohen, A. H., Mackler, S. A., and Selzer, M. E. (1988). Behavioral recovery following spinal transections: functional regeneration in the lamprey CNS. *Trends Neurosci.* 11, 227–231. doi: 10.1016/0166-2236(88)90131-2

Cooke, R. M., Luco, S., and Parker, D. (2012). Manipulations of spinal cord excitability evoke developmentally-dependent compensatory changes in the lamprey spinal cord. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 198, 25–41. doi: 10.1007/s00359-011-0683-0

Cooke, R. M., and Parker, D. (2009). Locomotor recovery after spinal cord lesions in the lamprey is associated with functional and ultrastructural changes below lesion sites. *J. Neurotrauma* 26, 597–612. doi: 10.1089/neu.2008.0660

Cornide-Petronio, M. E., Fernández-López, B., Barreiro-Iglesias, A., and Rodicio, M. C. (2014). Traumatic injury induces changes in the expression of the serotonin 1A receptor in the spinal cord of lampreys. *Neuropharmacology* 77, 369–378. doi: 10.1016/j.neuropharm.2013.10.017

Courtine, G., Gerasimenko, Y., van den Brand, R., Yew, A., Musienko, P., Zhong, H., et al. (2009). Transformation of nonfunctional spinal circuits into functional states after the loss of brain input. *Nat. Neurosci.* 12, 1333–1342. doi: 10.1038/nn.2401

Dale, N., and Grillner, S. (1986). Dual-component synaptic potentials in the lamprey mediated by excitatory amino acid receptors. *J. Neurosci.* 6, 2653–2661.

D'Amico, J., Murray, K., Li, Y., Chan, K., Finlay, M., Bennett, D., et al. (2013). Constitutively active 5-HT2/a1 receptors facilitate muscle spasms after human spinal cord injury. *J. Neurophysiol.* 109, 1473–1484. doi: 10.1152/jn.00821.2012

Dietz, V. (2002). Proprioception and locomotor disorders. *Nat. Rev. Neurosci.* 3, 781–790. doi: 10.1038/nrn939

Edgerton, V., Leon, R., Harkema, S., Hodgson, J., London, N., Reinkensmeyer, D., et al. (2001). Retraining the injured spinal cord. *J. Physiol.* 533, 15–22. doi: 10.1111/j.1469-7793.2001.0015b.x

El Manira, A., Zhang, W., Svensson, E., and Bussiéres, N. (1997). 5-HT inhibits calcium current and synaptic transmission from sensory neurons in lamprey. *J. Neurosci.* 17, 1786–1794.

Gackière, F., and Vinay, L. (2014). Serotonergic modulation of post-synaptic inhibition and locomotor alternating pattern in the spinal cord. *Front. Neural Circuits* 8:102. doi: 10.3389/fncir.2014.00102

Gimenez y Ribotta, M., Orsal, D., Feraboli-Lohnherr, D., and Privat, A. (1998). Recovery of locomotion following transplantation of monoaminergic neurons in the spinal cord of paraplegic rats. *Ann. N Y Acad. Sci.* 860, 393–411. doi: 10.1111/j.1749-6632.1998.tb09064.x

Giroux, N., Rossignol, S., and Reader, T. (1999). Autoradiographic study of alpha1- and alpha2-noradrenergic and serotonin1A receptors in the spinal cord of normal and chronically transected cats. *J. Comp. Neurol.* 406, 402–414. doi: 10.1002/(sici)1096-9861(19990412)406:3<402::aid-cne8>3.3.co;2-6

Goel, A., and Buonomano, D. (2013). Chronic electrical stimulation homeostatically decreases spontaneous activity, but paradoxically increases evoked network activity. *J. Neurophysiol.* 109, 1824–1836. doi: 10.1152/jn.00612.2012

Grasso, R., Ivanenko, Y., Zago, M., Molinari, M., Scivoletto, G., Castellano, V., et al. (2004). Distributed plasticity of locomotor pattern generators in spinal cord injured patients. *Brain* 127, 1019–1034. doi: 10.1093/brain/awh115

Hains, B. C., Johnson, K. M., McAdoo, D. J., Eaton, M. J., and Hulsebosch, C. E. (2001). Engraftment of serotonergic precursors enhances locomotor function and attenuates chronic central pain behavior following spinal hemisection injury in the rat. *Exp. Neurol.* 171, 361–378. doi: 10.1006/exnr.2001.7751

Harkema, S. J. (2008). Plasticity of interneuronal networks of the functionally isolated human spinal cord. *Brain Res. Rev.* 57, 255–264. doi: 10.1016/j.brainresrev.2007.07.012

Harkema, S., Gerasimenko, Y., Hodes, J., Burdick, J., Angeli, C., Chen, Y., et al. (2011). Effect of epidural stimulation of the lumbosacral spinal cord on voluntary movement, standing and assisted stepping after motor complete paraplegia: a case study. *Lancet* 377, 1938–1947. doi: 10.1016/S0140-6736(11)60547-3

Harris-Warrick, R. M., and Cohen, A. H. (1985). Serotonin modulates the central pattern generator for locomotion in the isolated lamprey spinal cord. *J. Exp. Biol.* 116, 27–46.

Hashimoto, T., and Fukuda, N. (1991). Contribution of serotonin neurons to the functional recovery after spinal cord injury in rats. *Brain Res.* 539, 263–270. doi: 10.1016/0006-8993(91)91630-j

Hochman, S., Garraway, S., Machacek, D., and Shay, B. (2001). "5-HT receptors and the neuromodulatory control of spinal cord function," in *Motor Neurobiology of the Spinal Cord*, ed T. Cope (Boca Raton, Florida: CRC Press), 47–87.

Hoffman, N., and Parker, D. (2011). Interactive and individual effects of sensory potentiation and region-specific changes in excitability after spinal cord injury. *Neuroscience* 199, 563–576. doi: 10.1016/j.neuroscience.2011.09.021

Holderith, N., Lorincz, A., Katona, G., Rózsa, B., Kulik, A., Watanabe, M., et al. (2012). Release probability of hippocampal glutamatergic terminals scales with the size of the active zone. *Nat. Neurosci.* 15, 988–997. doi: 10.1038/nn.3137

Katz, P., and Edwards, D. (1999). "Metamodulation: the control and modulation of neuromodulation," in *Beyond Neurotransmission*, ed P. Katz (New York: Oxford University Press), 349–381.

Kemnitz, C., Strauss, T., Hosford, D., and Buchanan, J. (1995). Modulation of swimming in the lamprey, Petromyzon marinus, by serotonergic and dopaminergic drugs. *Neurosci. Lett.* 201, 115–118. doi: 10.1016/0304-3940(95)12147-1

Kong, X.-Y., Wienecke, J., Chen, M., Hultborn, H., and Zhang, M. (2011). The time course of serotonin 2A receptor expression after spinal transection of rats: an immunohistochemical study. *Neuroscience* 177, 114–126. doi: 10.1016/j.neuroscience.2010.12.062

Li, Y., and Bennett, D. J. (2003). Persistent sodium and calcium currents cause plateau potentials in motoneurons of chronic spinal rats. *J. Neurophysiol.* 90, 857–869. doi: 10.1152/jn.00236.2003

Li, Y., Harvey, P. J., Li, X., and Bennett, D. J. (2004). Spastic long-lasting reflexes of the chronic spinal rat studied in vitro. *J. Neurophysiol.* 91, 2236–2246. doi: 10.1152/jn.01010.2003

Lurie, D. I., and Selzer, M. E. (1991). Axonal regeneration in the adult lamprey spinal cord. *J. Comp. Neurol.* 306, 409–416. doi: 10.1002/cne.903060305

Martin, M. M. (2002). Changes in electrophysiological properties of lamprey spinal motoneurons during fictive swimming. *J. Neurophysiol.* 88, 2463–2476. doi: 10.1152/jn.00725.2001

Matsushima, T., and Grillner, S. (1992). Local serotonergic modulation of calcium-dependent potassium channels controls intersegmental coordination in the lamprey spinal cord. *J. Neurophysiol.* 67, 1683–1690.

McClellan, A. D. (1994). Time course of locomotor recovery and functional regeneration in spinal cord-transected lamprey: in vitro preparations. *J. Neurophysiol.* 72, 847–860.

Murray, K. C., Nakae, A., Stephens, M. J., Rank, M., D'Amico, J., Harvey, P. J., et al. (2010). Recovery of motoneuron and locomotor function after spinal cord injury depends on constitutive activity in 5-HT2C receptors. *Nat. Med.* 16, 694–700. doi: 10.1038/nm.2160

Nahar, J., Lett, K. M., and Schulz, D. J. (2012). Restoration of descending inputs fails to rescue activity following deafferentation of a motor network. *J. Neurophysiol.* 108, 871–881. doi: 10.1152/jn.00183.2012

Oliphint, P. A., Alieva, N., Foldes, A. E., Tytell, E. D., Lau, B. Y., Pariseau, J. S., et al. (2010). Regenerated synapses in lamprey spinal cord are sparse and small even after functional recovery from injury. *J. Comp. Neurol.* 18, 2854–2872. doi: 10.1002/cne.22368

Otoshi, C., Walwyn, W., Tillakaratne, N., Zhong, H., Roy, R., and Edgerton, V. (2009). Distribution and localization of 5-HT(1A) receptors in the rat lumbar spinal cord after transection and deafferentation. *J. Neurotrauma* 26, 575–584. doi: 10.1089/neu.2008.0640

Parker, D. (2003). Activity-dependent feedforward inhibition modulates synaptic transmission in a spinal locomotor network. *J. Neurosci.* 23, 11085–11093.

Parker, D. (2005). Pharmacological approaches to functional recovery after spinal injury. *Curr. Drug Targets CNS Neurol. Disord.* 4, 195–210. doi: 10.2174/1568007053544192

Parker, D. (2006). Complexities and uncertainties of neuronal network function. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 361, 81–99. doi: 10.1098/rstb.2005.1779

Parker, D. (2010). Neuronal network analyses: premises, promises and uncertainties. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 365, 2315–2328. doi: 10.1098/rstb.2010.0043

Parker, D., and Gilbey, T. (2007). Developmental differences in neuromodulation and synaptic properties in the lamprey spinal cord. *Neuroscience* 145, 142–152. doi: 10.1016/j.neuroscience.2006.11.050

Parker, D., and Srivastava, V. (2013). Dynamic systems approaches and levels of analysis in the nervous system. *Front. Physiol.* 4:15. doi: 10.3389/fphys.2013.00015

Pearson, K. G. (2001). Could enhanced reflex function contribute to improving locomotion after spinal cord repair? *J. Physiol.* 533, 75–81. doi: 10.1111/j.1469-7793.2001.0075b.x

Rossignol, S., and Frigon, A. (2011). Recovery of locomotion after spinal cord injury: some facts and mechanisms. *Ann. Rev. Neurosci.* 34, 413–440. doi: 10.1146/annurev-neuro-061010-113746

Rossignol, S., Giroux, N., Chau, C., Marcoux, J., Brustein, E., and Reader, T. (2001). Pharmacological aids to locomotor training after spinal injury in the cat. *J. Physiol.* 533, 65–74. doi: 10.1111/j.1469-7793.2001.0065b.x

Roy, R. R., Harkema, S. J., and Edgerton, V. R. (2012). Basic concepts of activity-based interventions for improved recovery of motor function after spinal cord injury. *Arch. Phys. Med. Rehabil.* 93, 1487–1497. doi: 10.1016/j.apmr.2012.04.034

Ruiz, Y., Pombal, M., and Megías, M. (2004). Development of GABA-immunoreactive cells in the spinal cord of the sea lamprey, P. marinus. *J. Comp. Neurol.* 470, 151–163. doi: 10.1002/cne.11032

Schmidt, B. J., and Jordan, L. M. (2000). The role of serotonin in reflex modulation and locomotor rhythm production in the mammalian spinal cord. *Brain Res. Bull.* 53, 689–710. doi: 10.1016/s0361-9230(00)00402-0

Shah, P. K., Garcia-Alias, G., Choe, J., Gad, P., Gerasimenko, Y., Tillakaratne, N., et al. (2013). Use of quadrupedal step training to re-engage spinal interneuronal networks and improve locomotor function after spinal cord injury. *Brain* 136, 3362–3377. doi: 10.1093/brain/awt265

Svensson, E., Kim, O., and Parker, D. (2013). Altered GABA and somatostatin modulation of proprioceptive feedback after spinal cord injury in lamprey. *Neuroscience* 235, 109–118. doi: 10.1016/j.neuroscience.2013.01.017

Tanaka, E. M., and Ferretti, P. (2009). Considering the evolution of regeneration in the central nervous system. *Nat. Rev. Neurosci.* 10, 713–723. doi: 10.1038/nrn2707

Tillakaratne, N. J. K., de Leon, R. D., Hoang, T. X., Roy, R. R., Edgerton, V. R., and Tobin, A. J. (2002). Use-Dependent modulation of inhibitory capacity in the feline lumbar spinal cord. *J. Neurosci.* 22, 3130–3143.

Vajn, K., Suler, D., Plunkett, J., and Oudega, M. (2014). Temporal profile of endogenous anatomical repair and functional recovery following spinal cord injury in adult Zebrafish. *PLoS One* 9:e105857. doi: 10.1371/journal.pone.0105857

van den Brand, R., Heutschi, J., Barraud, Q., DiGiovanna, J., Bartholdi, K., Huerlimann, M., et al. (2012). Restoring voluntary control of locomotion after paralyzing spinal cord injury. *Science* 336, 1182–1185. doi: 10.1126/science.1217416

Verma, P., Garcia-Alias, G., and Fawcett, J. W. (2008). Spinal cord repair: bridging the divide. *Neurorehabil. Neural Repair* 22, 429–437. doi: 10.1177/1545968307313500

Wang, H., and Jung, R. (2002). Variability analyses suggest that supraspino-spinal interactions provide dynamic stability in motor control. *Brain Res.* 930, 83–100. doi: 10.1016/s0006-8993(02)02232-1

Wikström, M., Hill, R., Hellgren, J., and Grillner, S. (1995). The action of 5-HT on calcium-dependent potassium channels and on the spinal locomotor network in

lamprey is mediated by 5-HT1a-like receptors. *Brain Res.* 678, 191–199. doi: 10.1016/0006-8993(95)00183-q

Wolpaw, J. R., and Tennissen, A. M. (2001). Activity-dependent spinal cord plasticity in health and disease. *Ann. Rev. Neurosci.* 24, 807–843. doi: 10.1146/annurev.neuro.24.1.807

Yin, H. S., Mackler, S. A., and Selzer, M. E. (1987). The axon reaction of lamprey spinal interneurons. *Brain Res.* 421, 48–56. doi: 10.1016/0006-8993(87)91273-x

Zucker, R. S., and Regehr, W. G. (2002). Short-term synaptic plasticity. *Ann. Rev. Physiol.* 64, 355–405. doi: 10.1146/annurev.physiol.64.092501.114547

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The role of the serotonergic system in locomotor recovery after spinal cord injury

***Mousumi Ghosh*[1,2]* and *Damien D. Pearse*[1,2,3,4]**

[1] *The Miami Project to Cure Paralysis, University of Miami Miller School of Medicine, Miami, FL, USA*
[2] *Department of Neurological Surgery, University of Miami Miller School of Medicine, Miami, FL, USA*
[3] *The Neuroscience Program, University of Miami Miller School of Medicine, Miami, FL, USA*
[4] *The Interdisciplinary Stem Cell Institute, University of Miami Miller School of Medicine, Miami, FL, USA*

Edited by:
Hans Hultborn, University of Copenhagen, Denmark

Reviewed by:
Deborah Baro, Georgia State University, USA
Michael A. Lane, University of Florida, USA
Hans Hultborn, University of Copenhagen, Denmark

***Correspondence:**
Mousumi Ghosh, The Miami Project to Cure Paralysis and Department of Neurological Surgery, University of Miami Miller School of Medicine, Lois Pope LIFE Center, P.O. Box 016960, Mail locator R-48, Miami, FL 33101, USA
e-mail: mghosh@med.miami.edu

Serotonin (5-HT), a monoamine neurotransmitter synthesized in various populations of brainstem neurons, plays an important role in modulating the activity of spinal networks involved in vertebrate locomotion. Following spinal cord injury (SCI) there is a disruption of descending serotonergic projections to spinal motor areas, which results in a subsequent depletion in 5-HT, the dysregulation of 5-HT transporters as well as the elevated expression, super-sensitivity and/or constitutive auto-activation of specific 5-HT receptors. These changes in the serotonergic system can produce varying degrees of locomotor dysfunction through to paralysis. To date, various approaches targeting the different components of the serotonergic system have been employed to restore limb coordination and improve locomotor function in experimental models of SCI. These strategies have included pharmacological modulation of serotonergic receptors, through the administration of specific 5-HT receptor agonists, or by elevating the 5-HT precursor 5-hydroxytryptophan, which produces a global activation of all classes of 5-HT receptors. Stimulation of these receptors leads to the activation of the locomotor central pattern generator (CPG) below the site of injury to facilitate or improve the quality and frequency of movements, particularly when used in concert with the activation of other monoaminergic systems or coupled with electrical stimulation. Another approach has been to employ cell therapeutics to replace the loss of descending serotonergic input to the CPG, either through transplanted fetal brainstem 5-HT neurons at the site of injury that can supply 5-HT to below the level of the lesion or by other cell types to provide a substrate at the injury site for encouraging serotonergic axon regrowth across the lesion to the caudal spinal cord for restoring locomotion.

Keywords: serotonin receptor agonists, serotonin, spinal cord, locomotion control, central pattern generators (CPG)

INTRODUCTION

Spinal cord injury (SCI) is a devastating condition affecting approximately 273,000 individuals in the US, with 12,000 new cases occurring annually (National Spinal Cord Injury Statistical Center, University of Alabama, https://www.nscisc.uab.edu). Damage to the spinal cord results in the impairment of specific functions controlled by the nerves located at, or below, the level of SCI. According to the presence or absence of motor function, human SCI can be classified as complete or incomplete. Following incomplete injury, a certain degree of movement and sensation below the level of injury may be retained depending upon the severity of the injury and the corresponding extent of axonal preservation. Complete SCI results in paralysis, due to the lack of sensory and motor function below the level of injury, though rarely is SCI anatomically complete in man. One of the major consequences resulting from trauma to the spinal cord is the disruption of the dynamic interactions among the spinal neuronal network, supraspinal pathways and peripheral sensory inputs, which results in an impairment of locomotor function (Kiehn, 2006; Rossignol et al., 2006). This dysfunction is permanent due to the subsequent loss of motor neuron excitability and the inability of the central nervous system (CNS) to mount a robust reparative response endogenously. Although the complete ablation of direct supraspinal projections to regions below the injury results in the cessation of the voluntary control of movements, some residual motor function may remain from the spontaneous reorganization and recovery of neuronal network excitability from plastic changes within any spared ascending and descending systems (Ghosh et al., 2009; Tansey, 2010; Martinez and Rossignol, 2011; Rossignol and Frigon, 2011; D'Amico et al., 2014; El Manira, 2014; Filli et al., 2014).

To repair the injured spinal cord and restore voluntary motor function various strategies have been employed. Among the different approaches used, those that have elicited the sprouting or re-growth of serotonergic fibers caudal to the site of SCI have often been associated with an ensuing improvement in locomotor

function (Bregman et al., 2002; Engesser-Cesar et al., 2007). The reactivation of the central pattern generator (CPG), an important spinal cord center for locomotor output, and stimulation of the locomotor neural network through (1) the exogenous application of 5-HT (Cazalets et al., 1992; Madriaga et al., 2004; Thompson et al., 2011) or its precursor, L-5-hydroxytryptophan (5-HTP; Hayashi et al., 2010; Meehan et al., 2012); (2) through the administration of agonists for specific serotonergic receptor subsets, either alone (Antri et al., 2002; Landry and Guertin, 2004) or in combination with the stimulation of the dopaminergic (DA) and the noradrenergic (NA) systems (Brustein and Rossignol, 1999; Musienko et al., 2011) or; (3) the transplantation of serotonergic embryonic neurons that can innervate these regions (Privat et al., 1989; Rajaofetra et al., 1989a; Gimenez y Ribotta et al., 1998), have provided convincing evidence in support of an essential and indispensable role for the serotonergic system in promoting the restoration of locomotor function after SCI. Though it has been shown that concurrent stimulation of all the monoaminergic systems, NA, DA and serotonergic (Jordan et al., 2008), is important in the generation of locomotion, the present review will focus specifically on the function of the different components of the serotonergic pathway in regulating motor function output with attention to how these components are altered after SCI and lead to locomotor dysfunction. In addition, the therapeutic modalities that have been employed to modulate the serotonergic system in the CNS, in an attempt to restore locomotor function after experimental SCI, are discussed.

5-HT AND ANATOMICAL LOCALIZATION OF ITS NEURAL PATHWAYS

5-HT is a monoamine neurotransmitter synthesized from tryptophan, an essential amino acid, by a subset of neurons referred to as serotonergic neurons that are present in the CNS as well as by enterochromaffin cells in the gastrointestinal tract (Li et al., 2014). The anatomical localization of 5-HT pathways in the CNS was initially delineated in the rat brain by Dahlstroem and Fuxe (1964), who demonstrated that serotonergic neurons were largely concentrated in the raphe nuclei of the brainstem. Almost all the 5-HT axons found within the mammalian spinal cord originate supraspinally from neurons located in the brainstem (Takeuchi et al., 1982), primarily from three main regions, the medullary raphe pallidus, raphe obscuris and the raphe magnus (Azmitia, 1999), as well as part of the reticular formation that encompasses the pyramidal tract at these levels. The 5-HT axon terminals originating from descending brainstem projections exist at all levels of the spinal cord (Rajaofetra et al., 1989b; Hornung, 2003) and are localized to the dorsal horn, ventral horn and intermediate area (Ballion et al., 2002). Serotonergic projections innervating the dorsal horn predominantly arise from the raphe magnus via the dorsolateral funiculus, which also has sparse projections in the ventral horn. The neurons in the raphe obscuris and pallidus project to the ventrolateral white matter as well as terminate onto motoneurons in the ventral horn and in the intermediate gray via the ventral and ventrolateral funiculi, respectively (Azmitia and Gannon, 1986). The axon collaterals from a single raphe neuron are able to innervate both the sensory and motor nuclei of the autonomic system at different spinal levels, including both cervical and lumbar regions of the spinal cord (Martin et al., 1981).

Previously, Rajaofetra et al. (1992a) demonstrated in adult baboons that serotonergic innervation of the Onuf's nucleus, located in the ventrolateral part of the sacral spinal cord, was of both supraspinal and intraspinal origin. Supraspinal innervation was found to exist throughout the whole nucleus, with a predominance in the dorsal half, while the intraspinal innervation was primarily associated with the ventral half of the nucleus. Subsequently, Branchereau et al. (2002) reported in organotypic spinal cord cultures, which lacked descending serotonergic input, the expression of 5-HT in intraspinal neurons; these 5-HT intraspinal neurons were able to compensate for the lack of the descending supraspinal 5-HT fibers and contribute to the development of spontaneous locomotor activity. The 5-HT expressed by these intraspinal neurons was dependent upon the absence of 5-HT fibers as 5-HT from the descending input repressed expression of 5-HT from these intraspinal neurons. In the human spinal cord, Perrin et al. (2011) have mapped 5-HT profiles in the thoracic and lumbar segments, finding a similar neuroanatomical localization to that of rodents and non-human primates where serotonergic processes were identified primarily within the ventral horn surrounding motoneurons as well as also in the intermediolateral region and in the superficial part of the dorsal horn.

FUNCTION OF THE SEROTONERGIC PATHWAY IN LOCOMOTION

Multiple descending tracts from the brainstem function in the initiation and regulation of locomotion, including the glutamatergic, NA, DA and 5-HT pathways. These functions are mediated through the action of various neurotransmitters such as glutamate, NA, 5-HT and DA, which induce spinal motor activity, stimulate rhythmic activity and control segmental reflexes (Humphreys and Whelan, 2012; Beliez et al., 2014; Sławińska et al., 2014a). The role of 5-HT in locomotion remains, however, only partially understood. Experimental work from a number of research laboratories have provided convincing evidence that 5-HT regulates the rhythm and coordination of movements through the CPG. The CPG constitutes a major anatomical component of locomotion comprised of neurons distributed within a neural network in the thoraco-lumbar spinal cord that drives motoneuron output to generate simple rhythmic patterns, such as locomotion (Grillner and Walleń, 1985; Kiehn and Kullander, 2004). 5-HT has been recognized as a potent neuromodulator of CPG activity (Feraboli-Lohnherr et al., 1997). The CPG in the lumbar spinal cord is regulated both by supraspinal descending inputs that originate in the raphe nucleus and terminate in the intermediate gray and the ventral horn (Carlsson et al., 1963; Ballion et al., 2002) as well as by sensory afferents (Rossignol et al., 1988).

The neuronal network of the CPG, in response to monoaminergic input from the brainstem, contributes to the regulation of postural muscle tone and locomotion by determining which components of the specific locomotor program are necessary at a required or specific moment with respect to velocity, magnitude

and duration (Takakusaki et al., 2004). Under normal conditions, the different motor programs existing in the brainstem remain in a state of inhibition during rest (Grillner, 2006; Hikosaka, 2007). Fornal et al. initially suggested that under these conditions serotonergic neurons are controlled by tonic feedback inhibition (Fornal et al., 1994). When locomotion is initiated, signals activating the locomotor network in the mesencephalic locomotor region (MLR) and the diencephalic locomotor region (DLR) of the midbrain converge on reticulospinal neurons in the brainstem, which determine the extent and duration of locomotor activity that is necessary to be generated by the CPG. Therefore, the MLR, when subjected to electrical or chemical stimulation, triggers bouts of locomotion and elicits movement by activating the reticulospinal pathways (Shik et al., 1966; Garcia-Rill et al., 1985; Sholomenko and Steeves, 1987; Steeves et al., 1987). The initial synaptic targets of the MLR are neurons in the medial pontomedullary reticular formation (MdRF), after which their axons descend as the reticulospinal tract within the ventrolateral funiculus of the spinal cord to synapse on CPG neurons within the cervical or lumbar segments (Steeves and Jordan, 1984; Garcia-Rill et al., 1985; Noga et al., 1991).

As first discussed by Jacobs and Fornal (1993), the primary function of 5-HT neurons in the brainstem is to facilitate motor output during periods of tonic motor activity, such as postural shifts, or to control repetitive motor behaviors that are mediated by the spinal cord CPGs, such as locomotor speed. However, when the spinal cord is injured, there can be significant disruption or complete severing of the serotonergic projections, as well as other descending systems, to the CPG and loss of locomotor output. When exogenous 5-HT or selective 5-HT receptor agonists are supplied systemically or intraspinally after SCI, in combination with sufficient excitation by epidural electrical stimulation (EES) or glutamate, these locomotor behaviors can be re-elicited (Schmidt and Jordan, 2000; Antri et al., 2002; Landry et al., 2006; Courtine et al., 2009; Fouad et al., 2010).

5-HT participates directly in modulating motor function output through its binding to specific 5-HT receptors present upon the membrane of motoneurons. Depending on the specific receptor subtypes that are activated, either depolarization or hyperpolarization of the motoneurons occurs—thus 5-HT acts a control point in the regulation of spinal motoneuron excitability, leading to an amplification of synaptic excitation or inhibition (Perrier et al., 2013). Early studies employing selective 5-HT receptor agonists and antagonists provided evidence that the activation of 5-HT_{1A} receptors could mediate inhibitory responses, whereas excitation was produced by the activation of 5-HT_{2A} receptors (Bayliss et al., 1995). Excitatory neurotransmission by 5-HT occurs through the modulation of various ion channels which leads to a sustained depolarization due to the presence of persistent inward currents that are mediated by voltage sensitive Ca^{2+} and Na^{+} conductance to cause an amplification of synaptic input. Following SCI, there is a reduced input of brain stem-derived 5-HT which results in an altered membrane potential of the motoneurons causing an acute suppression of motoneuron excitability. This is attributed to various factors such as motoneuron hyperpolarization coupled to an acute disappearance of voltage-activated sodium and calcium persistent inward currents which prevents activation of action potentials. This inhibits motoneuron firing and increases presynaptic inhibition thereby rendering the motoneuron and spinal neuronal circuitry unexcitable acutely after the injury. 5-HT can also modulate motoneuron excitability indirectly through its effects on spinal interneurons where 5-HT can alter the action potential properties of these interneurons, especially following SCI. It has been observed in mouse lumbar V2a spinal interneurons (Husch et al., 2012) that an enhanced 5-HT super sensitivity occurs after SCI due to the elevated density of 5-HT_{2C} receptors on the cell membrane without promoting any significant changes in their level of excitability. 5-HT thus participates in regulating the firing frequency and excitability of spinal motor neurons, which corresponds to its ability to control the speed and amplitude of locomotion as well as alter the membrane properties of spinal interneurons (Harris-Warrick and Cohen, 1985; Zhang and Grillner, 2000; D'Amico et al., 2014; Wienecke et al., 2014) to promote increased motoneuron excitability. SCI-induced losses of 5-HT, however, can lead not only to an absence of motoneuron excitability and locomotor output but residual 5-HT or activity of its receptors may also produce aberrant motoneuron excitability that is involved in triggering spasticity and/or impaired motor output that can hinder normal locomotion (Perrier et al., 2013). This is an outcome observed following chronic SCI, where persistent inward currents are enhanced either due to compensatory over-expression of spontaneously active 5-HT_2 receptors (Murray et al., 2010) or as a result from a depolarized chloride reversal potential (Boulenguez et al., 2010).

5-HT RECEPTOR ACTIVATION AND LOCOMOTION

The different members of the 5-HT family of receptors are located within distinct areas of the central and the peripheral nervous systems, as well as in non-neuronal tissues, and are involved in a diversity of functions. The 5-HT receptor family represents one of the most complex families of neurotransmitter receptors that have been characterized to date. Studies conducted by a number of research groups have provided convincing experimental evidence that the descending serotonergic system modulates spinal reflexes and motor function/hind limb coordination through the activation of specific 5-HT receptors, which in turn cause an increase in motoneuron and interneuron excitability and the generation of CPG-mediated locomotor output (Schmidt and Jordan, 2000; Hochman et al., 2001; Pflieger et al., 2002).

A number of 5-HT receptor subtypes are expressed in high density on the membranes of motoneurons, including 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C} and 5-HT_{5A} (Perrier et al., 2013) as well as some spliced variants, 5-HT_3, 5-HT_4, 5-HT_6, 5-HT_7 and RNA edited isoforms, such as 5-HT_{2C} (Werry et al., 2008). These receptors mostly exist as homodimers, although they may also undergo heterodimerization (Renner et al., 2012). Dimerization occurs during their biosynthesis within the endoplasmic reticulum, which is important for their subsequent transport and expression in the plasma membrane as well as maximizes downstream coupling with the G-protein subunits (Herrick-Davis, 2013) that leads to motoneuron excitability. Delineating the signaling mechanisms regulated by 5-HT has been

difficult due to both the existence of multiple 5-HT receptor isoforms that are activated in the presence of 5-HT as well as the unavailability of highly selective pharmacological modulators that can act on single receptor subtypes. In the human brain, 13 5-HT GPCRs and one serotonin-gated ion channel receptor, 5-HT_3, have been identified (Millan et al., 2008; Lambe et al., 2011). Though 5-HT receptors have been classified under seven known subfamilies, 5-HT_1 through 5-HT_7, post-genomic modifications, such as alternative mRNA splicing or mRNA editing, have resulted in the identification of at least 30 distinct 5-HT receptor subtypes (Raymond et al., 2001). It has been demonstrated that the combined activation of more than one 5-HT receptor subtype is required for the generation of locomotor output, with signaling originating primarily from 5-HT_{1A}, 5-$HT_{2A/C}$ and 5-HT_7 receptor subtypes (Jordan et al., 2008). Work to date in characterizing 5-HT receptor activation involvement in real or fictive locomotor output have focused largely on motoneurons, as they are the direct effecters of locomotor activity, in addition to the spinal interneurons that comprise the CPG.

With the availability of agonists and antagonists that exhibit greater selectivity for specific 5-HT receptor subtypes, studies have begun to identify which 5-HT receptor subtypes mediate specific locomotor behaviors. Employing receptor-specific agonists and antagonists, which have varying binding affinities towards each of the 5-HT receptor subsets, researchers have identified 5-HT_{1A}, 5-$HT_{2A/2C}$ (Courtine et al., 2009), 5-HT_3 (Guertin and Steuer, 2005), and 5-HT_7 receptors (Liu et al., 2009) as important players in the regulation of the spinal locomotor network and which are able to generate locomotion in experimental models of SCI when employed in conjunction with the activation of other monoaminergic systems (Kim et al., 2001a; Antri et al., 2003; Fuller et al., 2005; Madriaga et al., 2004; Guertin and Steuer, 2005; Ung et al., 2008; Courtine et al., 2009; Liu et al., 2009; Musienko et al., 2011). Studies employing 5-HT receptor selective agonists and antagonists in rats following spinal transection have identified that 5-HT_2 receptors are predominantly responsible for mediating the depolarizing effects resulting from decreased potassium conductance in response to a 5-HT stimulus (Jacobs and Fornal, 1993; Harvey et al., 2006; Li et al., 2007; Perrier et al., 2013; Gackière and Vinay, 2014). In experiments carried out by Ung et al. (2008), in which behavioral and kinematic analyses were performed at 1 week after complete spinal cord transection in mice, they identified the involvement of specific 5-HT_2 receptor subtypes in locomotor-like movements through the use of selective antagonists. The non-selective 5-HT_2 receptor agonist quipazine was able to induce locomotor-like movements in the presence of the selective 5-HT_2 antagonists SB204741 and SB242084, which are inhibitory towards 5-HT_{2B} or 5-HT_{2C}, respectively. In contrast, quipazine failed to induce locomotor-like movements in animals that had been pretreated with MDL-100,907, a selective 5-HT_{2A} antagonist. This work provided evidence that 5-HT_{2A} receptors were involved in spinal locomotor network activation and the generation of locomotor-like movements induced by quipazine in transected animals. Antri et al. (2003) showed that the combined, daily stimulation of 5-HT_2 and 5-HT_{1A} receptors, using quipazine and 8-OHDPAT, respectively, after thoracic spinal cord transection in rats was more potent in restoring locomotion then when the 5-HT_2 agonist was employed alone, suggesting a cumulative effect of activating the two receptor subtypes in facilitating locomotion. 5-HT_{1A} receptors are localized throughout the CNS with high expression in limbic regions and in the dorsal raphe nucleus, while in the spinal cord, 5-HT_{1A} receptors are identified primarily within the ventral horn surrounding motoneurons, although 5-HT_{1A} receptors are more densely expressed at the lumbar level of the spinal cord. 5HT_{1A} receptors have been shown to produce hyperpolarization preferentially in a small subset of neurons, such as CA1 hippocampal neurons (Bobker and Williams, 1990; Levkovitz and Segal, 1997) and interneurons (Segal, 1990a,b). Therefore depending upon the specific receptor that 5-HT binds to, it can elicit either depolarizing or hyperpolarizing effects.

CAUDAL SEROTONERGIC AXON INNERVATION AND 5-HT LEVELS AFTER SCI AND THEIR EFFECTS ON RECOVERY

With 5-HT release within the spinal cord ventral horn playing a major role in mediating locomotor function, it is not surprising that following SCI, when there is severing of supraspinal serotonergic projections and depletion of 5-HT (Carlsson et al., 1963), that loss of 5-HT is one of the major limiting factors that prevents the recovery of motor function (Hashimoto and Fukuda, 1991). 5-HT loss after SCI is characterized by significant reductions or an absence of important enzymes such as tryptophan hydroxylase (TPH), which catalyzes the conversion of tryptophan to 5-hydroxytryptophan (5-HTP), that are necessary to generate 5-HT (Clineschmidt et al., 1971). Loss of 5-HT prevents the activation of the spinal locomotor CPG, interfering with the ability to evoke normal locomotion. When 5-HT levels are restored, locomotor function after SCI has been shown to be improved (Pearlstein et al., 2005).

Previous work by Hentall et al. (2006) characterized the spatial and temporal patterns of 5-HT release in the rodent lumbar spinal cord following electrical stimulation of the raphe magnus in SCI rats subjected to acute spinal cord transection at T6–T7. Their findings indicated that the action of monoamines in the spinal cord involves a combination of both synaptic neurotransmission as well as non-synaptic diffusion, similar to that previously observed (Bach-y-Rita, 1999). Noga et al. (2004) employed fast cyclic voltammetry, capable of measuring monoamines such as 5-HT at high spatial resolution in comparison to microdialysis-HPLC, to map the basal and steady-state extracellular distribution of monoamines released from tonically active, descending nerve terminals in the lumbar spinal cord of rats when at rest and after spinal cord transection. Their results demonstrated that at rest there was a greater concentration of 5-HT localized in specific regions of the dorsal and ventral horn of the lumbar cord as well as in the lateral region of the intermediate zone which varied within the different segments of the lumbar cord. Following thoracic spinal cord transection, a transient injury evoked increase in monoamine levels was noted briefly acutely within the distal stump of the transected cord. Maximal levels were measured in the superficial dorsal horn of the lumbar cord during this transient period after which there was a steady longer lasting decrease in monoamine levels (Noga et al., 2004).

Recently, Gerin et al. (2010) examined temporal alterations in the level of endogenous 5-HT release in the ventral horn of rats that were subjected to a sub-hemi-section of the spinal cord at the T9 vertebral level. Release of 5-HT was measured using a microdialysis probe and HPLC analysis, with results showing variations in the levels after SCI over a period of a month during the post-injury recovery period as well when the injured animals were subjected to treadmill exercise. It was found that a significant decrease in 5-HT levels occurred after SCI, which was <50% of that observed in non-lesioned animals, indicative of serotonergic denervation. With time post-SCI, 5-HT levels were found to rise, increasing by 202% at 18 days post injury compared to the levels observed at 8 days post-lesion, which correlated with the timing of motor function recovery. When treadmill exercise was performed, a ∼10% decrease in 5-HT levels was observed compared to rest at 18 and 34 days post-SCI.

ALTERATIONS IN 5-HT RECEPTOR ACTIVITY AFTER SCI

Previous studies using a complete spinal transection in the cat have shown that SCI leads to an immediate and dramatic reduction in motoneuron excitability (Hounsgaard et al., 1988; Miller et al., 1996). Spinal cord contusion in rodents, an incomplete SCI paradigm that is more clinically-relevant, leads to the preservation of a small number of serotonergic fibers that can undergo sprouting with time following an injury. The degree of locomotor function restoration observed after SCI has been reported to correlate to the density of sprouting of residual, uninjured serotonergic axons (Holmes et al., 2005; Nardone et al., 2014). The use of 5-HT receptor agonists in animals after an acute spinal transection has been shown to produce restoration of motoneuron excitability (Harvey et al., 2006; Li et al., 2007; Musienko et al., 2011; Sławińska et al., 2014a). Work by various researchers have demonstrated that locomotor-activated spinal neurons, which constitute the CPG network, are innervated by serotonergic fibers (Johnson et al., 2002; Perrin et al., 2011; Sławińska et al., 2014b) and that 5-HT is released within these sites during locomotor activity (Gerin et al., 2008). Together, these results have shown that serotonergic innervation and the availability of 5-HT in the lumbar cord (Antri et al., 2002) are critical for the restoration of hind limb motor function following SCI.

The loss of 5-HT can be overcome through the direct activation of 5-HT receptors, an approach which has been convincingly associated with the induction of locomotor output following SCI (Brustein and Rossignol, 1999; Feraboli-Lohnherr et al., 1999; Kim et al., 2001b). With the reduction or loss of 5-HT input caudal to the injury in rodent models of SCI, it has been demonstrated that there is a compensatory over-expression of 5-HT receptors (Fuller et al., 2005; Navarrett et al., 2012; Ren et al., 2013). In the lamprey, a vertebrate that exhibits locomotor recovery after complete SCI, a transient over-expression of the 5-HT_{1A} receptor transcript is observed for several weeks after SCI (Cornide-Petronio et al., 2014). In rats, Kong et al. (2010) reported a dramatic increase in 5-HT_{2A} receptor immunoreactivity in motoneurons caudal to the site of spinal cord transection, beginning 1 day after injury and persisting to maximal expression by 28 days which paralleled a concurrent reduction in the levels of 5-HT and the 5-HT transporter (Kong et al., 2011; Husch et al., 2012). Work by Otoshi et al. (2009) examined the influence of descending serotonergic input and sensory feedback on spinal 5-HT receptor expression using two paradigms. The first was a complete thoracic (T7–T8) spinal cord transection in adult rats, to eliminate supraspinal 5-HT input, while the second employed animals subjected to spinal cord isolation, so as to completely ablate both descending supraspinal as well as peripheral sensory input, completely eliminating any neuromuscular activity. They found that there was an enhanced expression of the 5-HT_{1A} receptor after transection that was dependent upon the presence of sensory input.

Murray et al. (2010) have provided findings that may explain how motoneurons are able to mediate some residual hind limb function in rodent models of complete spinal cord transection (S2, sacral spinal level) or with staggered hemisection, both of which remove the descending supraspinal projections caudal to the level of SCI. It appears that this residual function can arise as a result of alterations in 5-HT receptor expression and their constitutive activation as a compensatory mechanism. A SCI-induced alteration in the levels of the 5-HT_{2C} receptor has been correlated to a change the post-transcriptional editing of its mRNA that results in both elevated production and constitutive activation. SCI-mediated constitutive activation of 5-HT_{2C} receptors is able to restore large persistent calcium currents in motoneurons, which in turn is able to induce sustained muscle contractions and the restoration of motor function. Therefore, despite the non-availability of a serotonergic stimulus following SCI, which results in loss of motoneuron excitability acutely, a compensatory upregulation of spontaneously active 5-HT_2 receptors induced at chronic stages after SCI appears to be an adaptive mechanism which could be responsible for residual locomotor function.

The use of EES, applied onto the dorsal surface of the lumbar spinal cord or given intraspinally, has been used in decerebrated and acute spinal cord transected cats (Iwahara et al., 1992; Gerasimenko et al., 2007) as well as in rats (Ichiyama et al., 2005) to activate the spinal locomotor circuits so as to induce hind limb movements and locomotion (Gerasimenko et al., 2007; Courtine et al., 2009). The use of EES in SCI patients, to promote locomotor function, was first employed by Dimitrijevic et al. (1998), later optimized in paralyzed individuals in work by Shapkova et al. (Shapkova and Schomburg, 2001; Shapkova and Mushkin, 2002; Shapkova, 2004) and is currently being evaluated in clinics in the US (Harkema et al., 2011). Recently, Angeli et al. (2014) successfully used epidural stimulation to activate spinal circuitry in completely paralyzed American Spinal Injury Association Impairment Scale (AIS) A patients, opening a new avenue for therapeutic interventions to promote functional recovery after SCI. Through the use of immunohistochemistry, Noga et al. (2009) showed the co-localization of serotonergic axons with specific 5-HT receptor subtypes 5-HT_{1A}, 5-HT_{2A} and 5-HT_7, on c-Fos immunoreactive neurons within laminae VII and VIII of the thoraco-lumbar spinal cord segments following EES in pancuronium-paralyzed, decerebrate cats. The presence of these 5-HT receptor subtypes on these spinal neurons in T13–L7 spinal cord indicates that they likely play a role in 5-HT's modulation of locomotion.

To assess the roles of the specific 5-HT receptor subtypes after SCI that have been identified to be involved in locomotor

output function; 5-HT_{1A} (Courtine et al., 2009), 5-$HT_{2A/2C}$ (Courtine et al., 2009), 5-HT_3 (Guertin and Steuer, 2005) and 5-HT_7 (Liu et al., 2009), Musienko et al. (2011) conducted experiments using pharmacological manipulations with combinations of receptor specific agonists and/or antagonists. These studies were performed in adult rats subjected to a complete spinal cord transection at the mid-thoracic vertebral level (T7) and the effects of receptor manipulations on observed functional recordings were analyzed during locomotion enabled by EES. From these studies, specific functional roles for each of the 5-HT receptors in modulating motor function output were identified. It was shown that when transected animals were pretreated with the 5-HT_7 specific antagonist SB269970, prior to the administration of 8-OHDPAT, a dual agonist for 5-$HT_{1A/7}$, and used in conjunction with EES, a marked improvement in the animal's stepping patterns and inter-limb coordination was observed along with a reduction in kinematic variability and an increase in weight-bearing capacity. These improvements were not seen when EES was used alone, demonstrating an essential role of 5-HT_{1A} receptors in facilitating locomotion, as also reported by other groups (Antri et al., 2003; Courtine et al., 2009). Similarly, they observed that activation of 5-$HT_{2A/C}$ receptors with the general 5-HT_2 receptor agonist quipazine, in the presence of EES, produced facilitation of extension of the distal joints along with an improvement in weight-bearing capacity that was not observed with EES alone. When the 5-HT_3 receptor agonist SR57227A was used with EES, the animals exhibited both an enhancement in weight-bearing as well as a reduction in gait timing variability and paw dragging. The combined activation of 5-HT_7 and 5-HT_{1A} receptors with EES also elicited an improvement in stepping pattern and produced a potent facilitation in joint flexion, while combined activation of 5-HT_7 and 5-$HT_{2A/C}$ receptors improved inter-limb coordination, reduced the duration of dragging and substantially increased weight bearing capacity. Finally, the simultaneous activation of all four receptor subtypes, 5-HT_{1A}, 5-$HT_{2A/C}$ and 5-HT_7, with EES led to a significant increase in the EMG activity of the proximal extensor and flexor muscles compared with the stepping pattern elicited by EES alone. These results suggest that it may be necessary to target multiple 5-HT receptors in combination to obtain maximal efficacy in overall locomotor output in the presence of EES (Musienko et al., 2011), although it remains to be tested whether altering the sequential stimulation of different receptor subtypes will be needed to obtain optimal locomotor output.

In addition to pharmacological modification of specific 5-HT receptor subtypes, more recent work has focused on the use of molecular tools to examine receptor function using knockout approaches in transgenic animals. In mice lacking the 5-HT_{1A} receptor, work by Scullion et al. (2013) confirmed the important role of 5-HT_{1A} receptors in the generation of locomotion. 5-HT_{1A} knockout mice showed higher movement thresholds and smaller motor maps compared with wild-type animals. Increased use of transgenic animals, particularly conditional knockouts, to study the role of specific receptor subtypes in the spinal cord should be undertaken to provide a more specific approach than pharmacological agents for dissecting the function different 5-HT receptors play in the generation of motor output, both under normal locomotion and in the context of locomotor recovery following SCI.

DIFFERENTIAL ACTIVITY OF 5-HT RECEPTOR SUBTYPES IN INDUCING LOCOMOTION AFTER SCI

Although 5-HT acts on its multiple receptor subtypes, it can trigger different signaling pathways that may have excitatory (Cazalets et al., 1992; MacLean et al., 1998) or inhibitory (Schmidt and Jordan, 2000) effects on motor output. Depending on which 5-HT receptors are expressed and activated as well as their cellular and/or specific regional localization within the injured spinal cord, the serotonergic system can produce differential effects on locomotion (Landry et al., 2006; Courtine et al., 2009). Such a variation in response has been noted in phrenic motoneurons where 5-HT displays a differential effect elicited by the 5-HT_{1B} and 5-HT_{2A} receptor subtypes, where by one acts in a stimulatory capacity and the other is inhibitory (Holtmanj et al., 1986; Lalley, 1986). Work by Di Pasquale et al. (1997) showed that while 5-HT_{2A} activation mediated neuronal depolarization of phrenic motoneurons, pharmacological activation of the 5-HT_{1B} receptor resulted in the opposite effect, with inhibition of phrenic activity. This inhibition could be prevented by pre-treatment with 5-HT_{1B} receptor specific antagonists, indicating that opposite effects could be evoked in response to the activation of these two receptor subtypes. Thus 5-HT is able to regulate phrenic motoneuron firing via activation of spinal postsynaptic 5-HT_{2A} and presynaptic 5-HT_{1B} receptors, which in turn function to protect the diaphragm from overstimulation during a non-respiratory action such as vomiting or defecation. Similar differential effects of 5-HT elicited by different 5-HT receptor subtypes have also been demonstrated in spinalized rats, where monosynaptic transmission is inhibited or stimulated with the activation of 5-HT_{1A} and 5-$HT_{2A/2C}$ receptors, respectively (Hasegawa and Ono, 1996). Using specific agonists for 5-HT_1 and 5-HT_2 receptor subtypes, differential effects on hind limb locomotor activity in adult mice after complete thoracic spinal cord transection were reported (Landry and Guertin, 2004). While 5-$HT_{2A/2C}$ receptor agonists facilitated locomotion, stimulating 5-$HT_{2B/2C}$ or 5-HT_{1B} receptors not only failed to induce locomotor-like movements, but also abated the hind limb movements induced by the non-specific 5-HT receptor agonist quipazine, when combined with L-DOPA. The involvement of 5-HT_2 receptors in mediating excitation has also been shown in the completely transected cat spinal cord where restoration of extensor reflex excitability could be achieved only with 5-$HT_{2A/2C}$ specific agonists in contrast to 5-HT_{1A} and 5-$HT_{1B/D}$ receptors, which were determined to be ineffective (Miller et al., 1996).

SERTs AND LOCOMOTION AFTER SCI

Neurotransmission between two or more cell types occurs primarily through volume or synaptic transmission where, with the former, the point of release of the neurotransmitter might be at a distant location from that of the target cells. Volume transmission is associated with nonsynaptic interneuronal

communication involving a widespread activation of extrasynaptic as well as intrasynaptic receptors (Agnati et al., 1986). This produces a longer duration in the transmission of the signal and requires the diffusion of the neurotransmitter. In comparison, synaptic transmission occurs within a relatively constrained region of the synaptic cleft, between the pre- and the post-synaptic neurons. The duration of 5-HT signaling is regulated by the function of two types of transporters. The vesicular monoamine transporter (VMAT) which pumps 5-HT from the cytoplasm into synaptic vesicles and thereby controls the release of 5-HT from the presynaptic neurons. The other is the serotonin re-uptake transporter (SERT) which exists in the presynaptic plasma membrane of the serotonergic neurons and re-absorbs extracellular 5-HT back into the cytoplasm (Iversen, 1971; Blakely et al., 1991; Rudnick and Clark, 1993; Torres et al., 2003; Murphy et al., 2004) resulting in termination of 5-HT neurotransmission and preventing extrasynaptic transmission (Blakely et al., 1991; Schloss et al., 1992; Lesch et al., 1993). The regulation of serotonergic signaling by SERTs plays a key role in the regulation and the functioning of the serotonergic system in locomotion (Hayashi et al., 2010; Husch et al., 2012).

Various allosteric modulators of SERTs are currently gaining significant clinical importance as a way to enhance the functional capacity of these transport proteins when concentrations of 5-HT are rate limiting, such as observed following SCI. Work conducted by Hayashi et al. (2010) has suggested that even if 5-HT receptors are up-regulated following spinal cord transection and are thus available for the generation of locomotion when activated by specific agonists, the same approach may not be as effective in improving locomotor function in incomplete SCI models, such as a spinal cord contusion. Though spared supraspinal serotonergic projections are retained after incomplete SCI, there appears to be an absence of 5-HT receptor activation, despite an enhanced upregulation of 5-HT_{2C} receptors caudal to the injury. It was shown in this study that the administration of selective 5-HT_{2C} or 5-HT_{1A} receptor agonists, either alone or in combination after contusive SCI, was unable to produce receptor activation as evidenced from a lack of improvement in hind limb motor function. An improvement in motor function was only observed when there was broad receptor activation produced by the administration of the 5-HT precursor, 5-hydroxytryptophan (L-5-HTP), suggesting that an elevation in the levels of serotonergic receptors alone may not be sufficient to promote an improvement in motor function in models of incomplete injury. Even though there are spared serotonergic axons that are retained after contusive SCI, it has been suggested that they may have impaired function due to a dramatic reduction in the levels of SERTs, resulting in a dysregulation of 5-HT reuptake (Husch et al., 2012). Therefore prominent loss of SERT may be the causative reason for a reduced ability of the 5-HT agonists to improve locomotor outcome after an incomplete injury, indicating SERT as a critical limiting factor that is necessary to overcome locomotor impairment following SCI. Work to transgenetically overexpress SERTs in animals receiving SCI and the administration of selective 5-HT_1 and 5-HT_2 agonists would provide strong evidence for a role of SERTs in both SCI-mediated locomotor dysfunction and as a therapeutic modality for facilitating locomotor output.

TRANSPLANTATION PARADIGMS INVOLVING THE DELIVERY OF 5-HT EXPRESSING CELLS TO IMPROVE LOCOMOTOR OUTCOME AFTER SCI

Although a number of pharmacological approaches have been employed to activate the CPG to induce locomotion in models of SCI with varying degrees of success as described above, an alternative has been to introduce cells into the injured spinal cord that are capable of producing 5-HT. These exogenous 5-HT expressing cells can be used to replace the loss of monoaminergic supraspinal afferents and serve as a relay to restore serotonergic projections caudal to the level of the injury (Bregman, 1994). One such cell type that has been employed for this purpose is embryonic brainstem neurons or raphe neurons, which are able to extend serotonergic axons into adjacent host spinal tissue to deliver 5-HT following transplantation at, or below, the level of the injury (Privat et al., 1989; Rajaofetra et al., 1989a, 1992b; Gimenez y Ribotta et al., 1998; Ribotta et al., 2000). The intraspinal transplantation of these cells has been shown to reverse SCI-induced changes in 5-HT receptor densities and to restore hind limb weight support and locomotor activity on a treadmill, without the need for additional pharmacological interventions (Gimenez y Ribotta et al., 1996). Studies carried out by Feraboli-Lohnherr et al. (1997), employing the transplantation of embryonic brainstem neurons within the transected thoracic cord of chronically injured rats, showed that these neurons were able to provide serotonergic innervation to the lumbar cord. The projections from the transplanted brainstem neurons successfully activated the CPG to facilitate locomotor activity and this effect was further improved when a 5-HT re-uptake inhibitor was introduced. Majczyński et al. (2005) performed grafting of embryonic raphe nuclei cells into the spinal cord at T12, below the level of a complete spinal cord transection at T9 in adult rats at 1 month post injury and showed that hind limb locomotor function could be restored. The transected animals receiving the cell grafts, when placed on a treadmill, were able to walk with regular, alternating hind limb movements and exhibited plantar contact with the ground during the stance phase as well as ankle dorsiflexion during the swing phase of each step cycle. These behaviors were not observed in the injured control animals that did not receive the grafts and reflected the establishment of new serotonergic innervation to the caudal cord by the grafted neurons. The observed improvement in locomotor activity with embryonic raphe nuclei cell grafts could be reduced in the presence of the 5-HT_2 antagonist cyproheptadine, and the loss of function regained with the 5-HT_2 agonist quipazine, suggesting a 5-HT_2 receptor mediated effect.

A similar improvement in locomotor performance was observed when embryonic raphe cells were transplanted into the spinal cord of chronically injured rats that had received a transection SCI at either T9 or T11 (Gimenez y Ribotta et al., 1996). The degree of locomotor recovery correlated to the level at which the SCI or the transplant were made, with a better outcome observed when these were performed at the T11 level, rather than

T9, due to the closer proximity to the lumbar CPG and a greater degree of serotonergic axon reinnervation of the lumbar cord. Later work showed that with this approach, greater efficacy for restoring locomotor activity could be obtained when embryonic serotonergic neuron transplantation after SCI was performed even closer to the CPG, at the level of lumbo-sacral spinal cord (Orsal et al., 2002). In addition to using brainstem neurons, Eaton et al. (2008) used the transplantation of hNT2.19 cells, a human neuronal cell line differentiated to serotonergic neurons capable of secreting 5-HT, into the severely contused spinal cord of rats 1 week after SCI to restore serotonergic neurotransmission. When employed with the combination of environmental enrichment, a significant improvement in locomotor function was observed, including reductions in foot exo-rotation and footfall errors on a gridwalk test. Recently, Sławińska et al. (2013) intraspinally grafted E14 fetal neurons obtained from the medulla, containing the B1, B2 and B3 serotonergic regions, which constitutes all the likely sources of descending 5-HT tracts from the caudal brainstem, after SCI. These fetal neurons were grafted at the T10/11 vertebra level of adult rats with a complete spinal cord transection at the T9/10 level and were shown to successfully restore coordinated plantar stepping and improve intra- and interlimb coordination. Using pharmacological antagonists, it was further demonstrated that the transplants mediated their effects through the activation of 5-HT_7 and 5-HT_2 receptors localized to different populations of spinal neurons. Furthermore, even when these neurons were transplanted at 1 month post-SCI there was a restoration of coordinated hind limb function evoked in response to tail stimulation.

Studies employing cellular therapies to replace lost 5-HT projections to regions below the level of the lesion, particularly to the CPG, provide a promising means for restoring locomotion after SCI. At the same time, this approach may be able to overcome some of the limitations that might be anticipated with a pharmacological based strategy, such as a lack of persistent activation of 5-HT receptors as a result of receptor desensitization to prolonged agonist exposure (Kelly et al., 2008) or side effects associated with the systemic administration of these drugs (Sławińska et al., 2013). Studies have begun to examine feasible sources of serotonergic neurons that could be used for transplantation paradigms, particularly the use of induced pluripotent stem cell (IPSC) and transcription factor cocktails for the generation of these neurons from either fibroblasts or embryonic stem cells (Shimada et al., 2012). It remains to be determined whether these cells can be used to promote improved locomotor output after SCI when employed in the same experimental paradigms where embryonic brainstem neurons have demonstrated benefit.

CELL THERAPEUTIC STRATEGIES TO PROMOTE REGENERATION OF SEROTONERGIC AXONS AND FUNCTIONAL RECOVERY AFTER SCI

Though there are adaptive mechanisms of endogenous plasticity within the injured spinal cord and reorganization at the level of the brain that compensates for the loss of transmission from multiple descending pathways (Hawthorne et al., 2011; Zörner et al., 2014), with severe injuries such mechanisms are not able to reverse paralysis and restore locomotor function without the use of additional therapeutic interventions. In experimental SCI paradigms a range of therapeutic approaches have been shown to limit tissue damage, promote axon growth and plasticity and improve the degree of functional recovery (Pearse et al., 2004; Boulenguez and Vinay, 2009). Of the most effective approaches to improve locomotor outcome after SCI, many have demonstrated a significant enhancement of growth and regeneration of descending serotonergic axons caudal to the lesion site (Menei et al., 1998; Tobias et al., 2003; Pearse et al., 2004; Ramer et al., 2004; Oatway et al., 2005; Boido et al., 2009; Ghosh et al., 2012; Hodgetts et al., 2013; Hou et al., 2013; Kanno et al., 2014). It has been shown in different SCI models, in mice and rats, that there is some degree of endogenous axon growth/sprouting of serotonergic axons that occurs (Bregman et al., 1997; Ramón-Cueto et al., 1998), though the extent of such growth is only short distance (Bregman et al., 1997) unless an exogenous strategy is employed. The use of cell therapeutic approaches (Tetzlaff et al., 2011), such as the implantation of olfactory ensheathing cells (OECs) in rats with a complete transection SCI and Schwann cell (SC) bridging, has shown that significant long distance regeneration of serotonergic axons across a bridged SCI gap and into the contiguous caudal cord can be obtained (Ramón-Cueto et al., 2000); similar findings with OECs have been reported in other SCI paradigms (Lu et al., 2002; Plant et al., 2003). Though rats receiving OECs did exhibit modest improvements in hind limb function, they were unable to recover weight bearing capacity (Lu et al., 2002) and other neuronal populations, such as those of the corticospinal tract, failed to regenerate. Recently, Barbour et al. (2013) transplanted OEG and SCs sub-acutely, at 2 weeks, following spinal cord contusion in rat and reported an improvement in locomotor function that was accompanied by the growth of supraspinal projections originating from brainstem serotonergic regions.

The transplantation of genetically modified SCs, to secrete brain derived neurotrophic factor (BDNF; Menei et al., 1998) or D15A (Golden et al., 2007; Flora et al., 2013), a bifunctional neurotrophic molecule capable of activating both TrkB and C receptors, in transection or contusion SCI, respectively, have been shown also to promote considerable serotonergic axon regeneration at, and/or beyond, the injury level. Similarly, we have shown that genetic engineering of SCs, to enhance surface polysialic acid (PSA), also enhances their ability to support serotonergic growth across the lesion site and improve locomotor function when the SCs are transplanted into the contused spinal cord of rats (Ghosh et al., 2012). Recent work by Hodgetts et al. (2013) has reported that the transplantation of human mesenchymal precursor cells (Stro-1^+), obtained from SCI patients can improve functional recovery and tissue sparing in athymic nude rats subjected to a 200 kDyne contusion injury at the vertebral level of T9-T10. The promotion of significant serotonergic axon growth within and surrounding the transplanted cells was a key anatomical change, although evidence of these 5-HT axons reaching the CPG in the caudal cord was not investigated.

The use of cell grafts, not only as a source of serotonergic neurons to provide direct input to the CPG but also as a substrate for supporting the growth of severed supraspinal serotonergic

projections across the lesion to reinnervate the CPG, have thus shown potential for restoring locomotor function after SCI. Further enhancement of these approaches with regenerative factors to encourage greater axon growth into the caudal cord or with agonists of monoaminergic systems to enhance the functionality of caudal projections, may be able to provide more robust strategies for the recovery of function after SCI in which the spinal cord CPG remains intact.

CONCLUSION

The serotonergic pathway works in concert with other monoaminergic systems in the synaptic control of motoneuron excitability. 5-HT contributes to the initiation of locomotion and other rhythmic motor behaviors as well as to the modulation of synaptic transmission in specific reflex pathways through its action on presynaptic terminals. Binding of 5-HT to specific receptor subtypes is responsible for initiating diverse intracellular signaling cascades that can alter the properties of ligand gated voltage-sensitive ion channels and produce a subsequent rapid depolarization of the postsynaptic membrane potential of target neurons to increase their intrinsic excitability. One of the major changes that occurs after SCI is the loss of most, if not all, of the descending monoaminergic input to the spinal cord below the level of the lesion, particularly to the spinal CPG, resulting in immediate and persistent locomotor dysfunction. Although axotomized serotonergic axons have the capacity to undergo sprouting after SCI, endogenous recovery of locomotor function is limited.

Substantial progress has been made in our understanding of the roles of the diverse number of 5-HT receptor subtypes and how they coordinate with one another and receptor signaling through other monoaminergic systems to alter motor output following SCI. The use of either pharmacological or cellular approaches to modulate the activity of various receptor subtypes in response to 5-HT or to provide 5-HT input caudal to the lesion site, respectively, have shown that the restoration of serotonergic signaling can be an effective means for promoting motoneuron excitability and restoring locomotor function after SCI. The use of genetic tools to investigate the role of specific 5-HT receptors in different aspects of motor output or in understanding the role of regenerating serotonergic axons in functional efficacy provided by different therapeutic modalities remains limited and should receive greater attention in future research.

Approaches to restore function after SCI through targeting 5-HT have shown some degree of success, however, a number of limitations remain to be resolved and questions answered. Though the use of 5-HT or specific 5-HT receptor agonists can stimulate CPG neuronal networks and induce locomotion, it is not clear whether such an approach can be employed for sustained functional recovery in light of receptor desensitization and/or loss of responses to the stimuli over time, a common phenomenon with GPCR family members. One strategy for achieving a sustained functional improvement using physiological levels of 5-HT could be through cellular therapies, where serotonergic neurons generated from various sources could be used to replace lost supraspinal serotonergic projections to the CPG below the level of SCI. Not only does a cell therapeutic approach eliminate the risk of side effects associated with systemic administration of high levels of 5-HT receptor agonists but also allows intraspinal delivery of 5-HT directly to where it is required after potentially a single intervention. Alternatively, the use of cell transplants, in combination with pro-regenerative therapies, can be used to bridge the injury site and provide a substrate for severed serotonergic axons to re-grow to their original caudal targets. Though the robustness and applicability of such approaches to a variety of SCI paradigms and injury levels has not yet been thoroughly investigated and many cell transplantation strategies to date have failed to promote the growth of serotonergic axons in significant numbers beyond the lesion into the contiguous caudal cord let alone beyond to the lumbar CPG. Another issue with cell therapeutic approaches is their limited long-term cell survival following transplantation due to the inflammatory environment of the injured spinal cord and rejection, potentially reducing their effectiveness in maintaining functional improvements over time. Therefore, extensive optimization of various cell therapeutic approaches is currently a priority in the field to maximize their therapeutic efficacy.

Another promising strategy to induce locomotor function after SCI in combination with 5-HT or its receptor agonists involves the activation of spinal locomotor networks via EES and data so far are encouraging, both in experimental SCI paradigms and clinically in complete SCI individuals. Similar to the use of pharmacological approaches alone is whether such a combination strategy can be implemented for the persistent restoration of function and whether additional interventions, including cell transplants, can enhance or prolong the benefits of this strategy. Further research at the molecular, cellular and functional level to trigger the necessary sequence of neurological events for complete restoration of locomotor function after human SCI continues to remain as a major challenge and a research area of primary focus. The promotion or facilitation of serotonergic signaling, so as to enhance motoneuron excitability, stimulate CPG activity and restore locomotor function, is one direction at the forefront of research for generating putative interventional approaches for the treatment of SCI.

REFERENCES

Agnati, L. F., Fuxe, K., Zoli, M., Ozini, I., Toffano, G., and Ferraguti, F. (1986). A correlation analysis of the regional distribution of central enkephalin and beta-endorphin immunoreactive terminals and of opiate receptors in adult and old male rats. Evidence for the existence of two main types of communication in the central nervous system: the volume transmission and the wiring transmission. *Acta Physiol. Scand.* 128, 201–207. doi: 10.1111/j.1748-1716.1986.tb07967.x

Angeli, C. A., Edgerton, V. R., Gerasimenko, Y. P., and Harkema, S. J. (2014). Altering spinal cord excitability enables voluntary movements after chronic complete paralysis in humans. *Brain* 137, 1394–1409. doi: 10.1093/brain/awu038

Antri, M., Mouffle, C., Orsal, D., and Barthe, J. Y. (2003). 5-HT1A receptors are involved in short- and long-term processes responsible for 5-HT-induced locomotor function recovery in chronic spinal rat. *Eur. J. Neurosci.* 18, 1963–1972. doi: 10.1046/j.1460-9568.2003.02916.x

Antri, M., Orsal, D., and Barthe, J. Y. (2002). Locomotor recovery in the chronic spinal rat: effects of long-term treatment with a 5-HT2 agonist. *Eur. J. Neurosci.* 16, 467–476. doi: 10.1046/j.1460-9568.2002.02088.x

Azmitia, E. C. (1999). Serotonin neurons, neuroplasticity, and homeostasis of neural tissue. *Neuropsychopharmacology* 21, 33S–45S. doi: 10.1016/S0893-133X(99)00022-6

Azmitia, E. C., and Gannon, P. J. (1986). The primate serotonergic system: a review of human and animal studies and a report on macaca fasicularis. *Adv. Neurol.* 43, 407–468.

Bach-y-Rita, P. (1999). Theoretical aspects of sensory substitution and of neurotransmission-related reorganization in spinal cord injury. *Spinal Cord* 37, 465–474. doi: 10.1038/sj.sc.3100873

Ballion, B., Branchereau, P., Chapron, J., and Viala, D. (2002). Ontogeny of descending serotonergic innervation and evidence for intraspinal 5-HT neurons in the mouse spinal cord. *Brain Res. Dev. Brain Res.* 137, 81–88. doi: 10.1016/s0165-3806(02)00414-5

Barbour, H. R., Plant, C. D., Harvey, A. R., and Plant, G. W. (2013). Tissue sparing, behavioral recovery, supraspinal axonal sparing/regeneration following subacute glial transplantation in a model of spinal cord contusion. *BMC Neurosci.* 14:106. doi: 10.1186/1471-2202-14-106

Bayliss, D. A., Umemiya, M., and Berger, A. J. (1995). Inhibition of N- and P-type calcium currents and the after-hyperpolarization in rat motoneurones by serotonin. *J. Physiol. (Lond.)* 485, 635–647. doi: 10.1113/jphysiol.1995.sp020758

Beliez, L., Barrière, G., Bertrand, S. S., and Cazalets, J. R. (2014). Multiple monoaminergic modulation of posturo-locomotor network activity in the newborn rat spinal cord. *Front. Neural Circuits* 8:99. doi: 10.3389/fncir.2014.00099

Blakely, R. D., Berson, H. E., Fremeau, R. T. Jr., Caron, M. G., Peek, M. M., Prince, H. K., et al. (1991). Cloning and expression of a functional serotonin transporter from rat brain. *Nature* 354, 66–70. doi: 10.1038/354066a0

Bobker, D. H., and Williams, J. T. (1990). Serotonin-mediated inhibitory postsynaptic potential in guinea-pig prepositus hypoglossi and feedback inhibition by serotonin. *J. Physiol.* 422, 447–462. doi: 10.1113/jphysiol.1990.sp017994

Boido, M., Rupa, R., Garbossa, D., Fontanella, M., Ducati, A., and Vercelli, A. (2009). Embryonic and adult stem cells promote raphespinal axon outgrowth and improve functional outcome following spinal hemisection in mice. *Eur. J. Neurosci.* 30, 833–846. doi: 10.1111/j.1460-9568.2009.06879.x

Boulenguez, P., Liabeuf, S., Bos, R., Bras, H., Jean-Xavier, C., Brocard, C., et al. (2010). Downregulation of the potassium-chloride cotransporter KCC2 contributes to spasticity after spinal cord injury. *Nat. Med.* 16, 302–307. doi: 10.1038/nm.2107

Boulenguez, P., and Vinay, L. (2009). Strategies to restore motor functions after spinal cord injury. *Curr. Opin. Neurobiol.* 19, 587–600. doi: 10.1016/j.conb.2009.10.005

Branchereau, P., Chapron, J., and Meyrand, P. (2002). Ending 5-hydroxytryptamine raphe inputs repress the expression of serotonergic neurons and slow the maturation of inhibitory systems in mouse embryonic spinal cord. *J. Neurosci.* 22, 2598–2606.

Bregman, B. S. (1994). "Recovery of function after spinal cord injury: transplantation strategies," in *Functional Neural Transplantation*, eds S. B. Dunnett and A. Bjorklund (New York: Raven), 489–529.

Bregman, B. S., Coumans, J. V., Dai, H. N., Kuhn, P. L., Lynskey, J., McAtee, M., et al. (2002). Transplants and neurotrophic factors increase regeneration and recovery of function after spinal cord injury. *Prog. Brain Res.* 137, 257–273. doi: 10.1016/s0079-6123(02)37020-1

Bregman, B. S., McAtee, M., Dai, H. N., and Kuhn, P. L. (1997). Neurotrophic factors increase axonal growth after spinal cord injury and transplantation in the adult rat. *Exp. Neurol.* 148, 475–494. doi: 10.1006/exnr.1997.6705

Brustein, E., and Rossignol, S. (1999). Recovery of locomotion after ventral and ventrolateral spinal lesions in the cat. II. Effects of noradrenergic and serotoninergic drugs. *J. Neurophysiol.* 81, 1513–1530.

Carlsson, A., Magnusson, T., and Rosengren, E. (1963). 5-Hydroxytryptamine of the spinal cord normally and after transection. *Experimentia* 19, 359–406. doi: 10.1007/bf02152316

Cazalets, J. R., Sqalli-Houssaini, Y., and Clarac, F. (1992). Activation of the central pattern generators for locomotion by serotonin and excitatory amino acids in neonatal rat. *J. Physiol.* 455, 187–204. doi: 10.1113/jphysiol.1992.sp019296

Clineschmidt, B. V., Pierce, J. E., and Lovenberg, W. (1971). Tryptophan hydroxylase and serotonin in spinal cord and brain stem before and after chronic transection. *J. Neurochem.* 18, 1593–1596. doi: 10.1111/j.1471-4159.1971.tb00021.x

Cornide-Petronio, M. E., Fernández-López, B., Barreiro-Iglesias, A., and Rodicio, M. C. (2014). Traumatic injury induces changes in the expression of the serotonin 1A receptor in the spinal cord of lampreys. *Neuropharmacology* 77, 369–378. doi: 10.1016/j.neuropharm.2013.10.017

Courtine, G., Gerasimenko, Y., Van den Brand, R., Yew, A., Musienko, P., Zhong, H., et al. (2009). Transformation of nonfunctional spinal circuits into functional states after the loss of brain input. *Nat. Neurosci.* 12, 1333–1342. doi: 10.1038/nn.2401

Dahlstroem, A., and Fuxe, K. (1964). Evidence for the existence of monoamine containing neurons in the central nervous system. I. Demonstration of monamines in the cell bodies of brain stem neurons. *Acta Physiol. Scand. Suppl.* 62(Suppl. 232), 1–55.

D'Amico, J. M., Condliffe, E. G., Martins, K. J., Bennett, D. J., and Gorassini, M. A. (2014). Recovery of neuronal and network excitability after spinal cord injury and implications for spasticity. *Front. Integr. Neurosci.* 8:36. doi: 10.3389/fnint.2014.00036

Dimitrijevic, M. R., Gerasimenko, Y., and Pinter, M. M. (1998). Evidence for a spinal central pattern generator in humans. *Ann. N Y Acad. Sci.* 860, 360–376. doi: 10.1111/j.1749-6632.1998.tb09062.x

Di Pasquale, E., Lindsay, A. D., Feldman, J. L., Monteau, R., and Hilaire, G. (1997). Serotonergic inhibition of phrenic motoneuron activity: an in vitro study in neonatal rat. *Neurosci. Lett.* 230, 29–32. doi: 10.1016/s0304-3940(97)00469-2

Eaton, M. J., Pearse, D. D., McBroom, J. S., and Berrocal, Y. A. (2008). The combination of human neuronal serotonergic cell implants and environmental enrichment after contusive SCI improves motor recovery over each individual strategy. *Behav. Brain Res.* 194, 236–241. doi: 10.1016/j.bbr.2008.07.001

El Manira, A. (2014). Dynamics and plasticity of spinal locomotor circuits. *Curr. Opin. Neurobiol.* 29C, 133–141. doi: 10.1016/j.conb.2014.06.016

Engesser-Cesar, C., Ichiyama, R. M., Nefas, A. L., Hill, M. A., Edgerton, V. R., Cotman, C. W., et al. (2007). Wheel running following spinal cord injury improves locomotor recovery and stimulates serotonergic fiber growth. *Eur. J. Neurosci.* 25, 1931–1939. doi: 10.1111/j.1460-9568.2007.05469.x

Feraboli-Lohnherr, D., Barthe, J. Y., and Orsal, D. (1999). Serotonin-induced activation of the network for locomotion in adult spinal rats. *J. Neurosci. Res.* 55, 87–98. doi: 10.1002/(sici)1097-4547(19990101)55:1<87::aid-jnr10>3.3.co;2-r

Feraboli-Lohnherr, D., Orsal, D., Yakovleff, A., Giménez y Ribotta, M., and Privat, A. (1997). Recovery of locomotor activity in the adult chronic spinal rat after sublesional transplantation of embryonic nervous cells: specific role of serotonergic neurons. *Exp. Brain Res.* 113, 443–454. doi: 10.1007/pl00005597

Filli, L., Engmann, A. K., Zörner, B., Weinmann, O., Moraitis, T., Gullo, M., et al. (2014). Bridging the gap: a reticulo-propriospinal detour bypassing an incomplete spinal cord injury. *J. Neurosci.* 34, 13399–13410. doi: 10.1523/jneurosci.0701-14.2014

Flora, G., Joseph, G., Patel, S., Singh, A., Bleicher, D., Barakat, D. J., et al. (2013). Combining neurotrophin-transduced Schwann cells and rolipram to promote functional recovery from subacute spinal cord injury. *Cell Transplant.* 22, 2203–2217. doi: 10.3727/096368912x658872

Fornal, C. A., Litto, W. J., Metzler, C. W., Marrosu, F., Tada, K., and Jacobs, B. L. (1994). Single-unit responses of serotonergic dorsal raphe neurons to 5-HT1A agonist and antagonist drug administration in behaving cats. *J. Pharmacol. Exp. Ther.* 270, 1345–1358.

Fouad, K., Rank, M. M., Vavrek, R., Murray, K. C., Sanelli, L., and Bennett, D. J. (2010). Locomotion after spinal cord injury depends on constitutive activity in serotonin receptors. *J. Neurophysiol.* 104, 2975–2984. doi: 10.1152/jn.00499.2010

Fuller, D. D., Baker-Herman, T. L., Golder, F. J., Doperalski, N. J., Watters, J. J., and Mitchell, G. S. (2005). Cervical spinal cord injury upregulates ventral spinal 5-HT2A receptors. *J. Neurotrauma* 22, 203–213. doi: 10.1089/neu.2005.22.203

Gackière, F., and Vinay, L. (2014). Serotonergic modulation of post-synaptic inhibition and locomotor alternating pattern in the spinal cord. *Front. Neural Circuits* 8:102. doi: 10.3389/fncir.2014.00102

Garcia-Rill, E., Skinner, R. D., and Fitzgerald, J. A. (1985). Chemical activation of the mesencephalic locomotor region. *Brain Res.* 330, 43–54. doi: 10.1016/0006-8993(85)90006-x

Gerasimenko, Y. P., Ichiyama, R. M., Lavrov, I. A., Courtine, G., Cai, L., Zhong, H., et al. (2007). Epidural spinal cord stimulation plus quipazine administration enable stepping in complete spinal adult rats. *J. Neurophysiol.* 98, 2525–2536. doi: 10.1152/jn.00836.2007

Gerin, C. G., Hill, A., Hill, S., Smith, K., and Privat, A. (2010). Serotonin release variations during recovery of motor function after a spinal cord injury in rats. *Synapse* 64, 855–861. doi: 10.1002/syn.20802

Gerin, C., Teilhac, J. R., Smith, K., and Privat, A. (2008). Motor activity induces release of serotonin in the dorsal horn of the rat lumbar spinal cord. *Neurosci. Lett.* 436, 91–95. doi: 10.1016/j.neulet.2008.01.081

Ghosh, A., Sydekum, E., Haiss, F., Peduzzi, S., Zörner, B., Schneider, R., et al. (2009). Functional and anatomical reorganization of the sensory-motor cortex after incomplete spinal cord injury in adult rats. *J. Neurosci.* 29, 12210–12219. doi: 10.1523/jneurosci.1828-09.2009

Ghosh, M., Tuesta, L. M., Puentes, R., Patel, S., Melendez, K., El Maarouf, A., et al. (2012). Extensive cell migration, axon regeneration and improved function with polysialic acid-modified Schwann cells after spinal cord injury. *Glia* 60, 979–992. doi: 10.1002/glia.22330

Gimenez y Ribotta, M., Orsal, D., Feraboli-Lohnherr, D., Privat, A., Provencher, J., and Rossignol, S. (1998). Kinematic analysis of recovered locomotor movements of the hindlimbs in paraplegic rats transplanted with monoaminergic embryonic neurons. *Ann. N Y Acad. Sci.* 860, 521–523. doi: 10.1111/j.1749-6632.1998.tb09093.x

Gimenez y Ribotta, M. G., Roudet, C., Sandillon, F., and Privat, A. (1996). Transplantation of embryonic noradrenergic neurons in two models of adult rat spinal cord injury: ultrastructural immunocytochemical study. *Brain Res.* 707, 245–255. doi: 10.1016/0006-8993(95)01266-4

Golden, K. L., Pearse, D. D., Blits, B., Garg, M. S., Oudega, M., Wood, P. M., et al. (2007). Transduced Schwann cells promote axon growth and myelination after spinal cord injury. *Exp. Neurol.* 207, 203–217. doi: 10.1016/j.expneurol.2007.06.023

Grillner, S. (2006). Biological pattern generation: the cellular and computational logic of networks in motion. *Neuron* 5, 751–766. doi: 10.1016/j.neuron.2006.11.008

Grillner, S., and Wallén, P. (1985). Central pattern generators for locomotion, with special reference to vertebrates. *Annu. Rev. Neurosci.* 8, 233–261. doi: 10.1146/annurev.neuro.8.1.233

Guertin, P. A., and Steuer, I. (2005). Ionotropic 5-HT3 receptor agonist-induced motor responses in the hindlimbs of paraplegic mice. *J. Neurophysiol.* 94, 3397–3405. doi: 10.1152/jn.00587.2005

Harkema, S., Gerasimenko, Y., Hodes, J., Burdick, J., Angeli, C., Chen, Y., et al. (2011). Effect of epidural stimulation of the lumbosacral spinal cord on voluntary movement, standing and assisted stepping after motor complete paraplegia: a case study. *Lancet* 377, 1938–1947. doi: 10.1016/S0140-6736(11)60547-3

Harris-Warrick, R. M., and Cohen, A. H. (1985). Serotonin modulates the central pattern generator for locomotion in the isolated lamprey spinal cord. *J. Exp. Biol.* 116, 27–46.

Harvey, P. J., Li, X., Li, Y., and Bennett, D. J. (2006). 5-HT 2 Receptor activation facilitates a persistent sodium current and repetitive firing in spinal motoneurons of rats with and without chronic spinal cord injury. *J. Neurophysiol.* 96, 1158–1170. doi: 10.1152/jn.01088.2005

Hasegawa, Y., and Ono, H. (1996). Effects of 8-OH-DPAT, a 5-HT1Areceptor agonist and DOI, a 5-HT2A/2C agonist, on monosynaptic transmission in spinalized rats. *Brain Res.* 738, 158–161. doi: 10.1016/0006-8993(96)00991-2

Hashimoto, T., and Fukuda, N. (1991). Contribution of serotonin neurons to the functional recovery after spinal cord injury in rats. *Brain Res.* 539, 263–270. doi: 10.1016/0006-8993(91)91630-j

Hawthorne, A. L., Hu, H., Kundu, B., Steinmetz, M. P., Wylie, C. J., Deneris, E. S., et al. (2011). The unusual response of serotonergic neurons after CNS injury: lack of axonal dieback and enhanced sprouting within the inhibitory environment of the glial scar. *J. Neurosci.* 31, 5605–5616. doi: 10.1523/jneurosci.6663-10.2011

Hayashi, Y., Jacob-Vadakot, S., Dugan, E. A., McBride, S., Olexa, R., Simansky, K., et al. (2010). 5-HT precursor loading, but not 5-HT receptor agonists, increases motor function after spinal cord contusion in adult rats. *Exp. Neurol.* 221, 68–78. doi: 10.1016/j.expneurol.2009.10.003

Hentall, I. D., Pinzon, A., and Noga, B. R. (2006). Spatial and temporal patterns of serotonin release in the rat' s lumbar spinal cord following electrical stimulation of the nucleus raphe magnus. *Neuroscience* 142, 893–903. doi: 10.1016/j.neuroscience.2006.06.038

Herrick-Davis, K. (2013). Functional significance of serotonin receptor dimerization. *Exp. Brain Res.* 230, 375–386. doi: 10.1007/s00221-013-3622-1

Hikosaka, O. (2007). GABAergic output of the basal ganglia. *Prog. Brain Res.* 160, 209–226. doi: 10.1016/s0079-6123(06)60012-5

Hochman, S., Garraway, S. M., Machacek, D. W., and Shay, B. L. (2001). "5-HT receptors and the neuromodulatory control of spinal cord function," in *Motor Neurobiology of the Spinal Cord*, ed T. C. Cope (London: CRC Press), 47–87.

Hodgetts, S. I., Simmons, P. J., and Plant, G. W. (2013). A comparison of the behavioral and anatomical outcomes in sub-acute and chronic spinal cord injury models following treatment with human mesenchymal precursor cell transplantation and recombinant decorin. *Exp. Neurol.* 248, 343–359. doi: 10.1016/j.expneurol.2013.06.018

Holmes, G. M., Van Meter, M. J., Beattie, M. S., and Bresnahan, J. C. (2005). Serotonergic fiber sprouting to external anal sphincter motoneurons after spinal cord contusion. *Exp. Neurol.* 193, 29–42. doi: 10.1016/j.expneurol.2005.01.002

Holtmanj, J. R. Jr., Dickt, T. E., and Bergera, A. J. (1986). Involvement of serotonin in the excitation of phrenic motoneurons evoked by stimulation of the raphe obscurus. *J. Neurosci.* 6, 1185–1193.

Hornung, J. P. (2003). The human raphe nuclei and the serotonergic system. *J. Chem. Neuroanat.* 26, 331–343. doi: 10.1016/j.jchemneu.2003.10.002

Hou, S., Tom, V. J., Graham, L., Lu, P., and Blesch, A. (2013). Partial restoration of cardiovascular function by embryonic neural stem cell grafts after complete spinal cord transection. *J. Neurosci.* 33, 17138–17149. doi: 10.1523/jneurosci.2851-13.2013

Hounsgaard, J., Hultborn, H., Jespersen, B., and Kiehn, O. (1988). Bistability of alpha-motoneurones in the decerebrate cat and in the acute spinal cat after intravenous 5-hydroxytryptophan. *J. Physiol.* 405, 345–367. doi: 10.1113/jphysiol.1988.sp017336

Humphreys, J. M., and Whelan, P. J. (2012). Dopamine exerts activation-dependent modulation of spinal locomotor circuits in the neonatal mouse. *J. Neurophysiol.* 108, 3370–3381. doi: 10.1152/jn.00482.2012

Husch, A., Van Patten, G. N., Hong, D. N., Scaperotti, M. M., Cramer, N., and Harris-Warrick, R. M. (2012). Spinal cord injury induces serotonin supersensitivity without increasing intrinsic excitability of mouse V2a interneurons. *J. Neurosci.* 32, 13145–13154. doi: 10.1523/jneurosci.2995-12.2012

Ichiyama, R. M., Gerasimenko, Y. P., Zhong, H., Roy, R. R., and Edgerton, V. R. (2005). Hindlimb stepping movements in complete spinal rats induced by epidural spinal cord stimulation. *Neurosci. Lett.* 383, 339–344. doi: 10.1016/j.neulet.2005.04.049

Iversen, L. L. (1971). Role of transmitter uptake mechanisms in synaptic neurotransmission. *Br. J. Pharmacol.* 41, 571–591. doi: 10.1111/j.1476-5381.1971.tb07066.x

Iwahara, T., Atsuta, Y., Garsia-Rill, E., and Skinner, R. (1992). Spinal cord stimulation-induced locomotion in the adult cat. *Brain Res. Bull.* 28, 99–105. doi: 10.1016/0361-9230(92)90235-p

Jacobs, B. L., and Fornal, C. A. (1993). 5-HT and motor control: a hypothesis. *Trends Neurosci.* 16, 346–352. doi: 10.1016/0166-2236(93)90090-9

Johnson, D. A., Gartside, S. E., and Ingram, C. D. (2002). 5-HT1A receptor-mediated autoinhibition does not function at physiological firing rates: evidence from in vitro electrophysiological studies in the rat dorsal raphe nucleus. *Neuropharmacology* 43, 959–965. doi: 10.1016/s0028-3908(02)00116-8

Jordan, L. M., Liu, J., Hedlund, P. B., Akay, T., and Pearson, K. G. (2008). Descending command systems for the initiation of locomotion in mammals. *Brain Res. Rev.* 57, 183–191. doi: 10.1016/j.brainresrev.2007.07.019

Kanno, H., Pressman, Y., Moody, A., Berg, R., Muir, E. M., Rogers, J. H., et al. (2014). Combination of engineered Schwann cell grafts to secrete neurotrophin and chondroitinase promotes axonal regeneration and locomotion after spinal cord injury. *J. Neurosci.* 34, 1838–1855. doi: 10.1523/jneurosci.2661-13.2014

Kelly, E., Bailey, C. P., and Henderson, G. (2008). Agonist-selective mechanisms of GPCR desensitization. *Br. J. Pharmacol.* 153, S379–S388. doi: 10.1038/sj.bjp.0707604

Kiehn, O. (2006). Locomotor circuits in the mammalian spinal cord. *Annu. Rev. Neurosci.* 29, 279–306. doi: 10.1146/annurev.neuro.29.051605.112910

Kiehn, O., and Kullander, K. (2004). Central pattern generators deciphered by molecular genetics. *Neuron.* 41, 317–321. doi: 10.1016/s0896-6273(04)00042-x

Kim, D., Murray, M., and Simansky, K. J. (2001a). The serotonergic 5-HT (2C) agonist m-chlorophenylpiperazine increases weight-supported locomotion without development of tolerance in rats with spinal transections. *Exp. Neurol.* 169, 496–500. doi: 10.1006/exnr.2001.7660

Kim, D., Schallert, T., Liu, Y., Browarak, T., Nayeri, N., Tessler, A., et al. (2001b). Transplantation of genetically modified fibroblast expressing BDNF in adult rats with subtotal hemisection improves specific motor and sensory functions. *Neurorehabil. Neural Repair* 15, 141–150. doi: 10.1177/154596830101500207

Kong, X. Y., Wienecke, J., Chen, M., Hultborn, H., and Zhang, M. (2011). The time course of serotonin 2A receptor expression after spinal transection of rats: an immunohistochemical study. *Neuroscience* 177, 114–126. doi: 10.1016/j.neuroscience.2010.12.062

Kong, X. Y., Wienecke, J., Hultborn, H., and Zhang, M. (2010). Robust upregulation of serotonin 2A receptors after chronic spinal transection of rats: an immunohistochemical study. *Brain Res.* 1320, 60–68. doi: 10.1016/j.brainres.2010.01.030

Lalley, P. M. (1986). Serotoninergic and non-serotoninergic responses of phrenic motoneurones to raphe stimulation in the cat. *J. Physiol.* 380, 373–385. doi: 10.1113/jphysiol.1986.sp016291

Lambe, E. K., Fillman, S. G., Webster, M. J., and Weickert, S. C. (2011). Serotonin receptor expression in human prefrontal cortex: balancing excitation and inhibition across postnatal development. *PLoS One* 6:e22799. doi: 10.1371/journal.pone.0022799

Landry, E. S., and Guertin, P. A. (2004). Differential effects of 5-HT1 and 5-HT2 receptor agonists on hindlimb movements in paraplegic mice. *Prog. Neuro-Psychopharmacol. Biol. Psychiatry* 28, 1053–1060. doi: 10.1016/j.pnpbp.2004.05.001

Landry, E. S., Lapointe, N. P., Rouillard, C., Levesque, D., Hedlund, P. B., and Guertin, P. A. (2006). Contribution of spinal 5-HT1A and 5-HT7 receptors to locomotor-like movement induced by 8-OH-DPAT in spinal cord-transected mice. *Eur. J. Neurosci.* 24, 535–546. doi: 10.1111/j.1460-9568.2006.04917.x

Lesch, K. P., Wolozin, B. L., Estler, H. C., Murphy, D. L., and Riederer, P. (1993). Isolation of a cDNA encoding the human brain serotonin transporter. *J. Neural. Transm. Gen. Sect.* 91, 67–72. doi: 10.1007/bf01244919

Levkovitz, Y., and Segal, M. (1997). Serotonin 5HT1a receptors modulate hippocampal reactivity to afferent stimulation. *J. Neurosci.* 17, 5591–5598.

Li, H. J., Johnston, B., Aiello, D., Caffrey, D. R., Giel-Moloney, M., Rindi, G., et al. (2014). Distinct cellular origins for serotonin-expressing and enterochromaffin-like cells in the gastric corpus. *Gastroenterology* 146, 754.e3–764.e3. doi: 10.1053/j.gastro.2013.11.048

Li, X., Murray, K., Harvey, P. J., Ballou, E. W., and Bennett, D. J. (2007). Serotonin facilitates a persistent calcium current in motoneurons of rats with and without chronic spinal cord injury. *J. Neurophysiol.* 97, 1236–1246. doi: 10.1152/jn.00995.2006

Liu, J., Akay, T., Hedlund, P. B., Pearson, K. G., and Jordan, L. M. (2009). Spinal 5-HT7 receptors are critical for alternating activity during locomotion: in vitro neonatal and in vivo adult studies using 5-HT7 receptor knockout mice. *J. Neurophysiol.* 102, 337–348. doi: 10.1152/jn.91239.2008

Lu, J., Féron, F., Mackay-Sim, A., and Waite, P. M. (2002). Olfactory ensheathing cells promote locomotor recovery after delayed transplantation into transected spinal cord. *Brain* 125, 14–21. doi: 10.1093/brain/awf014

MacLean, J. N., Cowley, K. C., and Schmidt, B. J. (1998). NMDA receptor-mediated oscillatory activity in the neonatal rat spinal cord is serotonin dependent. *J. Neurophysiol.* 79, 2804–2808.

Madriaga, M. A., McPhee, L. C., Chersa, T., Christie, K. J., and Whelan, P. J. (2004). Modulation of locomotor activity by multiple 5-HT and dopaminergic receptor subtypes in the neonatal mouse spinal cord. *J. Neurophysiol.* 92, 1566–1576. doi: 10.1152/jn.01181.2003

Majczyński, H., Maleszak, K., Cabaj, A., and Sawińska, U. (2005). Serotonin-related enhancement of recovery of hind limb motor functions in spinal rats after grafting of embryonic raphe nuclei. *J. Neurotrauma* 22, 590–604. doi: 10.1089/neu.2005.22.590

Martin, G. F., Cabana, T., and Humbertson, A. O. Jr. (1981). Evidence for collateral innervation of the cervical and lumbar enlargements of the spinal cord by single reticular and raphe neurons. Studies using fluorescent markers in double-labeling experiments on the North American opossum. *Neurosci. Lett.* 24, 1–6. doi: 10.1016/0304-3940(81)90349-9

Martinez, M., and Rossignol, S. (2011). Changes in CNS structures after spinal cord lesions implications for BMI. *Prog. Brain Res.* 194, 191–202. doi: 10.1016/b978-0-444-53815-4.00007-8

Meehan, C. F., Grondahl, L., Nielsen, J. B., and Hultborn, H. (2012). Fictive locomotion in the adult decerebrate and spinal mouse in vivo. *J. Physiol.* 15, 289–300. doi: 10.1113/jphysiol.2011.214643

Menei, P., Montero-Menei, C., Whittermore, S. R., Bunge, M. B., and Bunge, R. P. (1998). Schwann cells genetically engineered to produce BDNF promote axonal regeneration of brainstem neurons across transected adult rat spinal cord. *Eur. J. Neurosci.* 10, 607–621. doi: 10.1046/j.1460-9568.1998.00071.x

Millan, M. J., Marin, P., Bockaert, J., and Mannoury la Cour, C. (2008). Signaling at G-protein-coupled serotonin receptors: recent advances and future research directions. *Trends Pharmacol. Sci.* 9, 454–464. doi: 10.1016/j.tips.2008.06.007

Miller, J. F., Paul, K. D., Lee, R. H., Rymer, W. Z., and Heckman, C. J. (1996). Restoration of extensor excitability in the acute spinal cat by the 5-HT2 agonist DOI. *J. Neurophysiol.* 75, 620–628.

Murphy, D. L., Lerner, A., Rudnick, G., and Lesch, K. P. (2004). Serotonin transporter: gene, genetic disorders and pharmacogenetics. *Mol. Interv.* 4, 109–123. doi: 10.1124/mi.4.2.8

Murray, K. C., Nakae, A., Stephens, M. J., Rank, M., D'Amico, J., Harvey, P. J., et al. (2010). Recovery of motoneuron and locomotor function after spinal cord injury depends on constitutive activity in 5-HT2C receptors. *Nat. Med.* 16, 694–700. doi: 10.1038/nm.2160

Musienko, P., van den Brand, R., Märzendorfer, O., Roy, R. R., Gerasimenko, Y., Edgerton, V. R., et al. (2011). Controlling specific locomotor behaviors through multidimensional monoaminergic modulation of spinal circuitries. *J. Neurosci.* 22, 9264–9278. doi: 10.1523/jneurosci.5796-10.2011

Nardone, R., Höller, Y., Thomschewski, A., Höller, P., Lochner, P., Golaszewski, S., et al. (2014). Serotonergic transmission after spinal cord injury. *J. Neural Transm.* doi: 10.1007/s00702-014-1241-z. [Epub ahead of print].

Navarrett, S., Collier, L., Cardozo, C., and Dracheva, S. (2012). Alterations of serotonin 2C and 2A receptors in response to T10 spinal cord transection in rats. *Neurosci. Lett.* 506, 74–78. doi: 10.1016/j.neulet.2011.10.052

Noga, B. R., Johnson, D. M., Riesgo, M. I., and Pinzon, A. (2009). Locomotor-activated neurons of the cat. I. Serotonergic innervation and co-localization of 5-HT7, 5-HT2A and 5-HT1A receptors in the thoraco-lumbar spinal cord. *J. Neurophysiol.* 102, 1560–1576. doi: 10.1152/jn.91179.2008

Noga, B. R., Kriellaars, D. J., and Jordan, L. M. (1991). The effect of selective brainstem or spinal cord lesions on treadmill locomotion evoked by stimulation of the mesencephalic or pontomedullary locomotor regions. *J. Neurosci.* 11, 1691–1700.

Noga, B. R., Pinzon, A., Mesigil, R. P., and Hentall, I. D. (2004). Steady-state levels of monoamines in the rat lumbar spinal cord: spatial mapping and the effect of acute spinal cord injury. *J. Neurophysiol.* 92, 567–577. doi: 10.1152/jn.01035.2003

Oatway, M. A., Chen, Y., Bruce, J. C., Dekaban, G. A., and Weaver, L. C. (2005). Anti-CD11d integrin antibody treatment restores normal serotonergic projections to the dorsal, intermediate and ventral horns of the injured spinal cord. *J. Neurosci.* 25, 637–647. doi: 10.1523/jneurosci.3960-04.2005

Orsal, D., Barthe, J. Y., Antri, M., Feraboli-Lohnherr, D., Yakovleff, A., Giménez y Ribotta, M., et al. (2002). Locomotor recovery in chronic spinal rat: long-term pharmacological treatment or transplantation of embryonic neurons? *Prog. Brain Res.* 137, 213–230. doi: 10.1016/s0079-6123(02)37018-3

Otoshi, C. K., Walwyn, W. M., Tillakaratne, N. J., Zhong, H., Roy, R. R., and Edgerton, V. R. (2009). Distribution and localization of 5-HT (1A) receptors in the rat lumbar spinal cord after transection and deafferentation. *J. Neurotrauma* 26, 575–584. doi: 10.1089/neu.2008.0640

Pearlstein, E., Ben-Mabrouk, F., Pflieger, J. F., and Vinay, L. (2005). Serotonin refines the locomotor-related alternations in the in vitro neonatal rat spinal cord. *Eur. J. Neurosci.* 21, 1338–1346. doi: 10.1111/j.1460-9568.2005.03971.x

Pearse, D. D., Pereira, F. C., Marcillo, A. E., Bates, M. L., Berrocal, Y. A., Filbin, M. T., et al. (2004). cAMP and Schwann cells promote axonal growth and functional recovery after spinal cord injury. *Nat. Med.* 10, 610–616. doi: 10.1038/nm1056

Perrier, J. F., Rasmussen, H. B., Christensen, R. K., and Petersen, A. V. (2013). Modulation of the intrinsic properties of motoneurons by serotonin. *Curr. Pharm. Des.* 19, 4371–4384. doi: 10.2174/1381612811319990341

Perrin, F. E., Gerber, Y. N., Teigell, M., Lonjon, N., Boniface, G., Bauchet, L., et al. (2011). Anatomical study of serotonergic innervation and 5-HT (1A) receptor in the human spinal cord. *Cell Death Dis.* 2:e218. doi: 10.1038/cddis.2011.98

Pflieger, J. F., Clarac, F., and Vinay, L. (2002). Postural modifications and neuronal excitability changes induced by short-term serotonin depletion during neonatal development in the rat. *J. Neurosci.* 22, 5108–5117.

Plant, G. W., Christensen, C. L., Oudega, M., and Bunge, M. B. (2003). Delayed transplantation of olfactory ensheathing glia promotes sparing/regeneration of supraspinal axons in the contused adult rat spinal cord. *J. Neurotrauma* 20, 1–16. doi: 10.1089/08977150360517146

Privat, A., Mansour, H., Rajaofetra, N., and Geffard, M. (1989). Intraspinal transplants of serotonergic neurons in the adult rat. *Brain Res. Bull.* 22, 123–129. doi: 10.1016/0361-9230(89)90136-6

Rajaofetra, N., Passagia, J. G., Marlier, L., Poulat, P., Pellas, F., Sandillon, F., et al. (1992a). Serotoninergic, noradrenergic and peptidergic innervation of Onuf's nucleus of normal and transected spinal cords of baboons (Papiopapio). *J. Comp. Neurol.* 318, 1–17. doi: 10.1002/cne.903180102

Rajaofetra, N., Poulat, P., Marlier, L., Proust, F., Drian, M. J., Passagia, J. G., et al. (1989a). First transplantation of embryonic serotonergic neurons in primate spinal cord. *C. R. Acad. Sci. III* 310, 81–88.

Rajaofetra, N., Poulat, P., Marlier, L., Sandillon, F., Drian, M. J., König, N., et al. (1992b). Transplantation of embryonic serotonin immunoreactive neurons into the transected spinal cord of adult monkey (Macacafascicularis). *Brain Res.* 572, 329–334. doi: 10.1016/0006-8993(92)90495-u

Rajaofetra, N., Sandillon, F., Geffard, M., and Privat, A. (1989b). Pre- and postnatal ontogeny of serotonergic projections to the rat spinal cord. *J. Neurosci. Res.* 22, 305–321. doi: 10.1002/jnr.490220311

Ramer, L. M., Au, E., Richter, M. W., Liu, J., Tetzlaff, W., and Roskams, A. J. (2004). Peripheral olfactory ensheathing cells reduce scar and cavity formation and promote regeneration after spinal cord injury. *J. Comp. Neurol.* 473, 1–15. doi: 10.1002/cne.20049

Ramón-Cueto, A., Cordero, M. I., Santos-Benito, F. F., and Avila, J. (2000). Functional recovery of paraplegic rats and motor axon regeneration in their spinal cords by olfactory ensheathing glia. *Neuron* 25, 425–435. doi: 10.1016/s0896-6273(00)80905-8

Ramón-Cueto, A., Plant, G. W., Avila, J., and Bunge, M. B. (1998). Long-distance axonal regeneration in the transected adult rat spinal cord is promoted by olfactory ensheathing glia transplants. *J. Neurosci.* 18, 3803–3815.

Raymond, J. R., Mukhin, Y. V., Gelasco, A., Turner, J., Collinsworth, G., Gettys, T. W., et al. (2001). Multiplicity of mechanisms of serotonin receptor signal transduction. *Pharmacol. Ther.* 92, 179–212. doi: 10.1016/s0163-7258(01)00169-3

Ren, L. Q., Wienecke, J., Chen, M., Møller, M., Hultborn, H., and Zhang, M. (2013). The time course of serotonin 2C receptor expression after spinal transection of rats: an immunohistochemical study. *Neuroscience* 236, 31–46. doi: 10.1016/j.neuroscience.2012.12.063

Renner, U., Zeug, A., Woehler, A., Niebert, M., Dityatev, A., Dityateva, G., et al. (2012). Heterodimerization of serotonin receptors 5-HT1A and 5-HT7 differentially regulates receptor signalling and trafficking. *J. Cell Sci.* 125, 2486–2499. doi: 10.1242/jcs.101337

Ribotta, M. G., Provencher, J., Feraboli-Lohnherr, D., Rossignol, S., Privat, A., and Orsal, D. (2000). Activation of locomotion in adult chronic spinal rats is achieved by transplantation of embryonic raphe cells reinnervating a precise lumbar level. *J. Neurosci.* 20, 5144–5152.

Rossignol, S., Dubuc, R. J., and Gossard, J. P. (2006). Dynamic sensorimotor interactions in locomotion. *Physiol. Rev.* 86, 89–154. doi: 10.1152/physrev.00028.2005

Rossignol, S., and Frigon, A. (2011). Recovery of locomotion after spinal cord injury: some facts and mechanisms. *Annu. Rev. Neurosci.* 34, 413–440. doi: 10.1146/annurev-neuro-061010-113746

Rossignol, S., Lund, J. P., and Drew, T. (1988). "The role of sensory inputs in regulating patterns of rhythmical movements in higher vertebrates. A comparison between locomotion, respiration and mastication," in *Neural Control of Rhythmic Movements in Vertebrates*, eds A. H. Cohen, S. Rossignol and S. Grillner (New York: Wiley), 201–283.

Rudnick, G., and Clark, J. (1993). From synapse to vesicle: the reuptake and storage of biogenic amine neurotransmitters. *Biochim. Biophys. Acta* 1144, 249–263. doi: 10.1016/0005-2728(93)90109-s

Schloss, P., Mayser, W., and Betz, H. (1992). Neurotransmitter transporters. A novel family of integral plasma membrane proteins. *FEBS Lett.* 307, 76–80. doi: 10.1016/0014-5793(92)80905-v

Schmidt, B. J., and Jordan, L. M. (2000). The role of serotonin in reflex modulation and locomotor rhythm production in the mammalian spinal cord. *Brain Res. Bull.* 53, 689–710. doi: 10.1016/s0361-9230(00)00402-0

Scullion, K., Boychuk, J. A., Yamakawa, G. R., Rodych, J. T., Nakanishi, S. T., Seto, A., et al. (2013). Serotonin 1A receptors alter expression of movement representations. *J. Neurosci.* 13, 4988–4999. doi: 10.1523/jneurosci.4241-12.2013

Segal, M. (1990a). Serotonin attenuates a slow inhibitory postsynaptic potentialin rat hippocampal neurons. *Neuroscience* 36, 631–641. doi: 10.1016/0306-4522(90)90006-p

Segal, M. (1990b). A subset of local interneurons generate slow inhibitory postsynaptic potentials in hippocampal neurons. *Brain Res.* 511, 163–164. doi: 10.1016/0006-8993(90)90236-5

Shapkova, E. Y. (2004). "Spinal locomotor capability revealed by electrical stimulation of the lumbar enlargement in paraplegic patients," in *Progress in Motor Control Volume Three*, eds M. Latash and M. Levin (Champaign, IL: Human Kinetics Publishers), 253–289.

Shapkova, E. Y., and Mushkin, L. A. (2002). Spinal cord stepping electrostimulation as a method for recovery of locomotor activity invertebrogenic myelopathies. *Med. Tekh.* 6, 29–32.

Shapkova, E. Y., and Schomburg, E. D. (2001). Two types of motor modulation underlying human stepping evoked by spinal cordelectrical stimulation (SCES). *Acta Physiol. Pharmacol. Bulg.* 26, 155–157.

Shik, M. L., Severin, F. V., and Orlovskiy, G. N. (1966). Control of walking and running by means of electric stimulation of the midbrain. *Biofizika* 11, 659–666.

Shimada, T., Takai, Y., Shinohara, K., Yamasaki, A., Tominaga-Yoshino, K., Ogura, A., et al. (2012). A simplified method to generate serotonergic neurons from mouse embryonic stem and induced pluripotent stem cells. *J. Neurochem.* 122, 81–93. doi: 10.1111/j.1471-4159.2012.07724.x

Sholomenko, G. N., and Steeves, J. D. (1987). Effects of selective spinal cord lesions on hind limb locomotion in birds. *Exp. Neurol.* 95, 403–418. doi: 10.1016/0014-4886(87)90148-8

Sławińska, U., Miazga, K., Cabaj, A. M., Leszczyńska, A. N., Majczyński, H., Nagy, J. I., et al. (2013). Grafting of fetal brainstem 5-HT neurons into the sublesional spinal cord of paraplegic rats restores coordinated hindlimb locomotion. *Exp. Neurol.* 247, 572–581. doi: 10.1016/j.expneurol.2013.02.008

Sławińska, U., Miazga, K., and Jordan, L. M. (2014a). 5-HT_2 and 5-HT_7 receptor agonists facilitate plantar stepping in chronic spinal rats through actions on different populations of spinal neurons. *Front. Neural Circuits.* 8:95. doi: 10.3389/fncir.2014.00095

Sławińska, U., Miazga, K., and Jordan, L. M. (2014b). The role of serotonin in the control of locomotor movements and strategies for restoring locomotion after spinal cord injury. *Acta Neurobiol. Exp. (Wars)* 74, 172–187.

Steeves, J. D., and Jordan, L. M. (1984). Autoradiographic demonstration of the projections from the mesencephaliclocomotor region. *Brain Res.* 307, 263–276. doi: 10.1016/0006-8993(84)90480-3

Steeves, J. D., Sholomenko, G. N., and Webster, D. M. (1987). Stimulation of the pontomedullary reticular formation initiates locomotion in decerebrate birds. *Brain Res.* 401, 205–212. doi: 10.1016/0006-8993(87)91406-5

Takakusaki, K., Saitoh, K., Harada, H., and Kashiwayanagi, M. (2004). Role of basal ganglia-brainstem pathways in the control of motor behaviors. *Neurosci. Res.* 50, 137–151. doi: 10.1016/j.neures.2004.06.015

Takeuchi, Y., Kimura, H., and Sano, Y. (1982). Immunohistochemical demonstration of serotonin neurons in the brainstem of the rat and cat. *Cell Tissue Res.* 224, 247–267. doi: 10.1007/bf00216872

Tansey, K. E. (2010). Neural plasticity and locomotor recovery after spinal cord injury. *PM R* 2(Suppl. 2), S220–S226. doi: 10.1016/j.pmrj.2010.10.007

Tetzlaff, W., Okon, E. B., Karimi-Abdolrezaee, S., Hill, C. E., Sparling, J. S., and Plemel, J. R. (2011). A systematic review of cellular transplantation therapies for spinal cord injury. *J. Neurotrauma* 28, 1611–1682. doi: 10.1089/neu.2009.1177

Thompson, C. K., Jayaraman, A., Kinnaird, C., and Hornby, T. G. (2011). Methods to quantify pharmacologically induced alterations in motor function in human incomplete SCI. *J. Vis. Exp.* 50, 1–7. doi: 10.3791/2148

Tobias, C. A., Shumsky, J. S., Shibata, M., Tuszynski, M. H., Fischer, I., Tessler, A., et al. (2003). Delayed grafting of BDNF and NT-3 producing fibroblasts into the injured spinal cord stimulates sprouting, partially rescues

axotomized red nucleus neurons from loss and atrophy and provides limited regeneration. *Exp. Neurol.* 184, 97–113. doi: 10.1016/s0014-4886(03)00394-7

Torres, G. E., Gainetdinov, R. R., and Caron, M. G. (2003). Plasma membrane monoamine transporters: structure, regulation and function. *Nat. Rev. Neurosci.* 4, 13–25. doi: 10.1038/nrn1008

Ung, R. V., Landry, E. S., Rouleau, P., Lapointe, N. P., Rouillard, C., and Guertin, P. A. (2008). Role of spinal 5-HT2 receptor subtypes in quipazine-induced hindlimb movements after a low-thoracic spinal cord transection. *Eur. J. Neurosci.* 28, 2231–2242. doi: 10.1111/j.1460-9568.2008.06508.x

Werry, T. D., Loiacono, R., Sexton, P. M., and Christopoulos, A. (2008). RNA editing of the serotonin 5-HT2C receptor and its effects on cell signalling, pharmacology and brain function. *Pharmacol. Ther.* 119, 7–23. doi: 10.1016/j.pharmthera.2008.03.012

Wienecke, J., Ren, L. Q., Hultborn, H., Chen, M., Møller, M., Zhang, Y., et al. (2014). Spinal cord injury enables aromatic L-amino acid decarboxylase cells to synthesize monoamines. *J. Neurosci.* 34, 11984–12000. doi: 10.1523/jneurosci.3838-13.2014

Zhang, W., and Grillner, S. (2000). The spinal 5-HT system contributes to the generation of fictive locomotion in lamprey. *Brain Res.* 879, 188–192. doi: 10.1016/s0006-8993(00)02747-5

Zörner, B., Bachmann, L. C., Filli, L., Kapitza, S., Gullo, M., Bolliger, M., et al. (2014). Chasing central nervous system plasticity: the brainstem's contribution to locomotor recovery in rats with spinal cord injury. *Brain* 137, 1716–1732. doi: 10.1093/brain/awu078

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Modulation of spontaneous locomotor and respiratory drives to hindlimb motoneurons temporally related to sympathetic drives as revealed by Mayer waves

Jacob Wienecke*[1,2], *Manuel Enríquez Denton*[1,3,4], *Katinka Stecina*[1,5], *Peter A. Kirkwood*[3] and *Hans Hultborn*[1]

[1] *Department of Neuroscience and Pharmacology, The Panum Institute, University of Copenhagen, Copenhagen, Denmark*
[2] *Department of Nutrition, Exercise and Sports, The Panum Institute, University of Copenhagen, Copenhagen, Denmark*
[3] *Sobell Department for Motor Neuroscience and Movement Disorders, University College London Institute of Neurology, London, UK*
[4] *Universidad del Valle de México, Mexico City, Mexico*
[5] *Department of Physiology and Pathophysiology, University of Manitoba, Winnipeg, MB, Canada*

Edited by:
Brian R. Noga, University of Miami, USA

Reviewed by:
C. J. Heckman, Northwestern University, USA
Ya-tang Li, California Institute of Technology, USA

****Correspondence:***
Hans Hultborn, Department of Neuroscience and Pharmacology, The Panum Institute, University of Copenhagen, bldg 33.3, Blegdamsvej 3, 2200 Copenhagen N, Denmark
e-mail: hhu@sund.ku.dk

In this study we investigated how the networks mediating respiratory and locomotor drives to lumbar motoneurons interact and how this interaction is modulated in relation to periodic variations in blood pressure (Mayer waves). Seven decerebrate cats, under neuromuscular blockade, were used to study central respiratory drive potentials (CRDPs, usually enhanced by added CO_2) and spontaneously occurring locomotor drive potentials (LDPs) in hindlimb motoneurons, together with hindlimb and phrenic nerve discharges. In four of the cats both drives and their voltage-dependent amplification were absent or modest, but in the other three, one or other of these drives was common and the voltage-dependent amplification was frequently strong. Moreover, in these three cats the blood pressure showed marked periodic variation (Mayer waves), with a slow rate (periods 9–104 s, mean 39 ± 17 SD). Profound modulation, synchronized with the Mayer waves was seen in the occurrence and/or in the amplification of the CRDPs or LDPs. In one animal, where CRDPs were present in most cells and the amplification was strong, the CRDP consistently triggered sustained plateaux at one phase of the Mayer wave cycle. In the other two animals, LDPs were common, and the occurrence of the locomotor drive was gated by the Mayer wave cycle, sometimes in alternation with the respiratory drive. Other interactions between the two drives involved respiration providing leading events, including co-activation of flexors and extensors during post-inspiration or a locomotor drive gated or sometimes entrained by respiration. We conclude that the respiratory drive in hindlimb motoneurons is transmitted via elements of the locomotor central pattern generator. The rapid modulation related to Mayer waves suggests the existence of a more direct and specific descending modulatory control than has previously been demonstrated.

Keywords: locomotion, respiration, central pattern generators, Mayer waves, motoneurons, sympathetic drive

INTRODUCTION

It is a common experience for those who work with decerebrate un-anesthetized preparations that they show a great deal of variability of "state," both between preparations and during the course of recording sessions. This may be expressed in different ways such as in the susceptibility of motoneurons to show persistent inward currents (PICs) or in the occurrence of a locomotor drive. Here our original aim was to describe the voltage-dependent amplification of CRDPs (Sears, 1964) in lumbar motoneurons in the decerebrate cat, following the demonstration by Kirkwood et al. (2002) that CRDPs may trigger plateau potentials (by PICs) in hindlimb motoneurons, even in barbiturate anesthetized preparations in which PICs are generally depressed. We set out to compare the amplification of the CRDPs with that of other physiologically derived motoneuron inputs, such as locomotor drive potentials (LDPs) (Brownstone et al., 1994) or stretch-evoked Ia excitation (Bennett et al., 1998).

Making direct comparisons between these different drives turned out to be a complicated issue, still unresolved, but during these initial experiments we unexpectedly came across evidence for one of the sources of the variability inherent in these preparations. We observed not only CRDPs, but also periods of spontaneous locomotor activity (with LDPs in the motoneurons) and a number of variable relations between the respiratory and locomotor activities as recorded in both muscle nerves and intracellularly in the motoneurons. Only some of these relations were evident to us during the experiments, which consisted of serial intracellular recordings from motoneurons, together with injections of depolarizing currents intended to reveal voltage-sensitive amplification of the various synaptic drives. On *post*

hoc analysis, however, it became evident that many of the motor patterns were related to intermittent increases in blood pressure. These spontaneous increases in blood pressure are called Mayer waves (Mayer, 1876; Montano et al., 1992; Julien, 2006) and are widely believed to be mediated by variation in sympathetic drive, though the origin of that variation is enigmatic (see Julien, 2006 for review). However, the systematic changes in respiratory drive, in activation of locomotor circuits, and in their interaction, provide new clues as to the spinal organization of the networks involved.

There are a large number of publications on how various types of physical activity (walking, running, and jumping) in humans and animals may entrain the respiratory cycles (see Discussion), but here it is the descending respiratory drive to the lumbar motoneurons which seemed to be the dominant rhythm in its inter-relationship with the spinal locomotor network. The relatively rapid modulation of this inter-relationship, and its association with the Mayer waves are the subjects of this communication. Some of the present results were briefly communicated in a congress report (Kirkwood et al., 2005).

METHODS

All surgery and experimental protocols were conducted in accordance with EU regulations (Council Directive 86/609/EEC) and with National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals* (NIH publication no. 86–23, revised 1985), and were approved by the Danish Animal Experimentation Inspectorate. Experiments were performed on seven adult cats of either sex weighing 2.9–3.6 kg. The present experiments were part of longer series of investigations into the respiratory drive recorded in motoneurons in various spinal cord segments, from which observations on phrenic and intercostal motoneurons have recently been published (Enríquez Denton et al., 2012). Many details of the methods can be found there. Here we will give a shorter version, with additional information on the specific arrangements for the hindlimb motoneuron/nerve preparation.

ANESTHESIA AND DECEREBRATION

After the induction of anesthesia with isoflurane–nitrous oxide (2–3% isoflurane, 70% N_2O, and 30% O_2), the animals were intubated, and cannulae were inserted into the right carotid artery and the right cephalic and jugular veins for the monitoring of blood pressure and the administration of fluid and drugs. The anesthetic gasses were administered first in an anesthetic chamber, then by a mask and finally via the tracheal cannula, maintained until decerebration was completed (see below).

Six of the seven cats were finally subjected to a mechanical decerebration at precollicular level, including removal of brain tissue rostral to the transection. This was carried out during anesthesia, at the end of the surgical procedure, after the animal had been transferred to the recording frame with a stereotactic head-holder (Gossard et al., 1994). In 5 of the 7 cats we initially performed an anemic decerebration by ligating the basilar and both common carotid arteries, a procedure that has been shown to produce a decerebration that includes all cerebral tissue above the pons and the anterior part of the cerebellum (Pollock and Davis, 1930; Crone et al., 1988; Geertsen et al., 2011). Following the transfer of the cat to the recording frame, the second carotid artery was ligated and the anesthetic removed. Decerebration was verified to be clinically complete by the development of tonic extensor muscle tone, lack of spontaneous movements, and large non-reactive pupils (Crone et al., 1988). However, in 4 of the 5 cats in this series of experiments, stereotyped stepping movements developed after the removal of anesthetics, so the anesthesia was immediately reinstated and the brain was removed rostral to a section at the level of the superior colliculi, just as for the two cats in which the mechanical decerebration was performed without preceding attempt of an anemic decerebration. Following these procedures, neuromuscular transmission was blocked with pancuronium bromide and artificial respiration was initiated (see below).

NERVE DISSECTION AND LAMINECTOMY

The following hindlimb nerves (left side) were dissected: posterior biceps and semitendinosus (PBSt), semimembranosus and anterior biceps (SmAB), the gastrocnemius and soleus (GS; in two preparations this nerve was left intact in continuity with the muscles to allow studies on the effect of muscle stretch), the tibial nerve (Tib) and the deep peroneal nerve (DP), which contains the tibial anterior and extensor digitorum nerves. These nerves were cut distally and freed from connective tissue for later mounting on bipolar silver hook electrodes for recording and stimulation (with the exception of the GS nerve in two cats as specified above). For stretch of triceps surae, a hole was drilled through the calcaneus bone, a heavy inelastic nylon cord was tied through the hole, and the bone was cut distally, leaving only a small bone chip attached to the Achilles tendon. The nylon cord was later tied to a muscle puller (310B, Cambridge) (see Bennett et al., 1998). Sinusoidal stretches were applied at 1 Hz. Laminectomy of L4–L6 vertebrae was performed to expose the dorsum of the spinal cord for later intracellular recording from hindlimb motoneurons. Mineral oil pools were fashioned from the loose skin at the laminectomy and hindlimb wound margins. A monopolar recording electrode was placed on the dorsal surface of the lumbar spinal cord to record the afferent incoming volley associated with electrical stimulation of the peripheral nerves. The C5 phrenic nerve was dissected and mounted on buried electrodes for recording efferent discharges. In one animal this recording failed early in the experiment and a T5 external intercostal nerve was prepared as an alternative.

MAINTENANCE OF THE PREPARATIONS

Atropine (0.1 mg kg^{-1}, s.c.) and solumedrol (2.5 mg kg^{-1}, i.v.) were administered at the beginning of the experiment and a buffer solution (10% glucose and 1.7% $NaHCO_3$) was infused continuously (~4.5 ml h^{-1}) after cannulation. Neuromuscular blockade was obtained with pancuronium bromide at a dose of 0.6 mg h^{-1} (i.v.) and animals were artificially ventilated with oxygen-enriched air, so as to bring the end-tidal CO_2 fraction initially to about 4%. A low stroke volume and a high pump rate (typically 1 s^{-1}) were employed, so that events related to the central respiratory drive could be distinguished from those due to movement-related afferent input. A bilateral pneumothorax was

performed and the end-expiratory pressure maintained at 2–3 cm H_2O. CO_2 was then added to the gas mixture to raise the end-tidal level to the value required. The actual value chosen was varied with the aim of controlling the central respiratory drive (end-tidal CO_2 fractions between 3 and 10%). Rectal temperature was maintained between 37 and 38°C by a servo-controlled warm air flow and a radiant heater. Blood pressure was measured from a cannulated common carotid artery. Mean values were above 80 mm Hg throughout. To assist maintenance of blood pressure Effortil (Etilifrin-hydrochlorid; Boehringer Ingelheim) was administered i.v. in five cats (though in two of them only near the end of the experiment). At the end of the experiment the animals were killed with an overdose of barbiturate.

RECORDING

AC-coupled recordings were made of the cord dorsum potentials for incoming afferent volleys and the electroneurograms (ENGs: the phrenic nerve; the external intercostal nerve in one cat; when appropriate, see below, the dissected hindlimb nerves as listed above). Intracellular recordings were DC-coupled, but a high gain output channel high pass filtered at 1 Hz was also included. Intracellular recordings were made from antidromically identified motoneurons, using an Axoclamp 2B amplifier (Axon Instruments) in either standard bridge mode, or in discontinuous current clamp (DCC) mode. Microelectrodes (typical impedance 5 MΩ) were filled with 2 M potassium acetate, and contained the local anesthetic derivative QX-314 (50 mm) to block actions potentials, so as to facilitate the study of the size of EPSPs at different membrane potentials. Note that in several of the records illustrated, a few action potentials survived, showing the QX-314 block to be incomplete at those times. DCC mode was used to allow for more accurate measurements of membrane potential despite changes in electrode resistance with injected current. The DCC cycling rate was typically around 3 kHz with optimal capacitance compensation. Most often slow depolarizing and hyperpolarizing ramps of currents were used (triangular current ramps), but some step changes of constant current levels were also employed. During many of the motoneuron recordings we also recorded efferent discharges from the hindlimb nerves via the same electrodes as used for antidromic identification purposes. This was rarely done in the early experiments, where the focus was on the voltage-dependent amplification of synaptic potentials, but once it was realized that a locomotor drive was sometimes present in the recordings, then these electrodes were switched to their recording mode as soon as antidromic identification had been confirmed. The ENG recordings were done with custom built amplifiers and analog filtering (1–10 kHz) and digitized at a rate of 10 kHz. Full wave rectification and additional filtering was done during analysis so that the onset and the offset of ENG bursts in each nerve were identified by visual inspection of ENG levels crossing a baseline defined by no activity periods. These onset and offset points were used during cycle-based averaging of ENG activity. The data were collected and analyzed with a Canadian software-based QNX-system, developed by the Winnipeg Spinal Cord Research Center to run under a real-time Unix personal computer, usually using separate runs of 200 s duration.

RESULTS

MAYER WAVES AND DISCHARGES IN HINDLIMB MUSCLE NERVES

The initial aim of the experiments was to investigate the occurrence of CRDPs in hindlimb motoneurons of decerebrates such as were previously reported to occur under anesthesia (Kirkwood et al., 2002; Ford and Kirkwood, 2006), concentrating in particular on their voltage dependency. Our monitor nerve discharge was therefore that of the phrenic (or, in one instance, an external intercostal nerve in lieu of the phrenic). In all preparations, CRDPs were common, almost all being of the expiratory decrementing (E_{dec}) type, as in Ford and Kirkwood (2006). In 4 out of the 7 experiments, the amplitudes of the CRDPs were relatively small, and often showing some amplification with depolarization, again similar to that previous study, although now with a higher occurrence of plateaux. When tested, no efferent discharges were present in the hindlimb nerves. In contrast, in the remaining three experiments, the respiratory drive in the hindlimb motoneurons was often strong, and additionally at some times during the experiment spontaneous LDPs were also seen. In the first of these experiments, the LDPs were only identified in the recordings *post hoc*, but in the other two experiments, the LDPs were confirmed as such by recording periodic (often flexor-extensor alternating) efferent discharges from the hindlimb nerves. These three animals were also characterized by periodic fluctuations in blood pressure, slower than the respiratory rate, with intervals between the blood pressure maxima ranging from 9 to 104 s, though at any moment during an experiment, the interval could be very variable. The mean interval was assessed in each of the computer runs where the fluctuations could be readily distinguished, the mean of these values being 39 s ± 17 SD ($n = 71$). We are taking these as Mayer waves, despite the frequencies mostly being slower than those classically recognized under this term (see Discussion). Here the Mayer waves appeared variably in the course of the recording sessions, often occurring as isolated phases of increased blood pressure on a steady background (e.g., see later in **Figures 5**, **9**). However, they also could occur, especially when the frequency was higher, as a continuously fluctuating waveform or, occasionally, as periodic dips in blood pressure from a steady high level (e.g., see later **Figure 3**).

Clinically, Mayer waves may be seen in pathological situations (e.g., in hypovolemia), but it should be noted that these three preparations were among those in apparently the best condition. One of them, which provides many of the illustrations here, was specifically noted as such in the experimental protocol, before any recordings had been made. In this animal there had been minimal blood loss during the decerebration procedure and it showed a brisk CO_2 response in the phrenic nerve, including hypocapnic apnea. An overall characteristic of these three preparations was a minute-to-minute variability of state, as reflected in the patterns of the Mayer waves, CRDPs, LDPs, and nerve discharges. However, a universal observation was that whenever the Mayer wave behavior was marked then either or both of the respiratory and locomotor drives were modulated in relation to the Mayer wave cycle. It is this modulation which is the subject of this report.

As illustrated in **Figure 1** (and later in **Figure 5**), most often the phrenic nerve discharge in the inspiratory phase just preceding the blood pressure rise was particularly intense while the

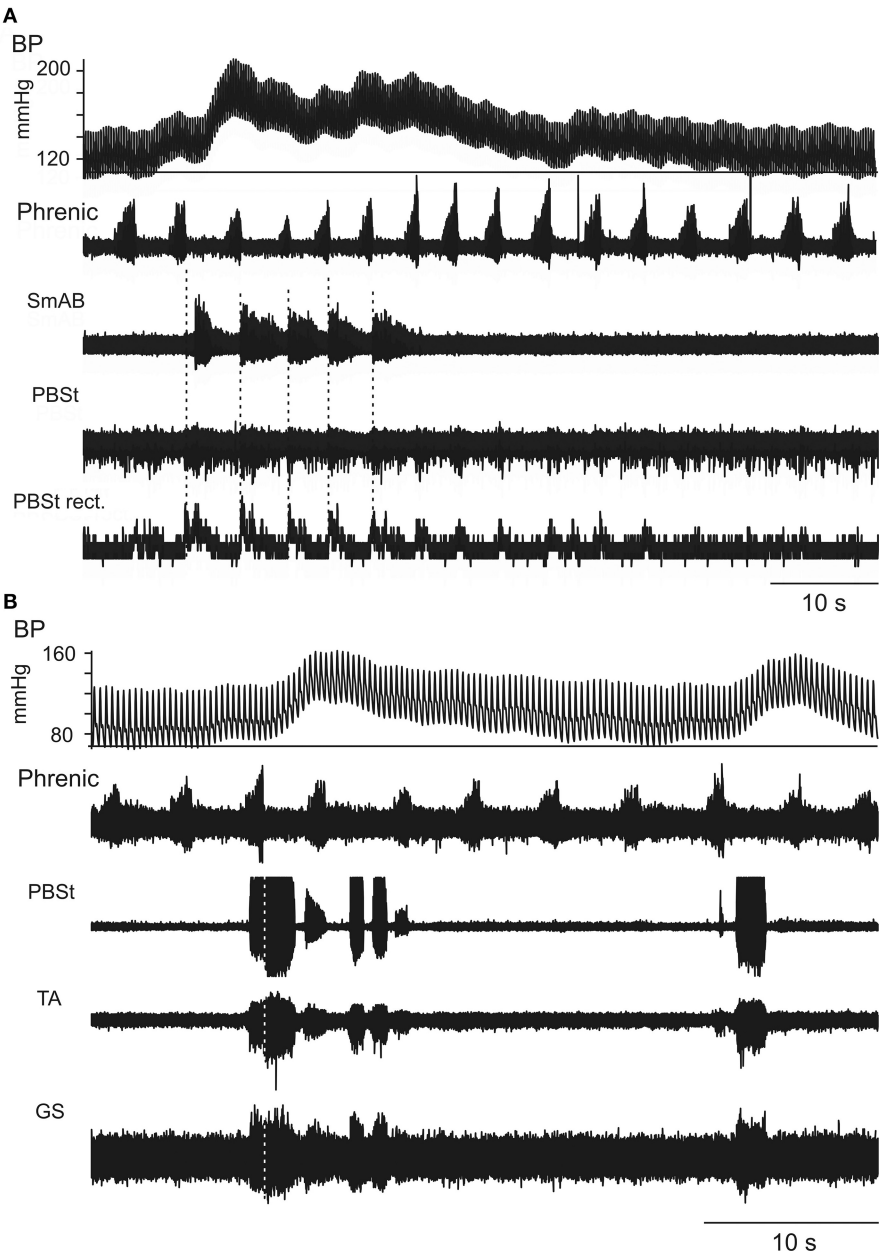

FIGURE 1 | Two different patterns of coupling between phrenic (inspiratory) discharges and hindlimb muscle nerve discharges. Appearance of one **(A)** or two **(B)** cycles of Mayer waves, as shown in the blood pressure (BP) traces and the phrenic nerve recordings (second traces). As was common, the phrenic nerve discharges in the respiratory cycle or cycles at or just before the hypertensive phase were more intense than in average respiratory cycles and the discharges during the hypertensive phase were less intense. In **(A)** the initial increase prior to the Mayer wave is difficult to judge as it occurred at the very beginning of the recording, but the less intense discharges during the blood pressure increase are easily seen. The following three traces show **(A)** the raw ENGs from SmAB and PBSt, together with the rectified and integrated version of the PBSt record, **(B)** the raw ENGs from PBSt, TA, and GS. As is typically the case, the intensity of the discharge in these nerves was highest during the hypertensive phase and was higher in the thigh and knee muscles [the PBSt record in **(B)** is saturated], while that in TA and especially in GS were weaker. Note that the typical expiratory decrementing pattern (E_{dec}) in **(A)** involved co-activation of flexors and extensors. **(B)** Shows a variant of this, where post-inspiratory bursts were present at the start of the hypertensive phases, but were accompanied by variable bursts during the end of inspiration and later into subsequent respiratory cycles. Vertical dotted lines indicate the end of inspiration.

phrenic nerve discharges in the following respiratory cycles were less intense than in steady state, a modulation with a similar phase difference to that shown by Preiss et al. (1975). **Figure 1** includes two different examples of the phrenic modulation, a relatively continuous variation in **Figure 1A**, and a modest increase of probably only one phrenic burst per Mayer-wave cycle in **Figure 1B**. Another variation, even closer to the behavior described by Preiss et al. (1975), was one where several respiratory

cycles showed an elevated phrenic discharge starting just before the blood pressure rise. An illustration of this was included in the Supplementary data of Enríquez Denton et al. (2012). **Figure 1** also illustrates one of the typical patterns of nerve discharges associated with the occurrence of Mayer waves, a co-activation of flexors and extensors. Most often, as in **Figure 1A**, this generalized nerve discharge occurred in the post-inspiratory phase, briefly after the end of the phrenic nerve discharge (Richter, 1982). However, with the first Mayer wave in **Figure 1B** the co-activation actually started during late inspiration and it was repeated a few times during the period of elevated blood pressure before falling silent. At the second Mayer wave in **Figure 1B** there was only a very brief and weak co-activation during inspiration, while the major discharge occurred, as usual, in the post-inspiratory phase. The two examples in **Figure 1** were typical of individual Mayer wave cycles in each of those two preparations in the particular states pertaining at the time (see later for a description of state variation). We describe below some of the variations seen in the other states.

In some cases the post-inspiratory co-activation of flexors and extensors occurred for each respiratory cycle for prolonged periods when Mayer waves were weak or absent, as seen in **Figure 2**.

The occurrence of Mayer waves was also frequently related to the initiation of short-lasting episodes of fictive locomotion. One such case is illustrated in **Figure 3A** in which locomotor-like activity is seen during the high-pressure periods in three Mayer wave cycles. At a faster time base in **Figure 3B** it is seen that the knee flexors PBSt alternated with the activity in the hip extensor SmAB and ankle extensor GS. However, the antagonist ankle flexor TA nerve activity coincided with the GS activity in this particular example (see further in the Discussion).

INTERFERENCE BETWEEN MAYER WAVE-RELATED HINDLIMB NERVE ACTIVITY AND SPONTANEOUS FICTIVE LOCOMOTION

One essential question emerging from the results in the preceding section is related to the identity of the spinal circuits mediating the respiratory-related discharge in the hindlimb muscle nerves. Are they part of the spinal CPG for locomotion or unrelated to that network? In the un-anesthetized decerebrate preparation locomotor-like activity sometimes develops spontaneously (Frigon and Gossard, 2009), as was the case for the three preparations, whose behavior we describe here. In these preparations we noted various examples of perturbations (resetting) and entrainment of the spontaneous locomotor rhythm by the central respiratory activity as monitored by the phrenic nerve discharge, and where the intracellular motoneuron recordings gave us particular insights.

The motoneuron recordings shown in **Figure 4** illustrate two aspects of the interaction between the respiratory and locomotor rhythms. In **Figure 4A** (a PBSt motoneuron) it can be seen firstly that, during the periods when the blood pressure was rising and maintained at a high level (right-hand side of the figure), every discharge in the phrenic nerve (marked by open arrowheads on top of the phrenic nerve ENG) was clearly related to a short interruption of PBSt nerve discharges (partly also seen in the SmAB nerve) together with a striking decrease of the flexor-related LDPs (filled arrows). During such a period, the PBSt discharge was exaggerated in the immediate post-inspiratory phase, as was the depolarization in the motoneuron. Secondly, in the relatively short periods of reduced blood pressure (and also during the decay phase of the preceding Mayer wave), the LDPs were not just interrupted or modulated, but are replaced by a post-inspiratory CRDP, the third cycle of which (immediately before the LDPs commence) appears to involve a plateau potential. A somewhat

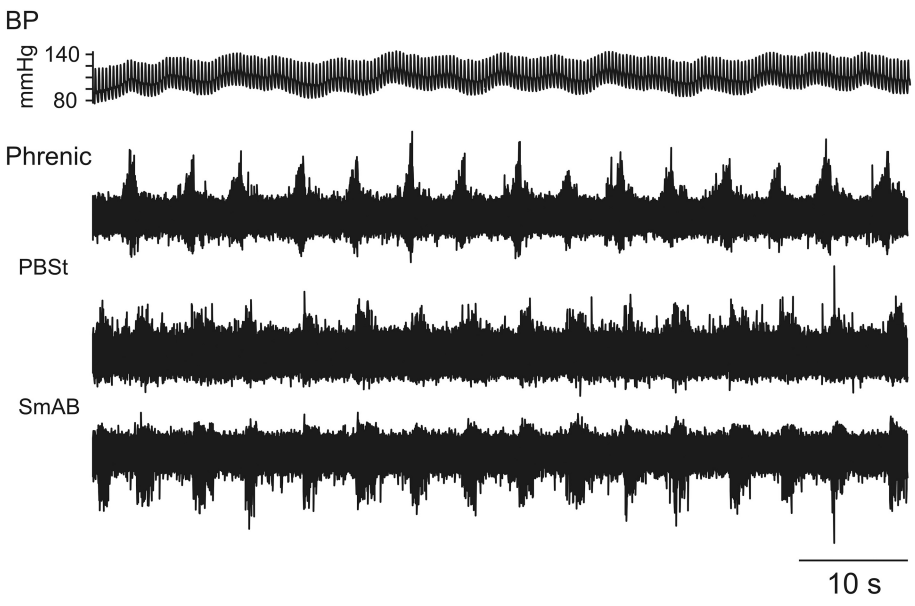

FIGURE 2 | Maintained post-inspiratory co-activation of hindlimb muscle nerves following every phrenic nerve discharge. In this case the maintained post-inspiratory co-activation of PBSt and SmAB occurred steadily in the absence of Mayer wave behavior.

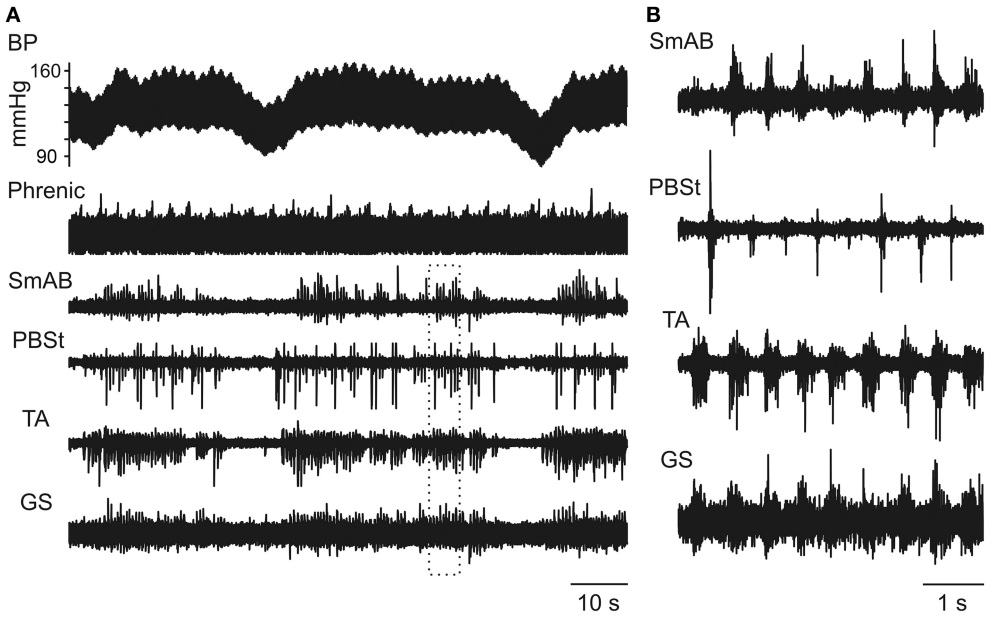

FIGURE 3 | Spinal locomotor-like activity may be initiated in relation to Mayer waves. (A) Three Mayer wave cycles are illustrated, where these consisted of relatively short-lasting hypotensive phases from a more steady value. Note that intermittent nerve discharges commence at the BP increase and cease at the BP drop. The alternation between discharges in the different nerves during these periods is seen better in the expanded sweep in **(B)** [from the period marked by the dotted rectangle in **(A)**]. Note that TA and GS were co-activated in this example, though alternating with PBSt. Also note that the intensity of the PBSt (and partly TA) discharges were also modulated according to the respiratory cycle [approximately one respiratory cycle included in **(B)**].

similar sequence is seen in **Figure 4B**, where the respiratory and locomotor rhythms again alternated according to the phase of the Mayer wave cycle. This can be seen in the nerve discharges, but is particularly clear in the (GS) motoneuron recording in this instance. At the beginning of the recording (10–20 s, during the decay-phase of the preceding Mayer wave and the period with low blood pressure), the nerve activity was dominated by the respiratory drive then followed by locomotor activity, corresponding to the rising blood pressure. The CRDP in this instance is dominated by a post-inspiratory inhibition (together with a PBSt discharge). This is significant, because it suggests that, at least in some instances when only a single (post-inspiratory) phase of the respiratory drive is evident in the hindlimb discharges, important components of the interneuron circuitry involving reciprocal inhibition appropriate to locomotion may nevertheless be recruited. These examples strongly suggest that the respiratory drive to the motoneurons not only interferes with, but may be mediated by, the spinal locomotor CPG circuits.

In **Figure 5A** there is an example of ongoing locomotor activity (seen as LDPs in the recorded SmAB motoneuron). In the middle of the recorded period there is a large inspiratory discharge (box) followed by a Mayer wave as in **Figure 1**. The phrenic nerve recording and the intracellular drive potential are shown at a faster time base in **Figure 5B**. In **Figure 5C** we show the variation—or rather stability—of the locomotor cycles, with the exception of cycles 10 and 11 marked below the motoneuron recording. It is obvious that in relation to the large phrenic nerve discharge just before the BP increase there is a very short cycle (i.e., the depolarising phase starts earlier than expected) followed by a very long duration for the next cycle. There is resetting of the locomotor rhythm here (the sum of cycles 10 and 11 is longer than any other pair of adjacent cycles), but it is relatively subtle: the timing of the rhythm recovered after a very brief interruption and it is not clear whether the respiratory input really caused an interference with the locomotor CPG rhythm-generator, or whether the modulation coinciding with the Mayer wave event simply slowed the locomotor rhythm and the large depolarisation reflected convergence at motoneuronal level.

The data of **Figure 5** come from a long period (about 3 h) in one cat, where a continuous locomotor rhythm was present (see later section). Despite the presence of isolated, relatively large Mayer waves as in **Figure 5**, there were also periods of minimal Mayer wave activity. We therefore looked carefully at these periods for indications of influence of the respiratory rhythm on the locomotor rhythm independent of Mayer wave occurrence. **Figure 6** illustrates one of these periods. In A the raw records are included from a part (33 s) of the 100 s long recording (in all 17 phrenic nerve discharge cycles; only few of them are illustrated in **Figure 6A**). Even from these raw records it can be seen that there is a tendency for the phrenic discharge to be followed closely by a flexor discharge (illustrated for PBSt), but there were also examples where this was not the case (e.g., the phrenic nerve discharges marked by asterisks in the upper rectified trace in the period illustrated in **Figure 6A**). The tendency of a post-inspiratory flexor discharge was formally tested for all 17 respiratory cycles. In **Figure 6B1** we show the discharges of the PBSt nerve from the recording shown in A, averaged in relation to the offset of the phrenic nerve burst, which demonstrated

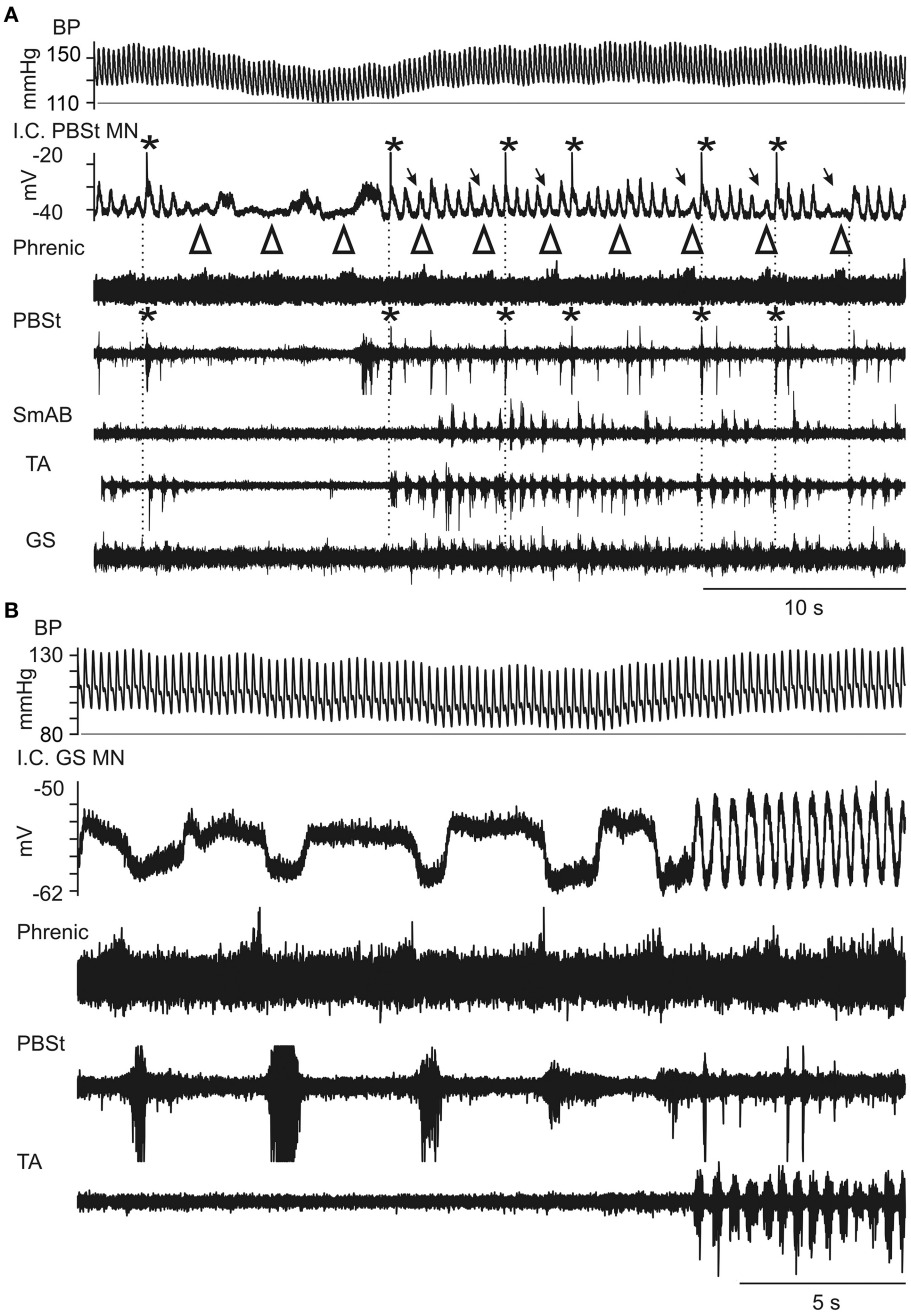

FIGURE 4 | Expanded records of Mayer wave-initiated locomotor-like activity during intracellular recording from two motoneurons. **(A)** From a PBST motoneuron. The largest bursts in the PBSt nerve occurred in the post-inspiratory phase of respiration, corresponding to the largest depolarizations of the LDP in a PBSt motoneuron (asterisks for both). These depolarizations often elicited spikes (truncated). During inspiration, indicated by open arrowheads above the phrenic recording (whose discharge appears weak, most likely a recording problem), there was a decreased amplitude of the LDP (arrows). Between the episodes of locomotor-like activity, the respiratory drive was evident as a CRDP in the PBSt motoneuron, consisting of rather variable depolarization in post-inspiration (arrowheads), and with a corresponding weak discharge, just detectable in the PBSt nerve. The prolonged depolarization visible in the intracellular record following the third arrowhead corresponds to the start of the locomotor episode and probably involved a plateau potential. Same nerve recordings as in **Figure 3**. **(B)** Similar behavior seen in a recording from the next motoneuron (GS) penetrated in the same preparation. Again, a CRDP is seen in the intracellular recording, in alternation with an LDP, according to the phase of the Mayer-wave. The GS nerve was silent at this time (not shown), but the apparent strong post-inspiratory inhibition in the motoneuron shows the alternation between GS and PBSt for both the CRDP and LDP, similar to that between TA and PBSt during the locomotor episodes. TA was silent during the respiratory episodes, but alternated with PBSt during the locomotor episodes. It is possible that persistent inward current contributed to the depolarizations in this cell, but periods of de- and hyperpolarizations (not shown) indicated that strong phasic inhibition of a tonic background excitation could be sufficient to explain the CRDP/LDP in this instance.

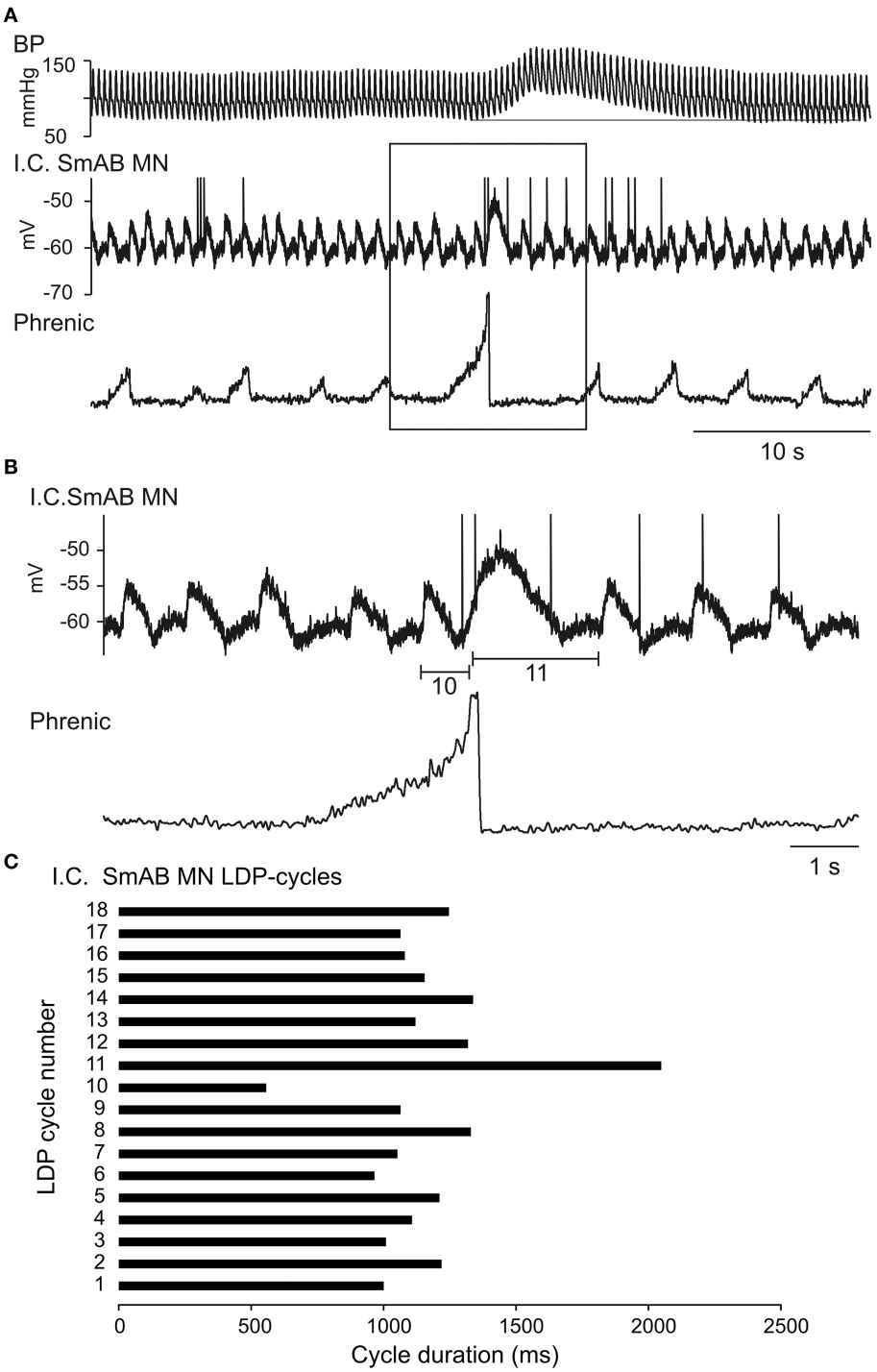

FIGURE 5 | Demonstration of the interaction between a regular LDP and a single large post-inspiratory depolarization at the beginning of a Mayer wave event. In **(A)** is shown a rather long-lasting recording of BP, intracellular recording from a SmAB motoneuron and the rectified phrenic nerve ENG with an incident of a large inspiratory discharge. In **(B)** is shown at an expanded time scale the part marked by the rectangle in **(A)**. In **(C)** is shown the cycle duration of the LDPs, the short (no 10) and long (no 11) duration cycles marked in **(B)**.

an apparent entrainment with a post-inspiratory flexor burst, and 4–5 locomotor cycles for each respiratory cycle. Note that the amplitude of the averaged waveform is about 60% of that of the raw integrated rectified PBSt discharge, thus indicating a rather strong phase-coupling. These features (including the same phase relation of the average to the phrenic discharge) were preserved, when this period of 17 cycles was split into two separate periods (7 and 9 cycles, data not shown). The

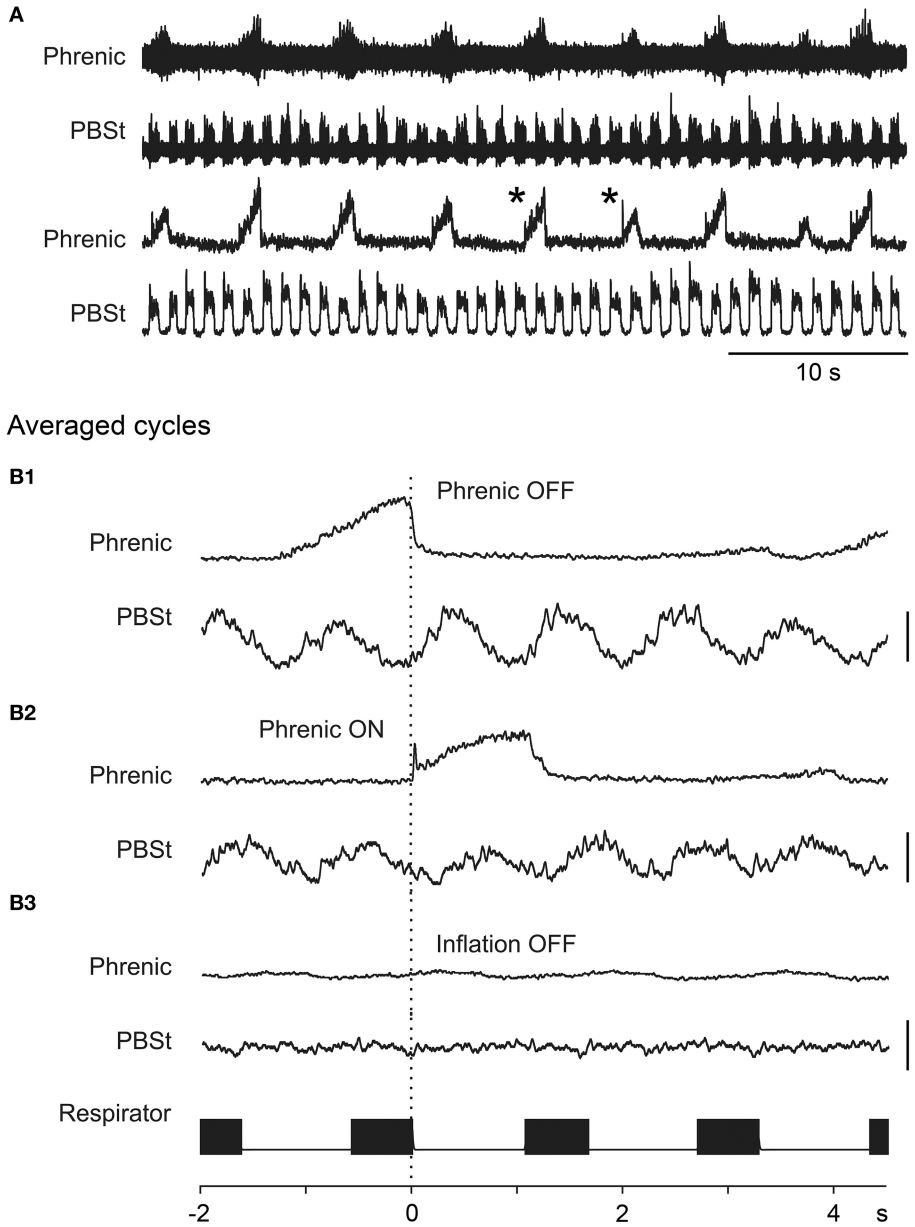

FIGURE 6 | Entrainment of the locomotor rhythm by the phrenic nerve discharge. (A) A segment of spontaneous ENG discharges in the phrenic and PBSt nerves. The lower two traces show the integrated rectified versions of the upper two traces. In **(B1–B3)** are shown the discharge in the PBSt nerve where the cycles were averaged in relation to the offset of the phrenic nerve burst **(B1)**, the onset of the phrenic nerve burst **(B2)**, and the offset of the inflation phase of the artificial respiration **(B3)**. The respirator signal was derived from a record of the opening of the inflation valve on this device (filled boxes in respirator trace indicate inflation phases). The averages were derived from a continuous recording, consisting of 17 respiratory cycles; in **(A)** is shown only a part of that recording session. The gains for the averaged traces are constant for **(B1–B3)**, for both the phrenic and the PBSt averages. Calibration bars for the PBSt averages indicates 50% of the average amplitude of individual PBSt integrated rectified bursts. The asterisks in **(A)** indicate two respiratory cycles where a phrenic burst was not followed immediately by a PBSt burst.

entrainment appears strongest when averaged in relation to the end of the phrenic discharge, most likely because the predominant excitatory event occurred in the post-inspiratory phase and the duration of the phrenic discharge was variable (cf. Richter, 1982). However, the averaged PBSt discharge is clearly visible also when triggered on the onset of the phrenic discharge (**Figure 6B2**).

The animals were paralyzed and artificially ventilated at a rather high frequency and low volume, with pneumothorax (cf. Methods). Nevertheless, it would have been possible that activation of thoracic, abdominal or vagal mechanoreceptors could have entrained the locomotor (and/or respiratory) rhythm. However, when the PBSt activity was averaged in relation to the pump (offset of inflation) there was absolutely no sign of

entrainment (**Figure 6B3**). Therefore, we conclude that in this case there is a strong entrainment of the locomotor rhythm by the intrinsic respiratory rhythm.

One other such period (20 phrenic cycles) showed the same effect (the same phase), but in five other periods analyzed, this was not really the case. The amplitude of the averaged PBSt ENG was very small suggesting the lack of a strong phase-locking, and thus a lack of evidence for entrainment. Overall, therefore, this long period of continuous locomotor discharge represented a wide spectrum of effects, with examples of a strong coupling and more often an independence between the two rhythm generators.

VOLTAGE-DEPENDENT AMPLIFICATION OF LDPs, MUSCLE STRETCH EVOKED EPSPs, AND CRDPs

A general finding in these experiments was that all of these drive potentials showed a strong voltage-dependent amplification in the voltage region where recruitment of PICs would be expected to contribute to an enhancement of their amplitudes. Such measurements are not always easy to obtain since reciprocal inhibition (through different inhibitory interneurons in between the excitatory periods) also will increase the peak-to-peak amplitude at increasingly depolarized levels. Furthermore, the membrane resistance is reduced at depolarized levels (both by opening of PIC channels and K^+ channels). Nevertheless, at least qualitative evidence for such amplification was commonly obtained. **Figure 7A** shows the voltage-dependent amplification of an LDP in a SmAB motoneuron in confirmation of Brownstone et al. (1994). This was demonstrated in 16 out of 16 motoneurons in the present experiments, although a sizeable inhibitory component was suspected in five of these). The decrease of the LDPs is best seen during the hyperpolarizing (descending) phase of the triangular current injection. During the peak of current injection the spikes inactivated and there was an obvious shunting. In **Figure 7B** (confirming Bennett et al., 1998) there is a similar potential-dependency of the EPSPs evoked by sinusoidal triceps surae stretches (in a GS motoneuron).

The voltage-dependent amplification of CDRPs (demonstrated in 12 out of 12 motoneurons) was sometimes very dramatic. In **Figure 8** we illustrate how a very small E_{dec} CRDP (in a GS motoneuron) was enhanced as the holding potential was increased from −82 to −75 mV and up to −62 mV. At the last level the depolarising phase triggered plateau potentials that usually fell off soon after initiation, and were fully terminated by a subsequent presumed inhibitory phase. These occurred in an all-or nothing fashion. The first two inspiratory discharges after the last depolarizing current step were not followed by plateaux, then the first plateau was not related to the inspiratory discharge, but appeared to be triggered spontaneously. At the very end of the trace the plateau potential was sustained for the whole respiratory cycle. In order to better visualize the potential-dependency we have averaged the intracellular recordings at the three different levels of current injection (−4 nA, +2 nA, and +6 nA) with reference to the beginning of inspiration (**Figure 8B**). The averages here show first, the presumed inhibitory component during inspiration, increasing, as expected, with depolarization, then, at +2 nA, a small, late depolarization occurred about 0.5 s into expiration.

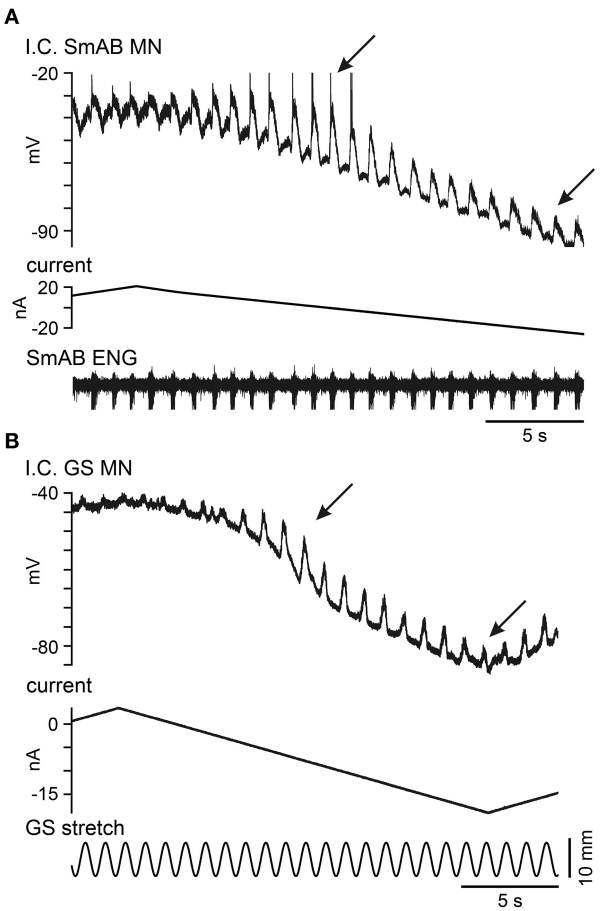

FIGURE 7 | Voltage dependent amplification of LDPs in a SmAB motoneuron and stretch-evoked Ia excitation in a GS motoneuron. (A) Recording from a SmAB motoneuron during spontaneous locomotor-like activity. The traces show the LDPs (top), the current injection (middle) and the SmAB ENG (bottom).The voltage dependent amplification of the LDPs (those between the arrowheads) are shown at the descending phase of a triangular current pulse. **(B)** Recordings from a GS motoneuron. The traces show the stretch-evoked EPSPs (top), the current injection (middle) and the sinusoidal muscle stretch of triceps surae (bottom). The arrowheads indicate the region where the amplification was present. At more depolarized levels the amplitude of the EPSP was smaller because of shunting (increased conductance), while the amplitude decreased at more hyperpolarized levels due to the voltage dependent amplification of the EPSP.

At +6 nA, the plateau-like depolarizations occurred at variable times between 0 and 0.5 s into expiration, as indicated by their initial spike-like components, which are clearly preserved in the average. However, because of this variability, the amplitude of the average plateau is attenuated as compared with the individual events.

EVIDENCE FOR CHANGES IN INTRINSIC MOTONEURON PROPERTIES IN RELATION TO MAYER WAVES

There is much evidence for Mayer waves reflecting an increased sympathetic drive at the postganglionic level (see Discussion). However, there is no direct evidence for a simultaneously

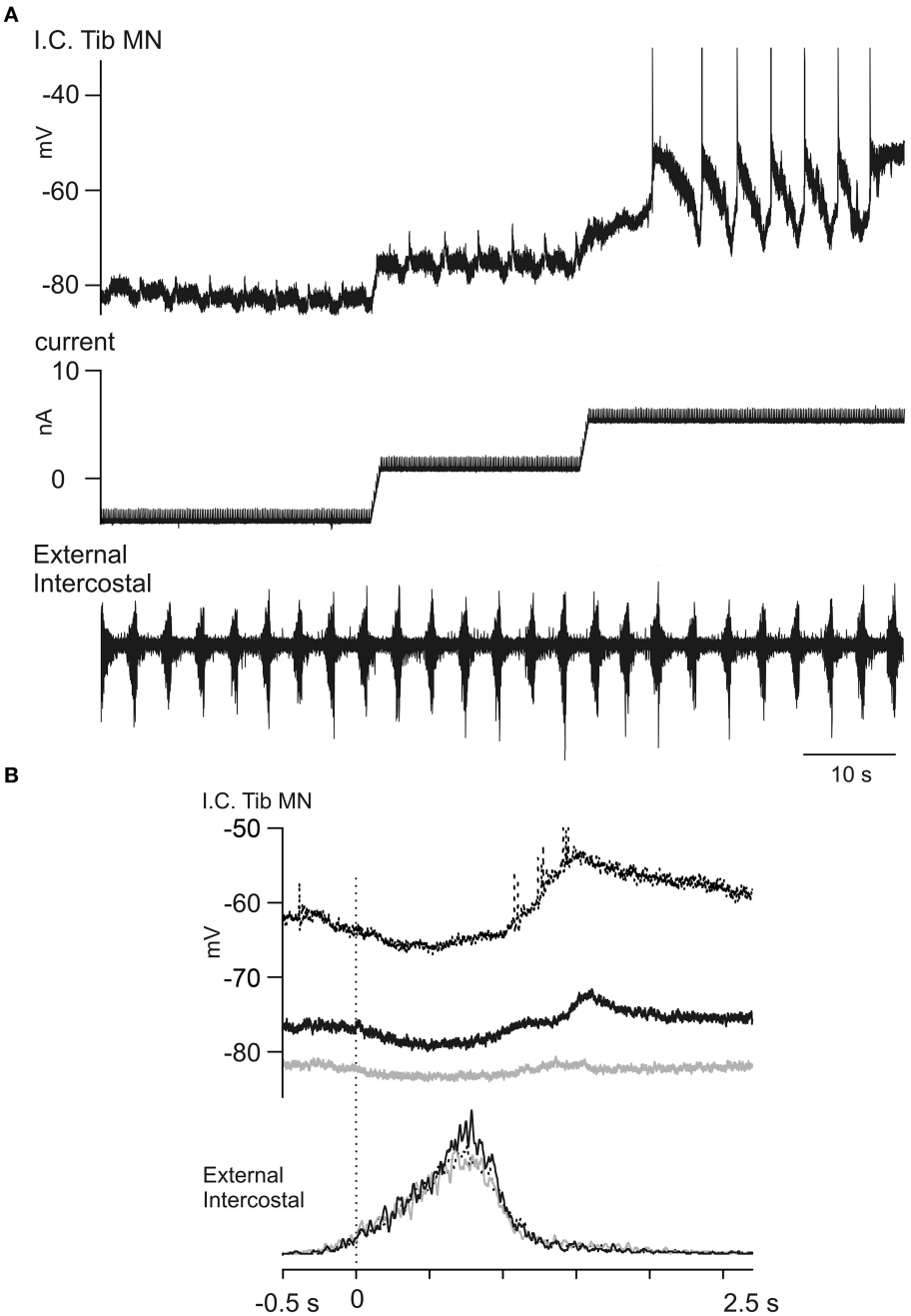

FIGURE 8 | Voltage dependent amplification of an E_{dec} CRDP. (A) The traces show a CRDP in a Tib motoneuron (top) during step-wise current injection (middle) and the T5 external intercostal ENG activity (bottom). The CRDP becomes larger at at more depolarized membrane potential levels. **(B)** The averaged CRDPs from **(A)**. The averages were performed separately at the three membrane potential levels (gray, black, and dotted, in increasing order), aligned to the start of inspiration. Note in the average the increased amplitude of the CRDP and the variable times of occurrence for the plateau-like events at +6 nA, which produces a relative attenuation in the averaged signal for this level ($n = 7$, 6, 7, respectively from low to high potential levels). Spikes truncated.

increased monoaminergic drive to the spinal cord, and we have not aimed to obtain direct evidence for such a drive in the current experiments (see further in Discussion). However, we have looked for signs of changes in intrinsic properties in the motoneurons during Mayer waves that are compatible with an increased monoaminergic drive onto them, promoting PICs and plateau properties. **Figure 9A** demonstrates how triangular current injections triggered plateau potentials (with a couple of initial spikes). The illustration shows a plateau potential that was initiated and terminated with lower current injection and at a more hyperpolarized membrane potential during the Mayer wave than during the first triangular current injection (no Mayer

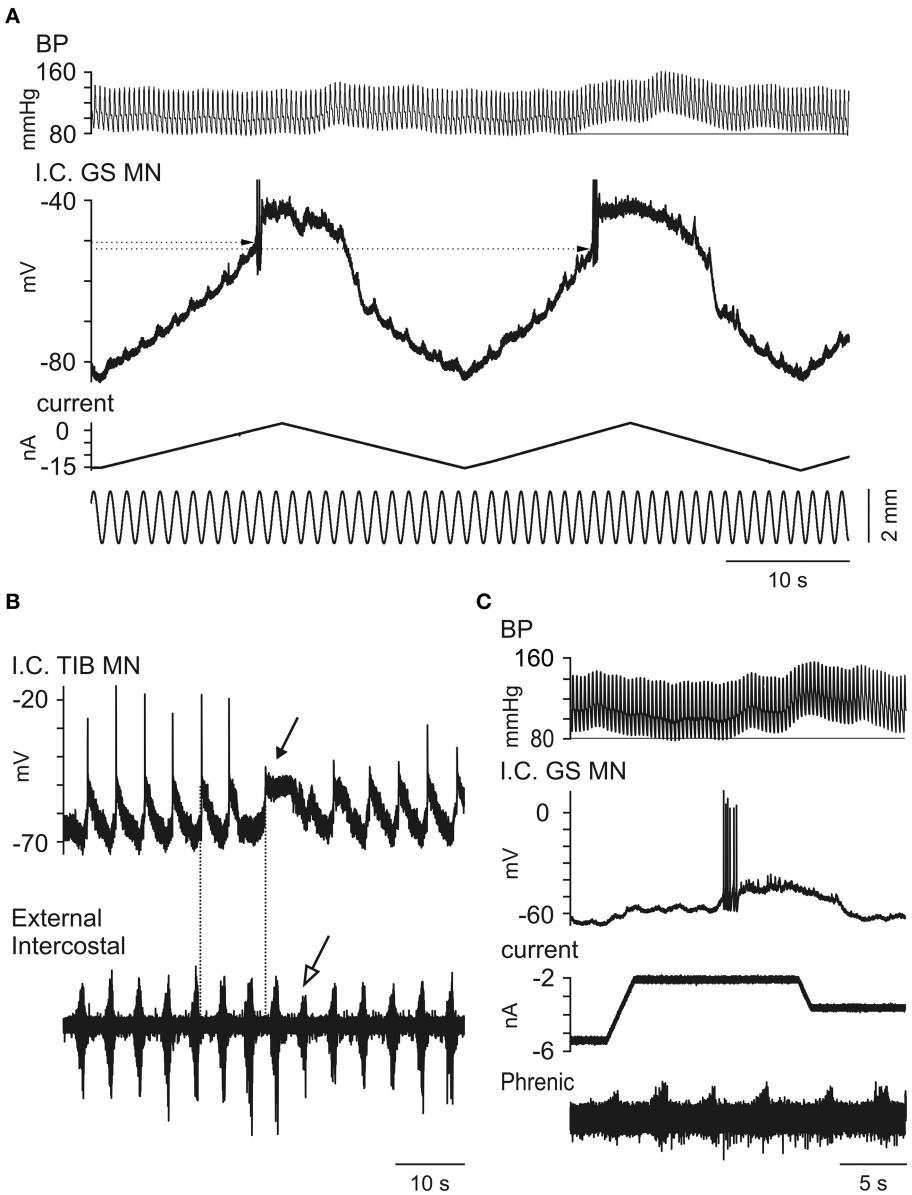

FIGURE 9 | Observations on changes in intrinsic motoneuronal properties in relation to Mayer waves. (A). Recurrent small depolarizations were evoked by small sinusoidal stretches of the triceps surae while triangular current injections were used to initiate plateau potentials. Note that at the beginning of the Mayer wave (at the second triangular increase of current) the threshold for evoking the plateau potential (dotted lines) is lower than during a lower-blood pressure period (i.e., during the first ramp). **(B)** CRDP recorded in a TIB MN. In this case the Mayer wave is inferred by the large external intercostal discharges followed by discharges with decreased amplitude (open arrow). Note the initiation of a plateau potential (filled arrow) even at a time when the CRDP is not expected (compare two dotted lines). **(C)** The traces show the BP, intracellular recording from a GS motoneuron, the injected current and the phrenic ENG. At a constant current injection period, minor potential fluctuations are visible and constant at the beginning of the recorded period, but shortly after the initiation of the Mayer wave one of these triggered both an intense firing and a plateau potential.

wave). The longer-lasting plateau during the descending phase during the Mayer wave is particularly striking, but from amplified records we could also confirm that the plateau was initiated at a 2.4 mV more hyperpolarized level (and with 3 nA less depolarizing current injection) than during the first trial before the onset of the Mayer wave. In another illustration, in **Figure 9B** it is noted that a spontaneous plateau potential was triggered "off-cycle," toward the end of the phase of increased inspiratory discharges (filled arrow) during the putative Mayer wave (in this case we did not record the blood pressure, but the large intercostal nerve discharges followed by a number of less intensive discharges (marked by an open arrow) at the following respiratory cycles were taken as an indirect indication of the Mayer wave cycle). This same sequence was repeated 5 times in this particular cell. In another motoneuron shown in **Figure 9C** it may be seen how the small potential fluctuations did not trigger a plateau potential

at normal blood pressure levels, but they did so when occurring at the very beginning of a Mayer wave. There were several examples of this kind, but as most of the Mayer waves appeared at irregular intervals a systematic investigation turned out to be difficult. Nevertheless, the present observations are highly suggestive of a neuromodulation that enhances the plateau-properties of motoneurons in relation to Mayer waves.

EVOLUTION OF THE GENERAL STATE OF EACH PREPARATION

In the preceding sections we have described a variety of different patterns of motor output. These did not occur at random, but each of the three preparations here showed a systematic change of state during the course of the experiment, though different for each preparation. Each of the observations reported in the preceding sections is only a snapshot, but each is nevertheless representative of longer recordings in that cell, and of the state. The recordings covered periods of 11, 7, and 9 h, respectively for the three preparations, and consisted of individual motoneuron (MN) recordings lasting from about 1 to 40 min. The states of each preparation can be summarized as follows.

Cat 1

Initial hindlimb nerve recordings were made in the absence of Mayer waves and showed PBSt discharges during post-Inspiration similar to those in **Figure 2** (although that illustration was actually derived from Cat 3). The initial 4 motoneurons yielded an uncertain picture as the phrenic nerve recording gradually failed. By the time an intercostal nerve had been prepared and intracellular recording resumed, Mayer waves were present and small amplitude presumed LDPs, amplified by depolarization, were detectable, sometimes alternating with CRDPs, according to the Mayer wave phase, which itself was clear from the intercostal nerve recording (as in **Figure 9B**). The amplitude of the Mayer waves was unknown (the blood pressure recording was not acquired by computer in this experiment), but was large enough by the time MN 10 was recorded to be noted in the protocol as an unusually large blood pressure variation being present. The CRDPs then became the dominant feature in the remaining motoneurons (up to MN 22). These CRDPs were strongly amplified by depolarization, involved plateau potentials, and were modulated in phase with the Mayer waves (**Figures 8**, **9B**).

Cat 2

Initial nerve hindlimb recordings were made during strong Mayer waves (amplitudes up to 45 mm Hg) with respiratory phased discharges in the nerves strongly modulated in phase with the Mayer waves (**Figure 1A**). During the early intracellular recordings, Mayer waves were initially absent, then returned (MNs 5, 6), then faded again (MNs 7–9). No LDPs and only very small CRDPS were present. The baseline blood pressure was steady at around 120/80. The phrenic discharge was only modulated when the Mayer wave amplitude was as high as 20 mm Hg. Between MNs 9 and 10 the blood pressure fell, but was restored by a single dose of Effortil (0.5 mg/kg). Following this, a strong locomotor drive became apparent, which persisted during the remaining 4 (relatively long) motoneuron recordings of this experiment. The Mayer wave amplitudes were smaller than in the initial runs (15–20 mm Hg) but nevertheless strong modulation of the motor discharges was apparent in phase with the Mayer waves (**Figures 3**, **4**). The Mayer waves gradually decreased in amplitude, but the modulation of the locomotor and respiratory discharges related to them persisted with amplitudes below 10 mm Hg.

Cat 3

This involved the largest number of motoneuron recordings (MN1–MN33). For the first 14 motoneuron recordings, only low amplitude Mayer waves were present, only low amplitude CRDPs and no LDPs. A weak respiratory-phased discharge was present in SmAB nerve in the one recording tested in this period. Modulation of the CRDPs and of the phrenic discharges in relation to the Mayer waves was only seen toward the latter part of this period (MNs 9–14) and only for larger amplitude Mayer waves (10–15 mm Hg). Then, for MNs 16–26, continuous LDPs were present, together with continuous locomotor discharges in the hindlimb nerves, as in **Figures 5**, **6**. Mayer waves were only sometimes present, generally of low amplitude ($\leq$15 mm Hg), and these were not accompanied by any obvious modulation of the motor output. However, occasional isolated large amplitude Mayer wave events (up to 40 mm Hg) also occurred, accompanied by resetting of the locomotor rhythm with regard to the respiratory cycle (**Figure 5**). For MNs 27–31, the Mayer waves became stronger and more regular, being accompanied first by a continuous respiratory drive in the hindlimb nerves in the periods between medium-amplitude Mayer wave events (**Figure 2**), then by the strongly modulated short (locomotor?) repetitive bursts synchronized to respiration and to large amplitude (45 mm Hg) Mayer waves (**Figure 1B**). For the last two motoneuron recordings, the Mayer wave amplitude decreased, as did the modulation of the locomotor discharges.

Overall, therefore, although not designed for this purpose, the structure of the experiments, which consisted of repeated measurements over many hours, revealed two or three different time scales of spontaneous modulatory variation. The first one, varying over hours to minutes, seemed to consist of the extent to which locomotor drives were present at all, a respiratory drive being present throughout (as it was in the four experiments not showing Mayer waves). The second one, varying over minutes to seconds determined, in phase with the Mayer waves, which of the two rhythms was transmitted to the motoneurones. Finally, the Mayer waves themselves waxed and waned independently of the locomotor drive, but over a similar time scale.

DISCUSSION

RELATIONS BETWEEN THE NEURONAL NETWORKS OF RESPIRATION AND LOCOMOTION AT LUMBAR LEVEL

In this report we have demonstrated that at spinal level there are intricate interrelations between the networks mediating a respiratory control of lower limb muscle activity and locomotor activity. In the decerebrate, unanesthetized and paralyzed preparation which we have used, the activation of these interneuronal circuits—and their interrelation—varied substantially with

the spontaneous occurrence of periods of increased blood pressure, i.e., the so called Mayer waves. During these periods the intrinsic behavior of motoneuron properties changed as might be expected if an increased monoaminergic drive to the spinal cord occurred in phase with the presumed sympathetic activity during the Mayer waves (cf. the examples in **Figure 9**, see below). It is well-established that monoamines are important transmitters in activating the spinal network underlying locomotor activity, the spinal locomotor CPG, as first demonstrated for the cat (administration of l-DOPA: Jankowska et al., 1967a,b; Grillner and Zangger, 1979, or the α_2 agonist clonidine, Forssberg and Grillner, 1973; Barbeau et al., 1987; Kiehn et al., 1992; Chau et al., 1998; Noga et al., 2006, 2007). In addition monoamines (NA; DA and 5-HT) have been used to activate spinal locomotion in the rabbit (Viala et al., 1979) and the marmoset monkey (Fedirchuk et al., 1998) as well as in adult rats (Iles and Nicolopoulos-Stounaras, 1996) and mice (Meehan et al., 2012). Monoamines, often in combination with NMDA receptor agonists, also activate the spinal CPG in the neonatal rat and mouse, (reviews; Schmidt and Jordan, 2000; Clarac et al., 2004; Miles and Sillar, 2011). Readers should also consult other articles in this Research Topic.

In general, the locomotor pattern evoked by the pharmacological cocktail is characterized by alternating flexor and extensor bursts, and by alternation between the two hindlimbs. However, at the start of the bursting activity it has been frequently noted that flexors and extensors were initially co-activated, as were sometimes the two limbs, and that the regularly alternating pattern only subsequently developed during the course of the recording session (Fedirchuk et al., 1998 in the marmoset monkey; Meehan et al., 2012 in the mouse; and more infrequently in the cat preparation, unpublished observation from Hultborn's laboratory). These observations are relevant for the interpretation of the pattern of co-activation of flexors and extensors in the post-inspiratory phase (**Figures 1**, **2**). One interpretation would be that the interneurons mediating such co-contraction are different from the locomotor circuits, but an alternative explanation would be that the respiratory drive is activating parts of the locomotor network in a functional configuration which causes a co-activation. As support for the latter explanation the prolonged locomotor periods in **Figures 3**, **4** actually display a co-activation of the ankle extensors (GS) and flexors (TA), although flexors and extensors otherwise were alternating. Even more convincing evidence for the respiratory drive working onto—and at least partly through—the locomotor circuits are the inhibitory components of the CRDP in **Figure 4B**, together with the possible resetting (**Figure 5**) and certain entrainment (**Figure 6**) of the locomotor rhythm by the respiratory one.

A correlation between actual motor activity and respiration has long been recognized, and is of course related to increased demand for oxygen and removal of CO_2. The regulation of the increased ventilation is complex and involves both feed-forward and feed-back mechanisms. It has been studied in humans (Krogh and Lindhard, 1913; Asmussen, 1983; Haouzi, 2006), as well as during the initiation of locomotion in the decerebrate cat (DiMarco et al., 1983). In relation to locomotion, several groups have established an entrainment of the respiratory rhythm by the locomotor activity (Kawahara et al., 1989), which to a large extent seems to originate from limb proprioceptive inputs (Potts et al., 2005; Giraudin et al., 2012), but there is also evidence for a central entrainment of the respiratory pattern by the spinal locomotor circuits (Perségol et al., 1988; Le Gal et al., 2014). The interactions between these two CPGs, the medullary respiratory one and the spinal locomotor one have been described at length in a series of papers from Viala and her colleagues, including also the likely cervical spinal respiratory oscillator, which is not of concern here. In nearly all of these papers the focus is on the dominance of the locomotor over the respiratory CPG, though in two publications from this group (Viala, 1986; Perségol et al., 1988), the authors allowed for the reverse to occur. In these papers (both including fictive activations) the authors describe how the dominance of one rhythm over the other can be varied. The present observations, which show an entrainment of the locomotor circuits by the respiratory rhythm, are closest to their data in the circumstances of a raised level of CO_2, which was also the circumstance of most of our observations. However, their preparations were different from ours: rabbits with, for the critical observations, a very caudal decerebration and locomotion induced pharmacologically. We suggest the dominance of the respiratory over the locomotor CPG was even stronger for our observations, because the high level of CO_2 was combined with only spontaneous locomotion. Some of our recordings, where a locomotor pattern alternated with a respiratory one were also remarkably similar to one previous publication in the cat (Figure 5B in Romaniuk et al., 1994). It may be proposed that by stimulating within the more caudal part of the "locomotor strip" (in the medulla) Romaniuk and his colleagues also gave only a relatively weak drive for locomotion. The only difference between their result and ours is that their forelimb nerve discharge showed an inspiratory rather than post-inspiratory pattern when the locomotor pattern did not prevail. However, this is quite compatible with previous observations of respiratory drives recorded intracellularly in forelimb (Enríquez Denton et al., 2012) or hindlimb (Ford and Kirkwood, 2006) motoneurons. By showing the linkage between the two CPGs our present observations also help to explain the many various earlier observations of respiratory influences on limb functions. Meyer-Lohman (1974) can be consulted for an early description of post-inspiratory effects in hindlimb motoneurons, and for even earlier references of respiratory effects.

The question of what function is served by these interactions remains. In addition to variable interactions between the central rhythm generators for spinal locomotor and respiratory control in the adult preparations, the coactivation has also been described in the fetal (Greer et al., 1992) and in the neonatal rat preparations (Morin and Viala, 2002). The coactivation of these CPGs and their variable coupling likely represents an "open-loop" operation of these networks with reduced "functional" control, as both feed-forward and feed-back mechanisms are depressed in the preparations used for studying them. In other words, the inter/descending/propriospinal-neuron networks coupling these two systems are hard-wired in the spinal-brainstem circuits very early during development, and they become very much under the control of sensory feedback and feed-forward signaling from the brain as the animals mature. Under such control, the role

of the interactions could be many and varied (see Discussion in Schomburg et al., 2003).

For interactions in the direction of locomotion to respiration, the function is the most obvious, and has been much discussed in terms of respiratory efficiency, or for minimum work in general, especially in situations such as galloping or, for birds, flying (Boggs, 2002). In the opposite direction, as suggested here, the function is less obvious. In this context, it should be remembered that the respiratory CPG, or the neurons within it, can be readily configured for other functions, which include vomiting, coughing, defecation, perhaps even mating (for references see Kirkwood and Ford, 2004). Thus, the influences we are reporting from decerebrate preparations may reflect the operation of connections evolved for motor acts other than respiration, and where the roles of the limbs may be more essential. These roles may also be different for the fore- and the hindlimbs. For the latter the connections may serve the functions just suggested, for the former they may serve accessory inspiratory actions. A large number of possible functions (largely still speculative) for the post-inspiratory (or E_{dec}) pattern of excitation in hindlimb motoneurons were discussed by Ford and Kirkwood (2006).

DO MAYER WAVES REFLECT AN INCREASED DESCENDING MONOAMINERGIC DRIVE TO THE SPINAL CORD?

First we will summarize some facts and questions about the so-called Mayer waves. Slow oscillations (6–9 cycles/min) in arterial pressure were described by Mayer in 1876 in anesthetized rabbits (Mayer, 1876). The review by Julien (2006) describes that the dominant frequency varies between species (∼0.1 Hz in humans; ∼0.3 Hz in rabbits and ∼0.4 Hz in rats and mice), and also that the value depends on the experimental circumstances such as anesthesia, body position and circulatory conditions. In experimental animals and in humans, simultaneous recordings of low frequency increases in arterial blood pressure (at much slower frequency than the respiratory movements themselves) and efferent sympathetic activity have revealed a strong correlation (see review by Julien, 2006). Nevertheless, the origin of these oscillations has been the subject of much experimental work and discussion, mainly focusing on two possible explanations which are not mutually exclusive; (1) the pacemaker theory assuming a central oscillator (in the brain stem) for the genesis of Mayer waves, and (2) the reflex theory emphasizing the baroreflex loop. A large number of experimental studies (reviewed by Julien, 2006) support both hypotheses. In animal experiments the baroreceptor loop can be easily opened either by surgical denervation or by pharmacological means, and this intervention certainly reduces the Mayer wave activity. On the other hand there are observations on slow waves of sympathetic nerve activity even when the blood pressure is clamped and the baroreceptor loop opened, thus emphasizing the presence of a central pacemaker (Preiss and Polosa, 1974; Preiss et al., 1975). As pointed out by Malpas (2002), evidence such as baroreceptor denervation reducing Mayer wave activity is very weak as to the baroreceptor role in an oscillating feedback loop, it only shows that these receptors provide a facilitatory input. However, experiments which show that the activity survives the opening of such a loop, such as those in Preiss and Polosa (1974), provide very strong evidence that Mayer waves can be generated purely centrally. Modern reviewers are dismissive of this evidence, because the Mayer wave frequency in those experiments was much lower and the amplitudes of the pressure variations were much higher than those usually now considered as Mayer waves, and also because Preiss and Polosa (1974) induced these waves by severe hypovolemia. In terms of amplitude and frequency of the blood pressure variation, our experiments are close to those of Preiss and Polosa, though, as mentioned in the first section of the Results, they did not need a hypovolemic stimulus. We also have independent evidence for a central origin of the Mayer waves here, in that the motor actions were fictive and, in the majority of cases where the Mayer waves appeared as isolated increases from a flat baseline, the first event was an increase in the phrenic discharge, before the rise in blood pressure occurred, similar to the phase difference reported by Preiss et al. (1975), also for fictive respiration in decerebrate preparations.

A different question is related to the identity of the descending pathways mediating the activation of the preganglionic sympathetic neurons. It has been demonstrated that neurons of the caudal raphe nuclei project to the intermediolateral cell column (Bacon et al., 1990), but investigations on the serotonergic actions on the sympathetic activity has been dogged by methodological problems (cf. review by Lovick, 1997), although there is evidence for a 5-HT-2-mediated excitation (Orer et al., 1996). There is also evidence for Mayer-wave related modulation in several of the caudal raphe neurons. Bruinstroop et al. (2012) have more recently documented a strong projection from the locus coeruleus onto the thoracic intermediolateral cell column where most of the cells are excited by α_1 receptors (Lewis and Coote, 1990a,b; see also Samuels and Szabadi, 2008). There is, however, no direct evidence for locus coeruleus neurons to be activated in relation to the Mayer waves.

Even though Mayer waves are correlated to efferent sympathetic activity there is so far no direct evidence for an increased descending monoaminergic drive to the spinal cord during these waves. It would be plausible that the brainstem activity during the Mayer waves is activating the preganglionic neurons in the intermediolateral cell column via descending glutamatergic pathways, which may also activate the spinal locomotor circuits. It is well-established that monoaminergic innervation of the spinal cord affect the intrinsic properties of the motoneurons, enhancing their PICs and thus their plateau properties (see e.g., Hounsgaard et al., 1988; Hounsgaard and Kiehn, 1989 for original articles and several subsequent reviews: Powers and Binder, 2001; Heckman et al., 2003, 2005, 2009; Hultborn et al., 2004; ElBasiouny et al., 2010; Zhang et al., 2012). However, the facilitation of the "plateau properties" in the motoneurons could possibly also be explained by activation of glutamatergic NMDA receptors (cf. Delgado-Lezama et al., 1997; Guertin and Hounsgaard, 1998; Enríquez Denton et al., 2012). It thus now seems warranted to directly investigate the monoaminergic levels during Mayer waves e.g., with voltametric techniques (cf. Hentall et al., 2006).

Further investigation is also warranted as to whether the behavior reported here represents a physiological mechanism or an epiphenomenon. The new observation here of the modulation of CPG interaction, and probably also of the motoneuron intrinsic properties, by a fast-acting descending, naturally occurring

drive, is certainly a mechanism which could be physiologically useful. This drive is related, via the Mayer-waves to the control of blood pressure. In broad terms, control of respiration and blood pressure are regarded by Holstege as part of the "emotional motor system," which he describes as that involved in survival behaviors (Holstege, 1991). In this context, an association with limb motor control should be of no surprise. One particular aspect of that, related to plateau potentials evoked by the respiratory drive, has already been suggested (Kirkwood and Ford, 2004), but there could be many others. In the decerebrate preparation without any specific stimulation, only some features of that association are likely to be detectable.

AUTHOR CONTRIBUTIONS

Conception and design of study: Jacob Wienecke, Manuel Enríquez Denton, Peter A. Kirkwood, Hans Hultborn. Acquisition, analysis and/or interpretation of data: Jacob Wienecke, Manuel Enríquez Denton, Katinka Stecina, Peter A. Kirkwood, Hans Hultborn. Drafting the ms.: Katinka Stecina, Peter A. Kirkwood, Hans Hultborn. Critical review of the ms. and its final approval: Jacob Wienecke, Manuel Enríquez Denton, Katinka Stecina, Peter A. Kirkwood, Hans Hultborn.

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REFERENCES

Asmussen, E. (1983). Control of ventilation in exercise. *Exerc. Sport Sci. Rev.* 11, 24–54. doi: 10.1249/00003677-198301000-00002

Bacon, S. J., Zagon, A., and Smith, A. D. (1990). Electron microscopic evidence of a monosynaptic pathway between cells in the caudal raphé nuclei and sympathetic preganglionic neurons in the rat spinal cord. *Exp. Brain. Res.* 79, 589–602. doi: 10.1007/BF00229327

Barbeau, H., Julien, C., and Rossignol, S. (1987). The effects of clonidine and yohimbine on locomotion and cutaneous reflexes in the adult chronic spinal cat. *Brain Res.* 437, 83–96. doi: 10.1016/0006-8993(87)91529-0

Bennett, D. J., Hultborn, H., Fedirchuk, B., and Gorassini, M. (1998). Synaptic activation of plateaus in hindlimb motoneurons of decerebrate cats. *J. Neurophysiol.* 80, 2023–2037.

Boggs, D. F. (2002). Interactions between locomotion and ventilation in tetrapods. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 133, 269–288. doi: 10.1016/S1095-6433(02)00160-5

Brownstone, R. M., Gossard, J. P., and Hultborn, H. (1994). Voltage-dependent excitation of motoneurones from spinal locomotor centres in the cat. *Exp. Brain Res.* 102, 34–44. doi: 10.1007/BF00232436

Bruinstroop, E., Cano, G., Vanderhorst, V. G., Cavalcante, J. C., Wirth, J., Sena-Esteves, M., et al. (2012). Spinal projections of the A5, A6 (locus coeruleus), and A7 noradrenergic cell groups in rats. *J. Comp. Neurol.* 520, 1985–2001. doi: 10.1002/cne.23024

Chau, C., Barbeau, H., and Rossignol, S. (1998). Effects of intrathecal α_1- and α_2-noradrenergic agonists and norepinephrine on locomotion in chronic spinal cats. *J. Neurophysiol.* 79, 2941–2963.

Clarac, F., Brocard, F., and Vinay, L. (2004). The maturation of locomotor networks. *Prog. Brain Res.* 143, 57–66. doi: 10.1016/S0079-6123(03)43006-9

Crone, C., Hultborn, H., Kiehn, O., Mazieres, L., and Wigstrom, H. (1988). Maintained changes in motoneuronal excitability by short-lasting synaptic inputs in the decerebrate cat. *J. Physiol.* 405, 321–343. doi: 10.1113/jphysiol.1988.sp017335

Delgado-Lezama, R., Perrier, J. F., Nedergaard, S., Svirskis, G., and Hounsgaard, J. (1997). Metabotropic synaptic regulation of intrinsic response properties of turtle spinal motoneurones. *J. Physiol.* 504, 97–102. doi: 10.1111/j.1469-7793.1997.097bf.x

DiMarco, A. F., Romaniuk, J. R., Von Euler, C., and Yamamoto, Y. (1983). Immediate changes in ventilation and respiratory pattern associated with onset and cessation of locomotion in the cat. *J. Physiol.* 343, 1–16. doi: 10.1113/jphysiol.1983.sp014878

ElBasiouny, S. M., Schuster, J. E., and Heckman, C. J. (2010). Persistent inward currents in spinal motoneurons: important for normal function but potentially harmful after spinal cord injury and in amyotrophic lateral sclerosis. *Clin. Neurophysiol.* 121, 1669–1679. doi: 10.1016/j.clinph.2009.12.041

Enríquez Denton, M., Wienecke, J., Zhang, M., Hultborn, H., and Kirkwood, P. A. (2012). Voltage-dependent amplification of synaptic inputs in respiratory motoneurones. *J. Physiol.* 590, 3067–3090. doi: 10.1113/jphysiol.2011.225789

Fedirchuk, B., Nielsen, J., Petersen, N., and Hultborn, H. (1998). Pharmacologically evoked fictive motor patterns in the acutely spinalized marmoset monkey (Callithrix jacchus). *Exp. Brain Res.* 122, 351–361. doi: 10.1007/s002210050523

Ford, T. W., and Kirkwood, P. A. (2006). Respiratory drive in hindlimb motoneurones of the anaesthetized female cat. *Brain Res. Bull.* 70, 450–456. doi: 10.1016/j.brainresbull.2006.07.003

Forssberg, H., and Grillner, S. (1973). The locomotion of the acute spinal cat injected with clonidine i.v. *Brain Res.* 50, 184–186. doi: 10.1016/0006-8993(73)90606-9

Frigon, A., and Gossard, J. P. (2009). Asymmetric control of cycle period by the spinal locomotor rhythm generator in the adult cat. *J. Physiol.* 587, 4617–4628. doi: 10.1113/jphysiol.2009.176669

Geertsen, S. S., Stecina, K., Meehan, C. F., Nielsen, J. B., and Hultborn, H. (2011). Reciprocal Ia inhibition contributes to motoneuronal hyperpolarisation during the inactive phase of locomotion and scratching in the cat. *J. Physiol.* 589, 119–134. doi: 10.1113/jphysiol.2010.199125

Giraudin, A., Le Bon-Jégo, M., Cabirol, M. J., Simmers, J., and Morin, D. (2012). Spinal and pontine relay pathways mediating respiratory rhythm entrainment by limb proprioceptive inputs in the neonatal rat. *J. Neurosci.* 32, 11841–11853. doi: 10.1523/JNEUROSCI.0360-12.2012

Gossard, J.-P., Brownstone, R. M., Barajon, I., and Hultborn, H. (1994). Transmission in a locomotor-related group Ib pathway from hindlimb extensor muscles in the cat. *Exp. Brain Res.* 98, 213–228. doi: 10.1007/BF00228410

Greer, J. J., Smith, J. C., and Feldman, J. L. (1992). Respiratory and Locomotor patterns generated in the fetal rat brain stem-spinal cord *in vitro*. *J. Neurophysiol.* 67, 996–999.

Grillner, S., and Zangger, P. (1979). On the central generation of locomotion in the low spinal cat. *Exp. Brain Res.* 34, 241–261. doi: 10.1007/BF00235671

Guertin, P. A., and Hounsgaard, J. (1998). NMDA-Induced intrinsic voltage oscillations depend on L-type calcium channels in spinal motoneurons of adult turtles. *J. Neurophysiol.* 80, 3380–3382.

Haouzi, P. (2006). Theories on the nature of the coupling between ventilation and gas exchange during exercise. *Respir. Physiol. Neurobiol.* 151, 267–279. doi: 10.1016/j.resp.2005.11.013

Heckman, C. J., Gorassini, M. A., and Bennett, D. J. (2005). Persistent inward currents in motoneuron dendrites: implications for motor output. *Muscle Nerve.* 31, 135–156. doi: 10.1002/mus.20261

Heckman, C. J., Lee, R. H., and Brownstone, R. M. (2003). Hyperexcitable dendrites in motoneurons and their neuromodulatory control during motor behavior. *Trends Neurosci.* 26, 688–695. doi: 10.1016/j.tins.2003.10.002

Heckman, C. J., Mottram, C., Quinlan, K., Theiss, R., and Schuster, J. (2009). Motoneuron excitability: the importance of neuromodulatory inputs. *Clin. Neurophysiol.* 120, 2040–2054. doi: 10.1016/j.clinph.2009.08.009

Hentall, I. D., Pinzon, A., and Noga, B. R. (2006). Spatial and temporal patterns of serotonin release in the rat's lumbar spinal cord following electrical

stimulation of the nucleus raphe magnus. *Neuroscience* 142, 893–903. doi: 10.1016/j.neuroscience.2006.06.038

Holstege, G. (1991). Descending motor pathways and the spinal motor system: limbic and non-limbic components. *Progr. Brain Res.* 87, 307–421. doi: 10.1016/S0079-6123(08)63057-5

Hounsgaard, J., Hultborn, H., Jespersen, B., and Kiehn, O. (1988). Bistability of alpha-motoneurones in the decerebrate cat and in the acute spinal cat after intravenous 5-hydroxytryptophan. *J. Physiol.* 405, 345–367. doi: 10.1113/jphysiol.1988.sp017336

Hounsgaard, J., and Kiehn, O. (1989). Serotonin-induced bistability of turtle motoneurones caused by a nifedipine-sensitive calcium plateau potential. *J. Physiol.* 414, 265–282. doi: 10.1113/jphysiol.1989.sp017687

Hultborn, H., Brownstone, R. B., Toth, T. I., and Gossard, J. P. (2004). Key mechanisms for setting the input-output gain across the motoneuron pool. *Prog. Brain Res.* 143, 77–95. doi: 10.1016/S0079-6123(03)43008-2

Iles, J. F., and Nicolopoulos-Stounaras, S. (1996). Fictive locomotion in the adult decerebrate rat. *Exp. Brain Res.* 109, 393–398. doi: 10.1007/BF00229623

Jankowska, E., Jukes, M. G., Lund, S., and Lundberg, A. (1967a). The effect of DOPA on the spinal cord. 5. Reciprocal organization of pathways transmitting excitatory action to alpha motoneurones of flexors and extensors. *Acta Physiol. Scand.* 70, 369-88. doi: 10.1111/j.1748-1716.1967.tb03636.x

Jankowska, E., Jukes, M. G., Lund, S., and Lundberg, A. (1967b). The effect of DOPA on the spinal cord. 6. Half-centre organization of interneurones transmitting effects from the flexor reflex afferents. *Acta Physiol. Scand.* 70, 389–402. doi: 10.1111/j.1748-1716.1967.tb03637.x

Julien, C. (2006). The enigma of Mayer waves: facts and models. *Cardiovasc. Res.* 70, 12–21. doi: 10.1016/j.cardiores.2005.11.008

Kawahara, K., Kumagai, S., Nakazono, Y., and Miyamoto, Y. (1989). Coupling between respiratory and stepping rhythms during locomotion in decerebrate cats. *J. Appl. Physiol.* 67, 110–115.

Kiehn, O., Hultborn, H., and Conway, B. A. (1992). Spinal locomotor activity in acutely spinalized cats induced by intrathecal application of noradrenaline. *Neurosci. Lett.* 143, 243–246. doi: 10.1016/0304-3940(92)90274-B

Kirkwood, P. A., and Ford, T. W. (2004). Do respiratory neurons control female receptive behavior: a suggested role for a medullary central pattern generator? *Prog. Brain Res.* 143, 105–114. doi: 10.1016/S0079-6123(03)43010-0

Kirkwood, P. A., Lawton, M., and Ford, T. W. (2002). Plateau potentials in hindlimb motoneurones of female cats under anaesthesia. *Exp. Brain Res.* 146, 399–403. doi: 10.1007/s00221-002-1163-0

Kirkwood, P., Enríquez Denton, M., Wienecke, J., Nielsen, J. B., and Hultborn, H. (2005). Physiological roles for persistent inward currents in motoneurones: insights from the central respiratory drive. *Biocybernet. Biomed. Eng.* 25, 31–38.

Krogh, A., and Lindhard, J. (1913). The regulation of respiration and circulation during the initial stages of muscular work. *J. Physiol.* 47, 112–136. doi: 10.1113/jphysiol.1913.sp001616

Le Gal, J. P., Juvin, L., Cardoit, L., Thoby-Brisson, M., and Morin, D. (2014). Remote control of respiratory neural network by spinal locomotor generators. *PLoS ONE* 9:e89670. doi: 10.1371/journal.pone.0089670

Lewis, D. I., and Coote, J. H. (1990a). Excitation and inhibition of rat sympathetic preganglionic neurones by catecholamines. *Brain Res.* 530, 229–234. doi: 10.1016/0006-8993(90)91287-Q

Lewis, D. I., and Coote, J. H. (1990b). The influence of 5-hydroxytryptamine agonists and antagonists on identified sympathetic preganglionic neurones in the rat, *in vivo*. *Br. J. Pharmacol.* 99, 667–672. doi: 10.1111/j.1476-5381.1990.tb12987.x

Lovick, T. A. (1997). The medullary raphe nuclei: a system for integration and gain control in autonomic and somatomotor responsiveness? *Exp. Physiol.* 82, 31–41. doi: 10.1113/expphysiol.1997.sp004013

Malpas, S. C. (2002). Neural influences on cardiovascular variability: possibilities and pitfalls. *Am. J. Physiol. Heart Circ. Physiol.* 282, H6–H20.

Mayer, S. (1876). Studien zur physiologie des herzens und der blutgefässe 6. abhandlung: über spontane blutdruckschwankungen. *Sitzungsber. Akad. Wiss. Wien Math. Naturwiss. Cl. Anat.* 174, 281–307.

Meehan, C. F., Grondahl, L., Nielsen, J. B., and Hultborn, H. (2012). Fictive locomotion in the adult decerebrate and spinal mouse *in vivo*. *J. Physiol.* 590, 289–300. doi: 10.1113/jphysiol.2011.214643

Meyer-Lohman, J. (1974). "Respiratory influences upon the lumbar extensor motor system of decerebrated cats," in *Central Rhythmic and Regulation*, eds W. Umbach and H. P. Koepchen (Stuttgart: Hippokrates Verlag), 334–341.

Miles, G. B., and Sillar, K. T. (2011). Neuromodulation of vertebrate locomotor control networks. *Physiology (Bethesda)* 26, 393–411. doi: 10.1152/physiol.00013.2011

Montano, N., Lombardi, F., Gnecchi Ruscone, T., Contini, M., Finocchiaro, M. L., Baselli, G., et al. (1992). Spectral analysis of sympathetic discharge, R-R interval and systolic arterial pressure in decerebrate cats. *J. Auton. Nerv. Syst.* 40, 21–31. doi: 10.1016/0165-1838(92)90222-3

Morin, D., and Viala, D. (2002). Coordinations of locomotor and respiratory rhythms *in vitro* are critically dependent of hindlimb sensory inputs. *J. Neurosci.* 22, 4756–4765.

Noga, B. R., Blythe, A. D., Brumley, M. R., Hentall, I. D., Kadam, B. H., and Sanchez, F. J. (2006). "Relation between monoamine levels and parameters of fictive locomotion," in *Neuroscience Meeting Planner*, Program No. 252.23.2006 (Atlanta, GA: Society for Neuroscience).

Noga, B. R., Blythe, A. D., Brumley, M. R., Hentall, I. D., Sanchez, F. J., and Taberner, A. M. (2007). "Modulation of spinal serotonin and norepinephrine levels during locomotion and at rest: real-time measurements with multielectrode cyclic voltammetry," in *Neuroscience Meeting Planner*, Program No. 924.13.2007 (San Diego, CA: Society for Neuroscience).

Orer, H. S., Clement, M. E., Barman, S. M., Zhong, S., Gebber, G. L., and McCall, R. B. (1996). Role of serotonergic neurons in the maintenance of the 10-Hz rhythm in sympathetic nerve discharge. *Am. J. Physiol.* 270, R174–R181.

Perségol, L., Jordan, M., Viala, D., and Fernandez, C. (1988). Evidence for central entrainment of the medullary respiratory pattern by the locomotor pattern in the rabbit. *Exp. Brain Res.* 71, 153–162. doi: 10.1007/BF00247530

Pollock, L. J., and Davis, L. (1930). The reflex activities of a decerebrate animal. *J. Comp. Neurol.* 50, 377–341. doi: 10.1002/cne.900500205

Potts, J. T., Rybak, I. A., and Paton, J. F. (2005). Respiratory rhythm entrainment by somatic afferent stimulation. *J. Neurosci.* 25, 1965–1978. doi: 10.1523/JNEUROSCI.3881-04.2005

Powers, R. K., and Binder, M. D. (2001). Input-output functions of mammalian motoneurons. *Rev. Physiol. Biochem. Pharmacol.* 143, 137–263. doi: 10.1007/BFb0115594

Preiss, G., Iscoe, S., and Polosa, C. (1975). Analysis of a periodic breathing pattern associated with Mayer waves. *Am. J. Physiol.* 228, 768–774.

Preiss, G., and Polosa, C. (1974). Patterns of sympathetic neuron activity associated with Mayer waves. *Am. J. Physiol.* 226, 724–730.

Richter, D. W. (1982). Generation and maintenance of the respiratory rhythm. *J. Exp. Biol.* 100, 93–107.

Romaniuk, J. R., Kasicki, S., Kazennikov, O. V., and Selionov, V. A. (1994). Respiratory responses to stimulation of spinal or medullary locomotor structures in decerebrate cats. *Acta Neurobiol. Exp. (Wars)* 54, 11–17.

Samuels, E. R., and Szabadi, E. (2008). Functional neuroanatomy of the noradrenergic locus coeruleus: its roles in the regulation of arousal and autonomic function part I: principles of functional organisation. *Curr. Neuropharmacol.* 6, 235–253. doi: 10.2174/157015908785777229

Schmidt, B. J., and Jordan, L. M. (2000). The role of serotonin in reflex modulation and locomotor rhythm production in the mammalian spinal cord. *Brain Res. Bull.* 53, 689–710. doi: 10.1016/S0361-9230(00)00402-0

Schomburg, E. D., Steffens, H., and Dembowsky, K. (2003). Rhythmic phrenic, intercostal and sympathetic activity in relation to limb and trunk motor activity in spinal cats. *Neurosci. Res.* 46, 229–240. doi: 10.1016/S0168-0102(03)00062-2

Sears, T. A. (1964). The slow potentials of thoracic respiratory motoneurones and their relation to breathing. *J. Physiol.* 175, 404–424. doi: 10.1113/jphysiol.1964.sp007524

Viala, D. (1986). Evidence for direct reciprocal interactions between the central rhythm generators for spinal "respiratory" and locomotor activities in the rabbit. *Exp. Brain Res.* 63, 225–232. doi: 10.1007/BF00236841

Viala, D., Vidal, C., and Freton, E. (1979). Coordinated rhythmic bursting in respiratory and locomotor muscle nerves in the spinal rabbit. *Neurosci. Lett.* 11, 155–196. doi: 10.1016/0304-3940(79)90119-8

Zhang, M., Hultborn, H., and Sukiasyan, N. (2012). "The distribution of calcium channel Cav1.3 in the central nervous system and its functions in relation to motor control," in *Calcium Channels: Properties, Functions and Regulation*, ed M. R. Figgins (Hauppauge, NY: Nova Science Publishers), 1–48.

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Spinal metaplasticity in respiratory motor control

Daryl P. Fields and Gordon S. Mitchell*

Department of Comparative Biosciences, University of Wisconsin-Madison, Madison, WI, USA

Edited by:
Hans Hultborn, University of Copenhagen, Denmark

Reviewed by:
Deborah Baro, Georgia State University, USA
David Parker, Cambridge University, UK
Carlos Mantilla, Mayo Clinic, USA

****Correspondence:***
Gordon S. Mitchell, Department of Comparative Biosciences, University of Wisconsin-Madison, 2015 Linden Drive, Madison, WI 53706, USA
e-mail: mitchell@svm.vetmed.wisc.edu

A hallmark feature of the neural system controlling breathing is its ability to exhibit plasticity. Less appreciated is the ability to exhibit metaplasticity, a change in the capacity to express plasticity (i.e., "plastic plasticity"). Recent advances in our understanding of cellular mechanisms giving rise to respiratory motor plasticity lay the groundwork for (ongoing) investigations of metaplasticity. This detailed understanding of respiratory metaplasticity will be essential as we harness metaplasticity to restore breathing capacity in clinical disorders that compromise breathing, such as cervical spinal injury, motor neuron disease and other neuromuscular diseases. In this brief review, we discuss key examples of metaplasticity in respiratory motor control, and our current understanding of mechanisms giving rise to spinal plasticity and metaplasticity in phrenic motor output; particularly after pre-conditioning with intermittent hypoxia. Progress in this area has led to the realization that similar mechanisms are operative in other spinal motor networks, including those governing limb movement. Further, these mechanisms can be harnessed to restore respiratory and non-respiratory motor function after spinal injury.

Keywords: respiratory control, respiratory plasticity, metaplasticity, spinal cord, motor neuron, intermittent hypoxia, phrenic motor neuron

INTRODUCTION

As with most neural systems, a hallmark of the neural system controlling breathing is its ability to express plasticity; defined as a persistent (>60 min) change in neural network function after an experience/stimulus has ended (Mitchell and Johnson, 2003). Respiratory plasticity is characteristic of development, continues throughout life, and is of considerable importance in preserving life when confronted with clinical disorders that compromise the ability to breathe, including diseases of the lung and chest wall, or neurological disorders such as spinal injury and motor neuron disease (Vinit et al., 2009; Dale-Nagle et al., 2010; Nichols et al., 2013; Dale et al., 2014).

Less well known is that respiratory plasticity itself adapts based on experience (Mitchell and Johnson, 2003), a phenomenon referred to as metaplasticity or "plastic plasticity" (Abraham and Bear, 1996). Although there are examples of respiratory plasticity and metaplasticity in sensory receptors, including the carotid body (CB) chemoreceptors (Kumar and Prabhakar, 2012); prominent examples of respiratory plasticity and metaplasticity are found at the other end of the respiratory control system, the motor nuclei (Mahamed and Mitchell, 2007; Dale-Nagle et al., 2010; Devinney et al., 2013).

In this brief review, we focus on known examples of spinal respiratory motor plasticity and metaplasticity, as well as implications for clinical application. We first define respiratory plasticity, metaplasticity and related concepts, and then the potential sites of respiratory plasticity, models of spinal respiratory plasticity/metaplasticity, possible mechanisms of metaplasticity, and finally, gaps in our knowledge that require additional research.

DEFINITIONS: (META) PLASTICITY AND (META) MODULATION

Modulation and plasticity are related but distinct properties that are frequently confused. Higher order properties such as metamodulation and metaplasticity are less frequently considered and even more often confused. Thus, brief definitions of these terms are provided in the context of respiratory motor control (Mitchell and Johnson, 2003); although they also apply to limb motor control (reviewed in Grau et al., 2014).

Modulation is a change in system behavior that fades rapidly (seconds to minutes) after the stimulus is removed. Neuromodulators often work through metabotropic G protein coupled receptors, which alter cell excitability through covalent modifications of membrane channels. Modulation does confer system flexibility and can initiate cellular mechanisms resulting in plasticity (Mitchell and Johnson, 2003), but the two are differentiated by what happens when the stimulating trigger is removed. For example, during a brief (5 to 30 min) hypoxic experience, phrenic nerve activity (and breathing) increases and returns to normal seconds/minutes after hypoxia has ceased. Alternatively, 3 successive 5-min hypoxic episodes give rise to a persistent increase in phrenic nerve activity lasting several hours after the final hypoxic episode has ended—an expression of plasticity (see below). In this example, modulation is the within episode (during hypoxia) augmentation of respiratory motor output, plasticity is the persistence of increased activity that lasts long after the hypoxia stimulus has ended.

Meta-modulation is a reversible change in the capacity or quality of modulation (Katz and Edwards, 1999; Mitchell and Johnson, 2003), and requires continued presence of

the meta-modulation trigger. Meta-modulating stimuli also frequently act through G protein coupled receptors, 2nd messengers and/or ion channels to augment the response of neurons to modulators (Katz and Edwards, 1999; Mesce, 2002; Ribeiro and Sebastiao, 2010), though metamodulation triggers have not yet been associated with long-term changes in gene expression. One interesting example of meta-modulation in respiratory control is the response of neurons in the nucleus of the solitary tract to concurrent serotonin and substance P application (Jacquin et al., 1989). Both serotonin and substance P alone positively modulate nucleus tractus solitarius (NTS) neurons. However, in the presence of substance P, serotonin becomes inhibitory (Jacquin et al., 1989). Thus, in these *in vitro* conditions, the impact of one modulator (serotonin) is augmented by concurrent application of another (substance P).

Plasticity (long-term) is a persistent (>60 min) change in function that outlasts the initiating stimulus (Mitchell and Johnson, 2003). Plasticity often requires new protein synthesis via translational and/or transcriptional regulation (Manahan-Vaughan et al., 2000; Mitchell and Johnson, 2003; Alberini, 2008), though it is not a prerequisite. A frequent initiating stimulus in many neural systems is neuronal activity, or activity-dependent synaptic plasticity (Malinow and Malenka, 2002; Wiegert and Bading, 2011); though activity-dependent plasticity is not characteristic of respiratory motor control (Mitchell and Johnson, 2003; Strey et al., 2013). Instead, neuromodulators frequently elicit respiratory plasticity through distinct signaling cascades induced by patterned metabotropic receptor activation.

One prominent model of plasticity in spinal respiratory motor control is phrenic long-term facilitation (pLTF), a long-lasting increase in phrenic motor output observed following acute intermittent hypoxia (AIH; Feldman et al., 2003; Mahamed and Mitchell, 2007; Dale-Nagle et al., 2010; Devinney et al., 2013). AIH elicits episodic serotonin release within the phrenic motor nucleus (Kinkead et al., 2001), activation of spinal serotonin receptors (Fuller et al., 2001; Baker-Herman and Mitchell, 2002) and a long-lasting enhancement of phrenic motor output (Mahamed and Mitchell, 2007; Devinney et al., 2013). Since this form of phrenic motor plasticity is initiated by intermittent, but not sustained hypoxia of similar cumulative duration, it is pattern sensitive (Baker and Mitchell, 2000; Devinney et al., 2013), similar to other forms of serotonin dependent plasticity (Sherff and Carew, 2002; Philips et al., 2013). In summary, modulatory experiences in themselves are not sufficient for plasticity as their effects fade once the trigger is removed. Alternatively, pattern specific modulation can be encoded through discrete signaling pathways to elicit a long-lasting augmentation of nerve output that persists after the triggering experience; i.e., plasticity/metaplasticity.

Metaplasticity is a change in the capacity for neuroplasticity (Abraham and Bear, 1996; Byrne, 1997). An important distinction between metaplasticity and metamodulation is that metaplasticity is expressed after triggering experiences (i.e., hypoxia) for both plasticity and metaplasticity are gone (Abraham, 2008). As in plasticity, metaplasticity often encodes previous experiences by altering gene expression (Mitchell and Johnson, 2003); therefore changing the ability of a system to respond to subsequent experiences. Through encoded memory, the effects of metaplasticity triggers can persists long after the stimulation has subsided; the focus of this review is to review how intermittent hypoxia training (a metaplasticity trigger) can augment subsequent spinal respiratory plasticity.

POTENTIAL SITES OF RESPIRATORY PLASTICITY AND METAPLASTICITY

Essential processes in control of breathing include respiratory rhythm generation, burst pattern formation (Feldman and Smith, 1989; Mitchell et al., 1990) as well as sensory feedback giving rise to chemoreflexes, mechanoreflexes, neuromodulation and neuroplasticity (Feldman et al., 2003; Mitchell and Johnson, 2003).

Respiratory plasticity and metaplasticity can occur in any component of the neural system controlling breathing (Mitchell and Johnson, 2003). Of particular interest to this review, plasticity and metaplasticity occur in spinal respiratory motor neurons (and/or interneurons) putting the final "touches" on burst pattern formation before the central nervous system (CNS) relays its command to breathe to respiratory muscles. Thus, plasticity and metaplasticity are important in sculpting motor output to individual respiratory muscles. Here, we focus on spinal respiratory motor plasticity, with less consideration given to plasticity in chemoreceptor feedback (Bisgard, 2000; Kumar and Prabhakar, 2012) or brainstem mechanisms of respiratory rhythm generation/pattern formation (Blitz and Ramirez, 2002; Feldman et al., 2003, 2005). We instead refer to several well-written reviews of carotid (Kumar and Prabhakar, 2012) and brainstem (Ramirez et al., 2012) plasticity, which can elicit upstream respiratory plasticity that is phenotypically similar to spinal respiratory plasticity.

One prominent source of modulatory input giving rise to plasticity originates from brainstem serotonergic raphe neurons that project to spinal and brainstem respiratory neurons. In particular, brainstem raphe neurons are known to modulate respiratory motor neurons and, under the right circumstances, initiate important forms of respiratory motor plasticity (Mitchell et al., 2001; Feldman et al., 2003). **Figure 1** outlines the neural network controlling breathing and identifies potential sites of respiratory neuroplasticity; all of which could also exhibit metaplasticity.

PHRENIC LONG-TERM FACILITATION IS A FORM OF SPINAL RESPIRATORY MOTOR PLASTICITY

The most extensively studied model of spinal respiratory motor plasticity is AIH induced pLTF; a persistent increase in phrenic nerve output following 3 × 5 min experiences of moderate hypoxia (35–45 mmHg PaO2; Hayashi et al., 1993; Bach and Mitchell, 1996; Mitchell et al., 2001; Feldman et al., 2003; Mahamed and Mitchell, 2007; Dale-Nagle et al., 2010; Devinney et al., 2013). In recent years we have come to use a generic term for long-lasting enhancement of phrenic motor output; phrenic motor facilitation (pMF). Whereas pMF may arise from a variety of triggers (i.e., inactivity, hypercapnia, pharmacology), pLTF

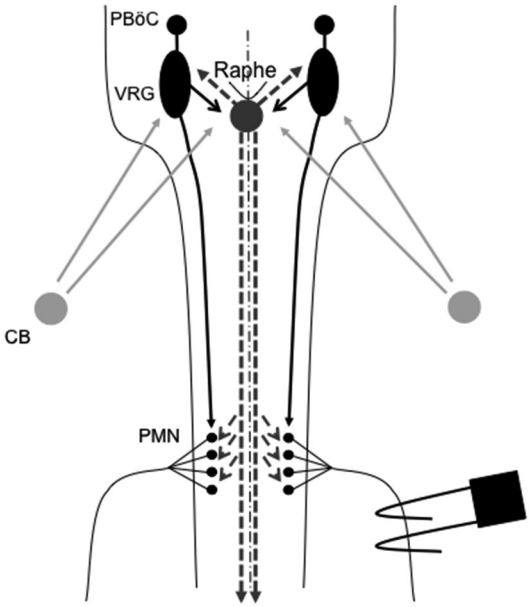

FIGURE 1 | Representation of brainstem and spinal cord regions critical for respiratory motor control. Respiratory rhythm generation requires a small region of the medulla known as the pre-Bötzinger complex (PBöC). The central rhythm is transmitted to brainstem respiratory pre-motor neurons of the ventral respiratory group (VRG). VRG pre-motor neurons subsequently relay respiratory drive projections to different respiratory motor neuron pools, including phrenic motor neurons (PMN). Sensory input to the respiratory system during episodic hypoxia is provided by the carotid body (CB) chemoreceptors in the neck, which project via chemoafferent neurons to the medullary nucleus of the solitary tract (not shown). These second order sensory neurons subsequently project (directly or indirectly) to multiple structures of importance in ventilatory control, including the PBöC, VRG and serotonergic neurons in the medullary raphe (raphe). Raphe serotonergic neurons play a key role in phrenic long-term facilitation (pLTF) following acute intermittent hypoxia (AIH) and, presumably, in metaplasticity of pLTF.

is a specific form of pMF elicited from AIH (Devinney et al., 2013).

In many instances, spinal signaling pathways that are necessary for pLTF are also independently sufficient to elicit pMF (reviewed by Dale-Nagle et al., 2010). For example, serotonin receptor antagonists applied to the C3–C5 cervical spine region during AIH abolishes pLTF; demonstrating that spinal serotonin receptor activation in the immediate vicinity of the phrenic motor nucleus is necessary for pLTF (Fuller et al., 2001; Baker-Herman and Mitchell, 2002). AIH-induced pLTF is also abolished by cervical spinal pre-treatment with siRNAs targeting BDNF mRNA (Baker-Herman et al., 2004) and neuronal nitric oxide synthase (nNOS; MacFarlane et al., 2014). Conversely, activation of cervical spinal serotonin type 2A, 2B and 7 receptors (MacFarlane and Mitchell, 2009; Hoffman and Mitchell, 2011; MacFarlane et al., 2011), TrkB receptors (the high affinity brain derived neurotrophic factor (BDNF) receptor; Baker-Herman et al., 2004), and nNOS (MacFarlane et al., 2014) can all give rise to phenotypically similar pMF in the absence of hypoxia (i.e., mimicking pLTF). Collectively, these experiments reveal important aspects of the signaling pathway giving rise to AIH-induced pLTF, but also identify important signaling checkpoints that are independently sufficient for pMF.

Though similar spinal signaling pathways can drive pLTF and other forms of pMF, it is important to differentiate between the two experimentally and conceptually (Dale-Nagle et al., 2010). For example, intrathecal drug application allows for the investigation of pMF mechanisms confined to the region of the phrenic motor nucleus. Conversely, moderate AIH-induced pLTF has global effects that influence other respiratory control sites (**Figure 1**) as well as non-respiratory motor pools within the thoracic and lumbar spinal segments. AIH-induced pLTF begins via hypoxia activation of peripheral (caotid body) chemoreceptors (Hayashi et al., 1993; Bach and Mitchell, 1996; Bavis and Mitchell, 2003), second order medullary neurons in the nucleus of the solitary tract, and then (indirectly) serotonergic neurons of the medullary raphe nuclei (Morris et al., 1996; Li et al., 1999; Mitchell et al., 2001; **Figure 1**). Subsequent episodic serotonin release on or near spinal respiratory motor neurons (Kinkead et al., 2001) initiates cellular cascades giving rise to moderate AIH (35–45 mmHg PaO2) induced pLTF (Fuller et al., 2001).

When elicited by more severe hypoxic episodes (25–30 mmHg PaO2) but otherwise the same AIH protocol (Nichols et al., 2012), AIH elicits pLTF through an entirely different mechanism; requiring spinal adenosine type 2A (A2A) receptor activation (Golder et al., 2008; Nichols et al., 2012). Our working model is that severe hypoxia triggers greater adenosine triphosphate (ATP) release from CNS cells, increasing ATP and adenosine concentrations. Elevated adenosine near phrenic motor neurons (PMN) activates A2A receptors and drives adenosine-dependent (serotonin independent) pLTF (Nichols et al., 2012). We have not yet identified the specific cellular source of adenosine for severe AIH induced pLTF. However, astrocytes increase their release of ATP and adenosine during hypoxia, and are a likely source for the relevant adenosine for severe AIH-induced pLTF (Kulik et al., 2010). Additional research is needed to confirm this hypothesis.

Although serotonin- and adenosine-dependent pLTF are phenotypically similar, they operate through distinct signaling pathways referred respectively as the Q and S pathways to pMF (Dale-Nagle et al., 2010); these pathways are named for the G proteins most often coupled to their initiating receptors (i.e., Gq for serotonin type 2 and Gs for adenosine 2A). Additionally, pMF can be induced by activation of Gq-coupled alpha-1 adrenergic receptors (Huxtable et al., 2014) or Gs-coupled 5-HT7 receptors (Hoffman and Mitchell, 2013). However, neither alpha-1 or 5-HT7 receptors are necessary for any form of AIH-induced pLTF (Hoffman and Mitchell, 2013; Huxtable et al., 2014). Thus, these receptors are sufficient for pMF, but not necessary for pLTF; they elicit pMF by convergent downstream signaling onto Q and S pathways.

The Q pathway to pMF consists of: spinal Gq-linked G protein coupled receptors activation (Fuller et al., 2001; Baker-Herman and Mitchell, 2002; MacFarlane et al., 2011; Huxtable et al., 2014), protein kinase C (PKC) activation (Devinney and Mitchell, unpublished observations), new synthesis of BDNF (Baker-Herman and Mitchell, 2002; Baker-Herman et al., 2004), TrkB

receptors (Baker-Herman et al., 2004; Dale et al., unpublished), and downstream signaling via ERK MAP kinases (Wilkerson and Mitchell, 2009; Hoffman et al., 2012). In contrast, the S pathway to pMF involves: spinal Gs-linked G protein coupled receptors (Hoffman and Mitchell, 2011; Nichols et al., 2012), adenylyl cyclase activation with synthesis of cyclic AMP, new protein synthesis of an immature TrkB isoform (vs. BDNF; Golder et al., 2008; Hoffman and Mitchell, 2011) and downstream signaling via Akt (vs. ERK; Golder et al., 2008; Hoffman et al., 2012).

The Q and S pathways interact in interesting and complex ways that we have defined as "cross-talk inhibition" (Dale-Nagle et al., 2010; Hoffman et al., 2010; Hoffman and Mitchell, 2013). Our working hypothesis is that manipulations of these cross-talk interactions during intermittent hypoxia training may underlie at least some forms of spinal respiratory metaplasticity (see below). During moderate AIH-induced pLTF, serotonin release activates abundant, high affinity 5-HT2 receptors; driving the dominant Q pathway to pLTF. Although concurrent activation of Gs protein linked A2A or 5-HT7 receptors is insufficient to trigger the S pathway at these levels of hypoxia (moderate AIH-induced pLTF is exclusively Q pathway dependent plasticity), sub-threshold activation of these Gs-linked receptors constrains the Q pathway and reduces pLTF magnitude. Inhibition of spinal A2A receptors (Hoffman et al., 2010) and/or 5-HT7 receptors (Hoffman and Mitchell, 2013) during moderate AIH eliminates this cross-talk constraint thereby enhancing moderate AIH-induced pLTF. We suspect that all Gs-linked G protein coupled receptors have a capacity to constrain the Q pathway to pMF since cross-talk inhibition is mediated by protein kinase A (PKA); a prominent effector of downstream Gs protein/cAMP signaling. Whereas spinal PKA inhibition relieves cross-talk inhibition and enhances moderate AIH induced pLTF (to a similar extent as A2A and 5-HT7 inhibition), PKA activation suppresses moderate AIH-induced pLTF (Hoffman and Mitchell, 2013). Since Gs-linked G protein coupled receptors are commonly expressed within many CNS cell types, we cannot yet state that cross-talk inhibition is operative within a single neuron; spinal motor neurons. It remains possible that elements of the Q and S pathways, and their interactions, arise from interneuron, astrocyte and/or microglia. The cellular localization of processes involved in spinal respiratory plasticity is an area that requires and warrants additional research.

Independent from the contributions of G protein coupled receptors, spinal activation of JAK2-coupled erythropoietin (EPO; Dale et al., 2012) or tyrosine kinase coupled vascular endothelial growth factor (VEGF; Dale-Nagle et al., 2011) receptors is sufficient to elicit pMF. EPO, VEGF, and their high affinity receptors are all oxygen-regulated proteins with increased expression following prolonged hypoxia (Semenza et al., 1991; Forsythe et al., 1996). We suspect that these proteins play a relatively greater role in phrenic motor plasticity during conditions of prolonged hypoxia experiences (days-months). Both VEGF- and EPO-induced pMF are Akt and ERK dependent, suggesting downstream divergence onto both the Q and S signaling pathways (Dale-Nagle et al., 2011; Dale et al., 2012). The diversity of hypoxia-induced mechanisms giving rise to phenotypically similar pMF most likely reflects the need to adapt to hypoxia that differs in quality (severity, pattern) and duration. Specifically, we suggest that differential responses with severity (i.e., Q to S pathways) and duration (serotonin/adenosine to VEGF/EPO; Dale et al., 2014) enable unique interactions for adaptive flexibility during hypoxia. Further, this diversity of responses may enable emergent plasticity properties, such as metaplasticity.

pLTF METAPLASTICITY INDUCED BY INTERMITTENT HYPOXIA PRECONDITIONING

CHRONIC INTERMITTENT HYPOXIA (CIH)

CIH in rodents simulates some aspects of the intermittent hypoxia experienced during obstructive sleep apnea (OSA) in humans (Lee et al., 2009). Thus, when rodents are preconditioned with CIH, pathogenesis ensues (Reeves and Gozal, 2006; Moraes et al., 2012; Prabhakar and Semenza, 2012; Navarette-Opazo and Mitchell, 2014), yet multiple distinct forms of respiratory plasticity can also be observed. These include: (1) increased baseline breathing (Ling et al., 2001); (2) increased baseline carotid sinus chemoafferent neuron activity (Peng and Prabhakar, 2003, 2004); (3) decreased synaptic strength from carotid chemoafferent neurons onto their NTS targets (Kline et al., 2007); and (4) increased short-term hypoxic phrenic response (Ling et al., 2001; Peng and Prabhakar, 2003, 2004). In addition, CIH also triggers metaplasticity, expressed as enhanced AIH-induced pLTF (Ling et al., 2001), increased tidal volume in response to AIH within unanesthetized rats (i.e., ventilatory LTF; McGuire et al., 2003, 2004), and sensory LTF of carotid chemoafferents neurons (sensory LTF); a form of plasticity observed only after CIH preconditioning (**Figure 2**; Peng and Prabhakar, 2003, 2004). In normal rodents, AIH does not elicit sensory LTF; after CIH pretreatment, robust sensory LTF is observed after the same AIH stimulus (i.e., persistent enhancement of carotid chemoafferent activity; Peng and Prabhakar, 2003, 2004). Although the newly revealed sensory LTF may contribute to the CIH-enhanced AIH-induced pLTF, considerable evidence suggests that this is not the entire story.

Enhanced pLTF could occur and/or be modified at any site in the neural network transmitting chemosensory activity to PMN; including chemoreceptors themselves, their afferent terminations in the nucleus of the solitary tract, neurons of the ventral respiratory column, raphe neurons, spinal phrenic interneurons and/or PMN (**Figure 1**). There is currently no published evidence that CIH-induced pLTF metaplasticity results from changes in brainstem modulatory neurons, although prolonged CIH (four, but not one week; Bach and Mitchell, unpublished observations) increases serotonergic terminal density in the phrenic motor nucleus; suggesting that anatomical plasticity of medullary raphe neuron projections may contribute to enhanced pLTF (McCrimmon et al., 1995; Kinkead et al., 1998). Further evidence for CIH-induced CNS plasticity (vs. at the peripheral chemoreceptors) and pLTF metaplasticity includes observations that CIH: (1) increases phrenic nerve responses to electrical activation of the cut, central end of the carotid sinus nerve, bypassing carotid chemoreceptors *per se* (Ling et al., 2001); (2) attenuates synaptic input into the nucleus of the solitary tract (i.e., wrong way to account for enahnced pLTF; Kline et al., 2007,

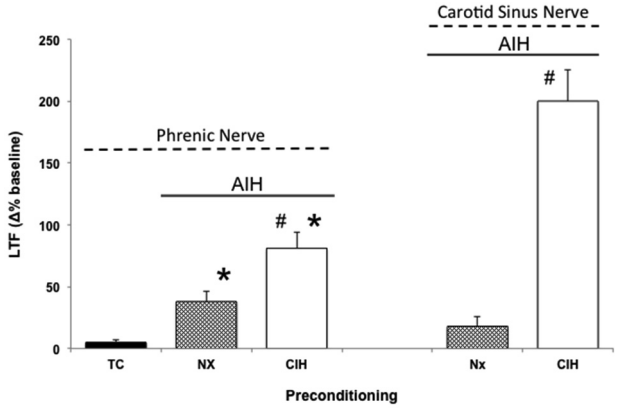

FIGURE 2 | Metaplasticity in AIH induced pLTF and carotid (chemo)sensory long-term facilitation (csLTF) following preconditioning with chronic intermittent hypoxia (CIH). On left, time controls (TC) are without AIH, and no pLTF is observed. In contrast, AIH elicits pLTF in rats pretreated with both normoxia (Nx) and CIH ($^{*}p < 0.05$ vs. TC). However, AIH-induced pLTF is greater in rats preconditioned with CIH vs. Nx ($^{\#}p < 0.05$ vs. Nx), demonstrating meta-plasticity in pLTF (data from Ling et al., 2001). On right, sensory recordings of the carotid sinus nerve reveal no csLTF in rats exposed to AIH following Nx preconditioning (post 60 min). However, after CIH preconditioning, robust csLTF is observed, revealing a form of plasticity not present in normal rats (i.e., metaplasticity; data from Peng and Prabhakar, 2003).

2009); and (3) strengthens spinal pathways to PMN following cervical injury (Fuller et al., 2003).

Although relatively little is known concerning sites and signaling mechanisms of CIH metaplasticity, CIH-enhanced moderate AIH-induced pLTF is still serotonin-dependent (Ling et al., 2001; McGuire et al., 2004). On the other hand, it is no longer dependent on 5-HT2 receptors alone (Ling et al., 2001; McGuire et al., 2004). Rather, the inhibitory actions of 5-HT7 receptors on moderate AIH-induced pLTF seen in normal rats (Hoffman and Mitchell, 2013) convert to excitatory actions; both 5-HT7 and 5-HT2 receptors now contribute to enhanced LTF in CIH trained rats (McGuire et al., 2004). Thus, CIH may disrupt normal cross-talk interactions between the Q and S pathways without compromising the respective Q and S contributions to pMF (enabling additive contributions). Mechanisms accounting for this apparent loss of cross-talk inhibition between 5-HT2 and 5-HT7 receptors are not known.

Because CIH elicits respiratory metaplasticity, we initially had contemplated CIH as a treatment option to restore respiratory control in clinical disorders that impair breathing capacity; such as cervical spinal injury (Fuller et al., 2003; Mitchell, 2007). Unfortunately, CIH has well-documented adverse consequences in multiple physiological systems; including systemic hypertension (Fletcher et al., 1992), metabolic syndrome (Li et al., 2005; Tasali and Ip, 2008) and hippocampal cell death with associated cognitive deficits (Row, 2007; Row et al., 2007). Thus, although CIH may have beneficial effects in restoring respiratory control in clinical disorders that compromise breathing, undesirable side effects limit its utility. An interesting corollary is that the increased sleep apnea prevalence in individuals with chronic spinal injuries (Sankari et al., 2014) may represent a form of "self medication," inducing spontaneous functional recovery. However, other factors associated with sleep apnea such as sleep fragmentation and systemic inflammation may over-ride any benefits (McGuire et al., 2008; Hakim et al., 2012; Zhang et al., 2012; Huxtable et al., 2013).

REPETITIVE ACUTE INTERMITTENT HYPOXIA (rAIH)

Less intensive rAIH preconditioning protocols retain the ability to elicit plasticity and metaplasticity without the known pathogenic effects of CIH (Lovett-Barr et al., 2012; Satriotomo et al., 2012; Navarette-Opazo and Mitchell, 2014). For example, daily acute intermittent hypoxia (dAIH; 10, 5 min hypoxic episodes for seven consecutive days) increases the strength of crossed-spinal synaptic pathways to PMN (Lovett-Barr et al., 2012) and enhances moderate AIH-induced LTF (Wilkerson and Mitchell, 2009). Similarly, AIH three times per week (3xwAIH; 10, 5 min hypoxic episodes, three times per week for four or 10 weeks) increases the expression of pro-plasticity molecules within PMN (Satriotomo et al., 2012), and enhances moderate AIH-induced pLTF (MacFarlane et al., 2010; Vinit et al., 2010). In contrast to CIH, neither dAIH (Wilkerson and Mitchell, 2009; Lovett-Barr et al., 2012) or 3xwAIH (Satriotomo et al., 2012) elicit detectable systemic hypertension, hippocampal cell death or reactive gliosis. With similar "low dose" intermittent hypoxia protocols, most reported systemic effects are in fact beneficial vs. pathogenic (Dale et al., 2014; Navarette-Opazo and Mitchell, 2014). Thus, rAIH may be a simple, safe and effective means of promoting motor plasticity and functional recovery of breathing (and non-respiratory motor) deficits caused by severe clinical disorders. For example, dAIH beginning one week after cervical spinal hemisection in rats promotes remarkable functional recovery of breathing capacity (Lovett-Barr et al., 2012). Even more startling, dAIH in combination with ladder walking induced prolonged restoration of forelimb function in rats (Lovett-Barr et al., 2012). The functional benefits of intermittent hypoxia therapy may be most notable when paired with task specific training. For example, dAIH for five days increased walking endurance by 18% in humans with chronic (>nine months), incomplete spinal injuries, but increased walking endurance 38% when combined with walking practice. Conversely, walking practice alone did not improve walking endurance (Hayes et al., 2014). Although detailed mechanisms underlying rAIH induced respiratory and limb functional recovery after spinal injury are not yet known, increased expression of molecules known to be associated with both the Q and S pathways suggests that both signaling pathways may be involved (Wilkerson and Mitchell, 2009; Lovett-Barr et al., 2012; Satriotomo et al., 2012; Dale et al., 2014). Furthermore, with transcriptome analysis of gene arrays from ventral cervical segments encompassing the phrenic motor nucleus, the most significant changes noted were associated with anti-inflammatory activities. Specifically, there was a pronounced reduction in activity of the pro-inflammatory transcription factors NfκB and Stat 1/2 (Small et al., 2014).There is little direct evidence to date concerning mechanisms of rAIH-induced pLTF metaplasticity; but these studies have provided several promising leads. Additional mechanistic studies are critical to understand

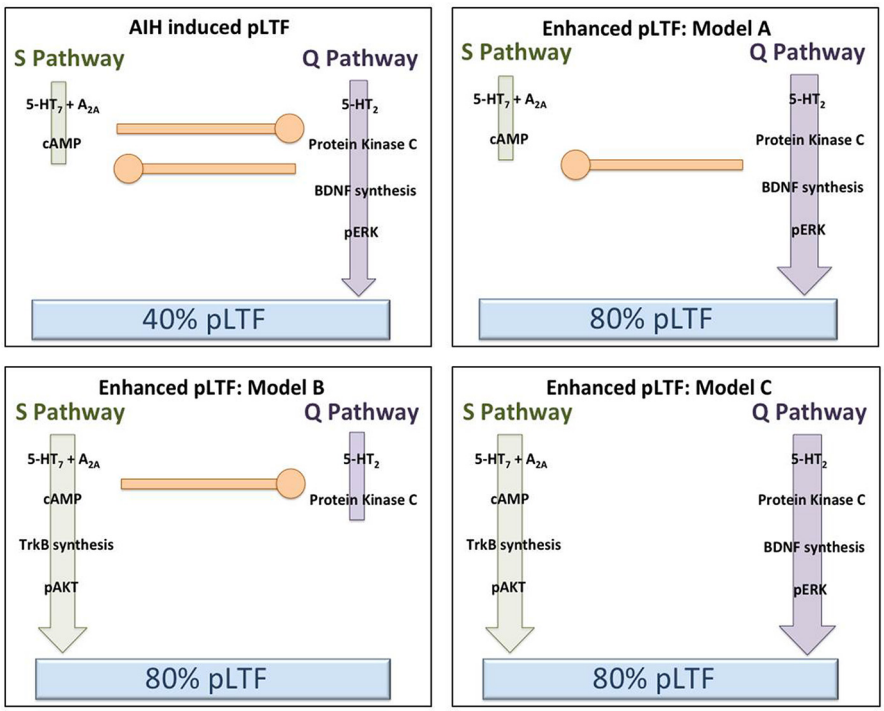

FIGURE 3 | Hypothetical models to explain pLTF metaplasticity (enhanced pLTF) after repetitive acute intermittent hypoxia (rAIH) preconditioning. In the upper left panel, our working model of Q and S pathway contributions to pLTF in normal rats is depicted (no preconditioning). Moderate AIH normally elicits ~40% pLTF, largely via dominant contributions from the Q pathway, with concurrent restraint from sub-threshold S pathway activation (i.e., cross-talk inhibition). In the remaining panels, rAIH preconditioning enhances AIH-induced pLTF, reaching ~80% facilitation. In the upper right (Model A), we illustrate the possibility that rAIH preconditioning enhances pLTF by enhancing the Q pathway to pLTF. This could be achieved by amplifying the Q pathway, or by removing inhibition from the S pathway (while leaving Q to S inhibition intact). For example, pLTF is doubled in normal rats when cross-talk inhibition from the S pathway is reduced from cervical spinal inhibition of A2A receptors, 5-HT7 receptors or PKA (see text for references). In either case, the Q pathway remains the dominant pathway to pLTF in this scenario, similar to enhanced pLTF following chronic cervical dorsal rhizotomy (CDR) or during end-stage amyotrophic lateral sclerosis (ALS) (see text for references). In the lower left panel (Model B), we illustrate the possibility that pLTF following rAIH preconditioning arises from a reversal to dominant S pathway contributions to pLTF vs. Q pathway-dependent pLTF found in normal rats. There is little available evidence to support this possibility; however, the precedent is provided by the greater S pathway-dependent pLTF resulting from severe AIH protocols (Nichols et al., 2012). In the lower right panel (Model C), we illustrate the possibility that rAIH preconditioning somehow eliminates cross-talk inhibition and uncouples the Q and S pathways to pMF; thus, each pathway is now able to contribute to an enhanced pLTF. This possibility is supported by available evidence concerning mechanisms of enhanced pLTF after CIH pre-conditioning, where both 5-HT2 and 5-HT7 receptors appear to make independent contributions (see text).

this highly novel and clinically translatable phenomenon. **Figure 3** outlines several possible changes in inter-pathway interactions that may enhance pLTF following rAIH training. A greater understanding of rAIH-induced metaplasticity will be important as we develop rAIH into a therapeutic tool to treat motor deficits caused by spinal injury (Lovett-Barr et al., 2012; Trumbower et al., 2012; Hayes et al., 2014) or motor neuron disease (Mitchell, 2007; Nichols et al., 2013).

OTHER MODELS OF pLTF METAPLASTICITY?

AIH-induced pLTF is modified by a number of other pre-treatments that enhance and diminish its expression (Mitchell et al., 2001). However, since it is not always possible to reverse the initiating factor/treatment (i.e., metaplasticity trigger), it is sometimes ambiguous if the change in pLTF reflects true metaplasticity vs. a persistent change in the initiating stimulus. Conditions altering AIH-induced pLTF that we suspect reflect models of metaplasticity include: (1) chronic cervical dorsal rhizotomy (CDR; Kinkead et al., 1998); (2) systemic inflammation (Vinit et al., 2011; Huxtable et al., 2013); (3) cervical spinal injury in a time-dependent manner (Golder and Mitchell, 2005); (4) ALS (Nichols and Mitchell, 2014); (5) age (Zabka et al., 2001); and (6) sex hormones (Zabka et al., 2006).

Of these, the best characterized is enhanced pLTF following CDR. Twenty-eight days following bilateral CDR, moderate AIH induced pLTF is doubled, with an equivalent increase in serotonin terminal density in the immediate vicinity of identified PMN (Kinkead et al., 1998). Increased serotonin terminal density is associated with enhanced pLTF in several models of pLTF metaplasticity (see above, McCrimmon et al., 1995; Kinkead et al., 1998; Mitchell et al., 2001; Satriotomo et al., 2006). Both normal and CDR-enhanced pLTF are abolished by the 5-HT2 receptor antagonist, ketanserin, demonstrating that CDR-enhanced pLTF arises from accentuation of normal Q pathway dependent pLTF (vs. combined Q/S pathway contributions following CIH training).

One week post-CDR, ventral spinal BDNF and neurotrophin-3 concentrations are increased (Johnson et al., 2000), as is the strength of crossed spinal synaptic pathways to PMN (Fuller et al., 2002). Similarly, end-stage motor neuron disease (ALS) is associated with: (1) increased BDNF and other growth/trophic factor expression in PMN (Satriotomo et al., 2006); (2) increased serotonin terminal density; and (3) enhanced Q pathway dependent pLTF (Nichols and Mitchell, 2014). Thus, enhanced pLTF may occur via accentuation of the normal Q pathway to pLTF (i.e., following CDR and ALS), or additive contributions from the Q and S pathways to pMF (e.g., following CIH). Several proposed mechanisms for enhanced, AIH-induced pLTF are outlined within **Figure 3**.

SIGNIFICANCE

Although considerable effort has been devoted to investigations of detailed cellular mechanisms giving rise to AIH-induced pLTF (Devinney et al., 2013), relatively little attention has been given to the equally important concept of respiratory metaplasticity. We still lack a fundamental understanding of when, where and how respiratory metaplasticity occurs. Even though metaplasticity confers a remarkable potential to amplify (and harness) existing mechanisms of plasticity, metaplasticity also offers promise to reveal new forms of plasticity not present in normal animals. Examples of the latter include sensory LTF after CIH preconditioning (Peng and Prabhakar, 2003, 2004), and dAIH-revealed, AIH-induced hypoglossal LTF in Brown Norway rats; a strain that does not normally exhibit hypoglossal LTF (Wilkerson and Mitchell, 2009). While hypoglossal LTF and pLTF are phenotypically similar (i.e., enhanced nerve activity following prior experience), the threshold for eliciting each differs based on triggering stimulus and strain/species (reviewed in Golder et al., 2005), strongly suggesting an adaptive change in plasticity capacity/threshold following dAIH training.

With our significant progress in understanding cellular mechanisms of AIH-induced pLTF, it is an advantageous model to study metaplasticity in respiratory motor control. Such studies are warranted from a basic science perspective, but also because rAIH is rapidly moving towards clinical application as a treatment for respiratory insufficiency in disorders that compromise breathing capacity (Lovett-Barr et al., 2012; Nichols et al., 2013) as well as motor deficits in non-respiratory motor systems (Lovett-Barr et al., 2012; Hayes et al., 2014). Principles elucidated by studies of plasticity and metaplasticity in respiratory motor control may be an essential guide for understanding the plasticity, metaplasticity and functional recovery of a diverse range of clinical disorders that compromise movement (Lovett-Barr et al., 2012; Dale et al., 2014; Hayes et al., 2014).

REFERENCES

Abraham, W. C. (2008). Metaplasticity: tuning synapses and networks for plasticity. *Nat. Rev. Neurosci.* 9:387. doi: 10.1038/nrn2356

Abraham, W. C., and Bear, M. F. (1996). Metaplasticity: the plasticity of synaptic plasticity. *Trends Neurosci.* 19, 126–130. doi: 10.1016/s0166-2236(96)80018-x

Alberini, C. M. (2008). The role of protein synthesis during the labile phases of memory: revisiting the skepticism. *Neurobiol. Learn. Mem.* 89, 234–246. doi: 10.1016/j.nlm.2007.08.007

Bach, K. B., and Mitchell, G. S. (1996). Hypoxia-induced long-term facilitation of respiratory activity is serotonin dependent. *Respir. Physiol.* 104, 251–260. doi: 10.1016/0034-5687(96)00017-5

Baker, T. L., and Mitchell, G. S. (2000). Episodic but not continuous hypoxia elicits long-term facilitation of phrenic motor output in rats. *J. Physiol.* 529, 215–219. doi: 10.1111/j.1469-7793.2000.00215.x

Baker-Herman, T. L., Fuller, D. D., Bavis, R. W., Zabka, A. G., Golder, F. J., Doperalski, N. J., et al. (2004). BDNF is necessary and sufficient for spinal respiratory plasticity following intermittent hypoxia. *Nat. Neurosci.* 7, 48–55. doi: 10.1038/nn1166

Baker-Herman, T. L., and Mitchell, G. S. (2002). Phrenic long-term facilitation requires spinal serotonin receptor activation and protein synthesis. *J. Neurosci.* 22, 6239–6246.

Bavis, R. W., and Mitchell, G. S. (2003). Intermittent hypoxia induces phrenic long-term facilitation in carotid-denervated rats. *J. Appl. Physiol. (1985)* 94, 399–409. doi: 10.1152/japplphysiol.00374.2002

Bisgard, G. E. (2000). Carotid body mechanisms in acclimatization to hypoxia. *Respir. Physiol.* 121, 237–246. doi: 10.1016/s0034-5687(00)00131-6

Blitz, D. M., and Ramirez, J. M. (2002). Long-term modulation of respiratory network activity following anoxia in vitro. *J. Neurophysiol.* 87, 2964–2971. doi: 10.1152/jn.00515.2001

Byrne, J. H. (1997). Synapses. Plastic plasticity. *Nature* 389, 791–792. doi: 10.1038/39746

Dale, E. A., Ben Mabrouk, F., and Mitchell, G. S. (2014). Unexpected benefits of intermittent hypoxia: enhanced respiratory and nonrespiratory motor function. *Physiology (Bethesda)* 29, 39–48. doi: 10.1152/physiol.00012.2013

Dale, E. A., Satriotomo, I., and Mitchell, G. S. (2012). Cervical spinal erythropoietin induces phrenic motor facilitation via extracellular signal-regulated protein kinase and Akt signaling. *J. Neurosci.* 32, 5973–5983. doi: 10.1523/JNEUROSCI.3873-11.2012

Dale-Nagle, E. A., Hoffman, M. S., MacFarlane, P. M., Satriotomo, I., Lovett-Barr, M. R., Vinit, S., et al. (2010). Spinal plasticity following intermittent hypoxia: implications for spinal injury. *Ann. N Y Acad. Sci.* 1198, 252–259. doi: 10.1111/j.1749-6632.2010.05499.x

Dale-Nagle, E. A., Satriotomo, I., and Mitchell, G. S. (2011). Spinal vascular endothelial growth factor induces phrenic motor facilitation via extracellular signal-regulated kinase and Akt signaling. *J. Neurosci.* 31, 7682–7690. doi: 10.1523/JNEUROSCI.0239-11.2011

Devinney, M. J., Huxtable, A. G., Nichols, N. L., and Mitchell, G. S. (2013). Hypoxia-induced phrenic long-term facilitation: emergent properties. *Ann. N Y Acad. Sci.* 1279, 143–153. doi: 10.1111/nyas.12085

Feldman, J. L., Mitchell, G. S., and Nattie, E. E. (2003). Breathing: rhythmicity, plasticity, chemosensitivity. *Annu. Rev. Neurosci.* 26, 239–266. doi: 10.1146/annurev.neuro.26.041002.131103

Feldman, J. L., Neverova, N. V., and Saywell, S. A. (2005). Modulation of hypoglossal motoneuron excitability by intracellular signal transduction cascades. *Respir. Physiol. Neurobiol.* 147, 131–143. doi: 10.1016/j.resp.2005.03.014

Feldman, J. L., and Smith, J. C. (1989). Cellular mechanisms underlying modulation of breathing pattern in mammals. *Ann. N Y Acad. Sci.* 563, 114–130. doi: 10.1111/j.1749-6632.1989.tb42194.x

Fletcher, E. C., Lesske, J., Behm, R., Miller, C. C. 3rd, Stauss, H., and Unger, T. (1992). Carotid chemoreceptors, systemic blood pressure and chronic episodic hypoxia mimicking sleep apnea. *J. Appl. Physiol. (1985)* 72, 1978–1984.

Forsythe, J. A., Jiang, B. H., Iyer, N. V., Agani, F., Leung, S. W., Koos, R. D., et al. (1996). Activation of vascular endothelial growth factor gene expression by hypoxia-inducible factor 1. *Mol. Cell. Biol.* 16, 4601–4613.

Fuller, D. D., Johnson, S. M., Johnson, R. A., and Mitchell, G. S. (2002). Chronic cervical spinal sensory denervation reveals ineffective spinal pathways to phrenic motoneurons in the rat. *Neurosci. Lett.* 323, 25–28. doi: 10.1016/s0304-3940(02)00121-0

Fuller, D. D., Johnson, S. M., Olson, E. B. Jr., and Mitchell, G. S. (2003). Synaptic pathways to phrenic motoneurons are enhanced by chronic intermittent hypoxia after cervical spinal cord injury. *J. Neurosci.* 23, 2993–3000.

Fuller, D. D., Zabka, A. G., Baker, T. L., and Mitchell, G. S. (2001). Phrenic long-term facilitation requires 5-HT receptor activation during but not following episodic hypoxia. *J. Appl. Physiol. (1985)* 90, 2001–2006.

Golder, F. J., and Mitchell, G. S. (2005). Spinal synaptic enhancement with acute intermittent hypoxia improves respiratory function after chronic cervical spinal cord injury. *J. Neurosci.* 25, 2925–2932. doi: 10.1523/jneurosci.0148-05.2005

Golder, F. J., Ranganathan, L., Satriotomo, I., Hoffman, M., Lovett-Barr, M. R., Watters, J. J., et al. (2008). Spinal adenosine A2a receptor activation elicits long-lasting phrenic motor facilitation. *J. Neurosci.* 28, 2033–2042. doi: 10.1523/JNEUROSCI.3570-07.2008

Golder, F. J., Zabka, A. G., Bavis, R. W., Baker-Herman, T., Fuller, D. D., and Mitchell, G. S. (2005). Differences in time-dependent hypoxia phreinc responses among inbred rat strains. *J. Appl. Physiol. (1985)* 98, 838–844. doi: 10.1152/japplphysiol.00984.2004

Grau, J. W., Huie, J. R., Lee, K. H., Hoy, K. C., Huang, Y. J., Turtle, J. D., et al. (2014). Metaplasticity and behavior: how training and inflammation affect plastic potential within the spinal cord and recovery after injury. *Front. Neural Circuits* 8:100. doi: 10.3389/fncir.2014.00100

Hakim, F., Gozal, D., and Kheirandish-Gozal, L. (2012). Sympathetic and catecholaminergic alterations in sleep apnea with particular emphasis on children. *Front. Neurol.* 3:7. doi: 10.3389/fneur.2012.00007

Hayashi, F., Coles, S. K., Bach, K. B., Mitchell, G. S., and Mccrimmon, D. R. (1993). Time-dependent phrenic nerve responses to carotid afferent activation: intact vs. decerebellate rats. *Am. J. Physiol.* 265, R811–R819.

Hayes, H. B., Jayaraman, A., Herrmann, M., Mitchell, G. S., Rymer, W. Z., and Trumbower, R. D. (2014). Daily intermittent hypoxia enhances walking after chronic spinal cord injury: a randomized trial. *Neurology* 82, 104–113. doi: 10.1212/01.wnl.0000437416.34298.43

Hoffman, M. S., Golder, F. J., Mahamed, S., and Mitchell, G. S. (2010). Spinal adenosine A2(A) receptor inhibition enhances phrenic long term facilitation following acute intermittent hypoxia. *J. Physiol.* 588, 255–266. doi: 10.1113/jphysiol.2009.180075

Hoffman, M. S., and Mitchell, G. S. (2011). Spinal 5-HT7 receptor activation induces long-lasting phrenic motor facilitation. *J. Physiol.* 589, 1397–1407. doi: 10.1113/jphysiol.2010.201657

Hoffman, M. S., and Mitchell, G. S. (2013). Spinal 5-HT7 receptors and protein kinase A constrain intermittent hypoxia-induced phrenic long-term facilitation. *Neuroscience* 250, 632–643. doi: 10.1016/j.neuroscience.2013.06.068

Hoffman, M. S., Nichols, N. L., Macfarlane, P. M., and Mitchell, G. S. (2012). Phrenic long-term facilitation after acute intermittent hypoxia requires spinal ERK activation but not TrkB synthesis. *J. Appl. Physiol. (1985)* 113, 1184–1193. doi: 10.1152/japplphysiol.00098.2012

Huxtable, A. G., Macfarlane, P. M., Vinit, S., Nichols, N. L., Dale, E. A., and Mitchell, G. S. (2014). Adrenergic alpha1 receptor activation is sufficient, but not necessary for phrenic long-term facilitation. *J. Appl. Physiol. (1985)* 116, 1345–1352. doi: 10.1152/japplphysiol.00904.2013

Huxtable, A. G., Smith, S. M., Vinit, S., Watters, J. J., and Mitchell, G. S. (2013). Systemic LPS induces spinal inflammatory gene expression and impairs phrenic long-term facilitation following acute intermittent hypoxia. *J. Appl. Physiol. (1985)* 114, 879–887. doi: 10.1152/japplphysiol.01347.2012

Jacquin, T., Denavit-Saubié, M., and Champagnat, J. (1989). Substance P and serotonin mutually reverse their excitatory effects in the rat nucleus tractus solitarius. *Brain Res.* 502, 214–222. doi: 10.1016/0006-8993(89)90616-1

Johnson, R. A., Okragly, A. J., Haak-Frendscho, M., and Mitchell, G. S. (2000). Cervical dorsal rhizotomy increases brain-derived neurotrophic factor and neurotrophin-3 expression in the ventral spinal cord. *J. Neurosci.* 20:RC77.

Katz, P. S., and Edwards, D. H. (1999). *Beyond Neurotransmission.* New York: Oxford University Press.

Kinkead, R., Bach, K. B., Johnson, S. M., Hodgeman, B. A., and Mitchell, G. S. (2001). Plasticity in respiratory motor control: intermittent hypoxia and hypercapnia activate opposing serotonergic and noradrenergic modulatory systems. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 130, 207–218. doi: 10.1016/S1095-6433(01)00393-2

Kinkead, R., Zhan, W. Z., Prakash, Y. S., Bach, K. B., Sieck, G. C., and Mitchell, G. S. (1998). Cervical dorsal rhizotomy enhances serotonergic innervation of phrenic motoneurons and serotonin-dependent long-term facilitation of respiratory motor output in rats. *J. Neurosci.* 18, 8436–8443.

Kline, D. D., Hendricks, G., Hermann, G., Rogers, R. C., and Kunze, D. L. (2009). Dopamine inhibits N-type channels in visceral afferents to reduce synaptic transmitter release under normoxic and chronic intermittent hypoxic conditions. *J. Neurophysiol.* 101, 2270–2278. doi: 10.1152/jn.91304.2008

Kline, D. D., Ramirez-Navarro, A., and Kunze, D. L. (2007). Adaptive depression in synaptic transmission in the nucleus of the solitary tract after in vivo chronic intermittent hypoxia: evidence for homeostatic plasticity. *J. Neurosci.* 27, 4663–4673. doi: 10.1523/jneurosci.4946-06.2007

Kulik, T. B., Aronhime, S. N., Echeverry, G., Beylin, A., and Winn, H. R. (2010). The relationship between oxygen and adenosine in astrocyte cultures. *Glia* 58, 1335–1344. doi: 10.1002/glia.21011

Kumar, P., and Prabhakar, N. R. (2012). Peripheral chemoreceptors: function and plasticity of the carotid body. *Compr. Physiol.* 2, 141–219. doi: 10.1002/cphy.c100069

Lee, E. J., Woodske, M. E., Zou, B., and O'donnell, C. P. (2009). Dynamic arterial blood gas analysis in conscious, unrestrained C57BL/6J mice during exposure to intermittent hypoxia. *J. Appl. Physiol. (1985)* 107, 290–294. doi: 10.1152/japplphysiol.91255.2008

Li, J., Grigoryev, D. N., Ye, S. Q., Thorne, L., Schwartz, A. R., Smith, P. L., et al. (2005). Chronic intermittent hypoxia upregulates genes of lipid biosynthesis in obese mice. *J. Appl. Physiol. (1985)* 99, 1643–1648. doi: 10.1152/japplphysiol.00522.2005

Li, Z., Morris, K. F., Baekey, D. M., Shannon, R., and Lindsey, B. G. (1999). Multimodal medullary neurons and correlational linkages of the respiratory network. *J. Neurophysiol.* 82, 188–201.

Ling, L., Fuller, D. D., Bach, K. B., Kinkead, R., and Olson, E. B. Jr. (2001). Chronic intermittent hypoxia elicits serotonin-dependent plasticity in the central neural control of breathing. *J. Neurosci.* 21, 5381–5388.

Lovett-Barr, M. R., Satriotomo, I., Muir, G. D., Wilkerson, J. E., Hoffman, M. S., Vinit, S., et al. (2012). Repetitive intermittent hypoxia induces respiratory and somatic motor recovery after chronic cervical spinal injury. *J. Neurosci.* 32, 3591–3600. doi: 10.1523/jneurosci.2908-11.2012

MacFarlane, P. M., and Mitchell, G. S. (2009). Episodic spinal serotonin receptor activation elicits long-lasting phrenic motor facilitation by an NADPH oxidase-dependent mechanism. *J. Physiol.* 587, 5469–5481. doi: 10.1113/jphysiol.2009.176982

MacFarlane, P. M., Vinit, S., and Mitchell, G. S. (2011). Serotonin 2A and 2B receptor-induced phrenic motor facilitation: differential requirement for spinal NADPH oxidase activity. *Neuroscience* 178, 45–55. doi: 10.1016/j.neuroscience.2011.01.011

MacFarlane, P. M., Vinit, S., and Mitchell, G. S. (2014). Spinal nNOS regulates phrenic motor facilitation by a 5-HT2B receptor and NADPH oxidase dependent mechanism. *Neuroscience* 269, 67–78. doi: 10.1016/j.neuroscience.2014.03.014

MacFarlane, P. M., Vinit, S., Roopra, A., and Mitchell, G. S. (2010). Enhanced phrenic long-term facilitation following repetitive acute intermittent hypoxia: role of glycolytic flux. *FASEB J.* 24, 799.15.

Mahamed, S., and Mitchell, G. S. (2007). Is there a link between intermittent hypoxia-induced respiratory plasticity and obstructive sleep apnoea? *Exp. Physiol.* 92, 27–37. doi: 10.1113/expphysiol.2006.033720

Malinow, R., and Malenka, R. C. (2002). AMPA receptor trafficking and synaptic plasticity. *Annu. Rev. Neurosci.* 25, 103–126. doi: 10.1146/annurev.neuro.25.112701.142758

Manahan-Vaughan, D., Kulla, A., and Frey, J. U. (2000). Requirement of translation but not transcription for the maintenance of long-term depression in the CA1 region of freely moving rats. *J. Neurosci.* 20, 8572–8576.

McCrimmon, D. R., Mitchell, G. S., and Dekin, D. K. (1995). *Glutamate, GABA and Serotonin in Ventilatory Control.* New York: Marcel-Dekker.

McGuire, M., Tartar, J. L., Cao, Y., McCarley, R. W., White, D. P., Strecker, R. E., et al. (2008). Sleep fragmentation impairs ventilatory long-term facilitation via adenosine A1 receptors. *J. Physiol.* 586, 5215–5229. doi: 10.1113/jphysiol.2008.158121

McGuire, M., Zhang, Y., White, D. P., and Ling, L. (2003). Chronic intermittent hypoxia enhances ventilatory long-term facilitation in awake rats. *J. Appl. Physiol. (1985)* 95, 1499–1508. doi: 10.1152/japplphysiol.00044.2003

McGuire, M., Zhang, Y., White, D. P., and Ling, L. (2004). Serotonin receptor subtypes required for ventilatory long-term facilitation and its enhancement after chronic intermittent hypoxia in awake rats. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 286, R334–R341. doi: 10.1152/ajpregu.00463.2003

Mesce, K. A. (2002). Metamodulation of the biogenic amines: second-order modulation by steroid hormones and amine cocktails. *Brain Behav. Evol.* 60, 339–349. doi: 10.1159/000067793

Mitchell, G. S. (2007). "Respiratory plasticity following intermittent hypoxia: a guide for novel therapeutic approaches to ventilatory control disorders," in *Genetic Basis for Respiratory Control Disorders*, ed C. Gaultier (New York: Springer Publishing Company), 291–311.

Mitchell, G. S., Baker, T. L., Nanda, S. A., Fuller, D. D., Zabka, A. G., Hodgeman, B. A., et al. (2001). Invited review: intermittent hypoxia and respiratory plasticity. *J. Appl. Physiol. (1985)* 90, 2466–2475.

Mitchell, G. S., Douse, M. A., and Foley, K. T. (1990). Receptor interactions in modulating ventilatory activity. *Am. J. Physiol.* 259, R911–R920.

Mitchell, G. S., and Johnson, S. M. (2003). Neuroplasticity in respiratory motor control. *J. Appl. Physiol. (1985)* 94, 358–374. doi: 10.1152/japplphysiol.00523.2002

Moraes, D. J., Zoccal, D. B., and Machado, B. H. (2012). Medullary respiratory network drive sympathetic overactivity and hypertensions in rats submitted to chronic intermittent hypoxia. *Hypertension* 60, 1374–1380. doi: 10.1161/HYPERTENSIONAHA.111.189332

Morris, K. F., Arata, A., Shannon, R., and Lindsey, B. G. (1996). Long-term facilitation of phrenic nerve activity in cats: responses and short term scale correlations of medullary neurones. *J. Physiol.* 490, 463–480. doi: 10.1113/jphysiol.1996.sp021158

Navarette-Opazo, A. A., and Mitchell, G. S. (2014). Therapeutic potential of intermittent hypoxia: a matter of dose. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 307, R1181–R1197. doi: 10.1152/ajpregu.00208.2014

Nichols, N. L., Dale, E. A., and Mitchell, G. S. (2012). Severe acute intermittent hypoxia elicits phrenic long-term facilitation by a novel adenosine-dependent mechanism. *J. Appl. Physiol. (1985)* 112, 1678–1688. doi: 10.1152/japplphysiol.00060.2012

Nichols, N. L., and Mitchell, G. S. (2014). Enhanced phrenic long-term facilitation in rats with motor neuron death from intrapleural CTB-saporin injections. *FASEB J.* 28, 713.1.

Nichols, N. L., Van Dyke, J., Nashold, L., Satriotomo, I., Suzuki, M., and Mitchell, G. S. (2013). Ventilatory control in ALS. *Respir. Physiol. Neurobiol.* 189, 429–437. doi: 10.1016/j.resp.2013.05.016

Peng, Y. J., and Prabhakar, N. R. (2003). Reactive oxygen species in the plasticity of respiratory behavior elicited by chronic intermittent hypoxia. *J. Appl. Physiol. (1985)* 94, 2342–2349. doi: 10.1152/japplphysiol.00613.2002

Peng, Y. J., and Prabhakar, N. R. (2004). Effect of two paradigms of chronic intermittent hypoxia on carotid body sensory activity. *J. Appl. Physiol. (1985)* 96, 1236–1242. doi: 10.1152/japplphysiol.00820.2003

Philips, G. T., Kopec, A. M., and Carew, T. J. (2013). Pattern and predicitability in memory formation: from molecular mechanisms to clinical relevance. *Neurobiol. Learn. Mem.* 105, 117–124. doi: 10.1016/j.nlm.2013.05.003

Prabhakar, N. R., and Semenza, G. L. (2012). Adaptive and maladaptive cardiorespiratory responses to continuous and intermittent hypoxia mediated by hypoxia-inducible factors 1 and 2. *Physiol. Rev.* 92, 967–1003. doi: 10.1152/physrev.00030.2011

Ramirez, J. M., Doi, A., Garcia, A. J. 3rd, Elsen, F. P., Koch, H., and Wei, A. D. (2012). The cellular building blocks of breathing. *Compr. Physiol.* 4, 2683–2731. doi: 10.1002/cphy.c110033

Reeves, S. R., and Gozal, D. (2006). Respiratory and metabolic responses to early postnatal chronic intermittent hypoxia and sustained hypoxia in the developing rat. *Peditr. Res.* 60, 680–686. doi: 10.1203/01.pdr.0000246073.95911.18

Ribeiro, J. A., and Sebastiao, A. M. (2010). Modulation and metamodulation of synapses by adenosine. *Acta Physiol. (Oxf.)* 199, 161–169. doi: 10.1111/j.1748-1716.2010.02115.x

Row, B. W. (2007). Intermittent hypoxia and cognitive function: implications from chronic animal models. *Adv. Exp. Med. Biol.* 618, 51–67. doi: 10.1007/978-0-387-75434-5_5

Row, B. W., Kheirandish, L., Cheng, Y., Rowell, P. P., and Gozal, D. (2007). Impaired spatial working memory and altered choline acetyltransferase (CHAT) immunoreactivity and nicotinic receptor binding in rats exposed to intermittent hypoxia during sleep. *Behav. Brain Res.* 177, 308–314. doi: 10.1016/j.bbr.2006.11.028

Sankari, A., Bascom, A., Oomman, S., and Badr, M. S. (2014). Sleep disordered breathing in chronic spinal cord injury. *J. Clin. Sleep Med.* 10, 65–72. doi: 10.5664/jcsm.3362

Satriotomo, I., Dale, E. A., Dahlberg, J. M., and Mitchell, G. S. (2012). Repetitive acute intermittent hypoxia increases expression of proteins associated with plasticity in the phrenic motor nucleus. *Exp. Neurol.* 237, 103–115. doi: 10.1016/j.expneurol.2012.05.020

Satriotomo, I., Nashold, L. J., Svendsen, C. N., and Mitchel, G. S. (2006). Enhancement of BDNF and serotonin terminal density in phreic and hypoglossal motor nuclei in a rat model of amyotrophic lateral sclerosis. *FASEB J.* 20, A1212.

Semenza, G. L., Nejfelt, M. K., Chi, S. M., and Antonarakis, S. E. (1991). Hypoxia-inducible nuclear factors bind to an enhancer element located 3′ to the human erythropoietin gene. *Proc. Natl. Acad. Sci. U S A* 88, 5680–5684. doi: 10.1073/pnas.88.13.5680

Sherff, C. M., and Carew, T. J. (2002). Coincident induction of long-term facilitation at sensory-motor synapses in Aplysia: presynaptic and postsynaptic factors. *Neurobiol. Learn. Mem.* 78, 498–507. doi: 10.1006/nlme.2002.4092

Small, A., Nikodemova, M., Doughtery, B., Mitchell, G. S., and Watters, J. J. (2014). Repetitive acute intermittent hypoxia differentially affects CD11b+ cell phenotypes after C2 spinal hemisection. *FASEB J.* 28, 713.6.

Strey, K. A., Baertsch, N. A., and Baker-Herman, T. L. (2013). Inactivity-induced respiratory plasticity: protecting the drive to breathe in disorders that reduce respiratory neural activity. *Respir. Physiol. Neurobiol.* 189, 384–394. doi: 10.1016/j.resp.2013.06.023

Tasali, E., and Ip, M. S. (2008). Obstructive sleep apnea and metabolic syndrome: alterations in glucose metabolism and inflammation. *Proc. Am. Thorac. Soc.* 5, 207–217. doi: 10.1513/pats.200708-139mg

Trumbower, R. D., Jayaraman, A., Mitchell, G. S., and Rymer, W. Z. (2012). Exposure to acute intermittent hypoxia augments somatic motor function in humans with incomplete spinal cord injury. *Neurorehabil. Neural Repair* 26, 163–172. doi: 10.1177/1545968311412055

Vinit, S., Lovett-Barr, M. R., and Mitchell, G. S. (2009). Intermittent hypoxia induces functional recovery following cervical spinal injury. *Respir. Physiol. Neurobiol.* 169, 210–217. doi: 10.1016/j.resp.2009.07.023

Vinit, S., Satriotomo, I., Macfarlane, P. M., and Mitchell, G. S. (2010). Enhanced phrenic long-term facilitation following repetitive (pLTF) acute intermittent hypoxia. *FASEB J.* 799.15.

Vinit, S., Windelborn, J. A., and Mitchell, G. S. (2011). Lipopolysaccharide attenuates phrenic long-term facilitation following acute intermittent hypoxia. *Respir. Physiol. Neurobiol.* 176, 130–135. doi: 10.1016/j.resp.2011.02.008

Wiegert, J. S., and Bading, H. (2011). Activity-dependent calcium signaling and ERK-MAP kinases in neurons: a link to structural plasticity of the nucleus and gene transcription regulation. *Cell Calcium* 49, 296–305. doi: 10.1016/j.ceca.2010.11.009

Wilkerson, J. E., and Mitchell, G. S. (2009). Daily intermittent hypoxia augments spinal BDNF levels, ERK phosphorylation and respiratory long-term facilitation. *Exp. Neurol.* 217, 116–123. doi: 10.1016/j.expneurol.2009.01.017

Zabka, A. G., Behan, M., and Mitchell, G. S. (2001). Long term facilitation of respiratory motor output decreases with age in male rats. *J. Physiol.* 531, 509–514. doi: 10.1111/j.1469-7793.2001.0509i.x

Zabka, A. G., Mitchell, G. S., and Behan, M. (2006). Conversion from testosterone to oestradiol is required to modulate respiratory long-term facilitation in male rats. *J. Physiol.* 576, 903–912. doi: 10.1113/jphysiol.2006.114850

Zhang, S. X., Wang, Y., and Gozal, D. (2012). Pathological consequences of intermittent hypoxia in the central nervous system. *Compr. Physiol.* 2, 1767–1777. doi: 10.1002/cphy.c100060

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REVIEW
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Sensory Activation of Command Cells for Locomotion and Modulatory Mechanisms: Lessons from Lampreys

Gheylen Daghfous[1,2], *Warren W. Green*[3†], *Simon T. Alford*[4], *Barbara S. Zielinski*[3] *and Réjean Dubuc*[1,2*]

[1] Groupe de Recherche en Activité Physique Adaptée, Département des Sciences de l'Activité Physique, Université du Québec à Montréal, Montréal, QC, Canada, [2] Groupe de Recherche sur le Système Nerveux Central, Département de Neurosciences, Université de Montréal, Montréal, QC, Canada, [3] Department of Biological Sciences and Great Lakes Institute for Environmental Research, University of Windsor, Windsor, ON, Canada, [4] Laboratory of Integrative Neuroscience, Department of Biological Sciences, University of Illinois at Chicago, Chicago, IL, USA

Sensorimotor transformation is one of the most fundamental and ubiquitous functions of the central nervous system (CNS). Although the general organization of the locomotor neural circuitry is relatively well understood, less is known about its activation by sensory inputs and its modulation. Utilizing the lamprey model, a detailed understanding of sensorimotor integration in vertebrates is emerging. In this article, we explore how the vertebrate CNS integrates sensory signals to generate motor behavior by examining the pathways and neural mechanisms involved in the transformation of cutaneous and olfactory inputs into motor output in the lamprey. We then review how 5-hydroxytryptamine (5-HT) acts on these systems by modulating both sensory inputs and motor output. A comprehensive review of this fundamental topic should provide a useful framework in the fields of motor control, sensorimotor integration and neuromodulation.

Keywords: sensorimotor, locomotion, modulation, reticulospinal neurons, lamprey, 5-HT

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****Correspondence:***
Réjean Dubuc
rejean.dubuc@gmail.com

†Present address:
Warren W. Green,
Department of Pharmacology and Therapeutics, University of Florida, College of Medicine, Gainesville, FL, USA

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INTRODUCTION

Locomotion is a rhythmic motor behavior involved in everyday functions. It requires the activation and coordination of the axial and/or appendicular musculature. Spinal neuronal networks called "central pattern generators" (CPGs) for locomotion generate the patterns of muscle activation that underlie propulsion during locomotion. Supraspinal structures, on the other hand, are required

Abbreviations: 5-HT, 5-hydroxytryptamine; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AOO, accessory olfactory organ; ARRN, anterior rhombencephalic reticular nucleus; CPG, central pattern generator; DR, dorsal root; dV, descending root of the trigeminal nerve; DCN, dorsal column nucleus; EPSC, excitatory post synaptic current; EPSP, excitatory post synaptic potential; MLR, mesencephalic locomotor region; MOE, main olfactory epithelium; MRN, mesencephalic reticular nucleus; MRRN, middle rhombencephalic reticular nucleus; ndV, nucleus of the descending root of the trigeminal nerve; NMDA, N-methyl-D-aspartate; NMDAR, N-methyl-D-aspartate receptor; OB, olfactory bulb; OLA, octavolateralis area; OSN, olfactory sensory neurons; PRRN, posterior rhombencephalic reticular nucleus; PT, posterior tuberculum; RS, reticulospinal; SC, spinal cord; SNAP-25, synaptosomal-associated protein 25; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; TTX, tetrodotoxin; VGCC, voltage-gated calcium channel; VR, ventral root.

for activating and controlling the spinal CPGs. Descending inputs trigger, maintain, and eventually stop locomotion. The brainstem reticulospinal (RS) cells act as command cells that constitute an important interface between supraspinal and spinal networks. As such, the activation of RS cells by sensory (sensory-evoked locomotion) or internal clues (goal-directed locomotion) will markedly influence spinal function.

In this review, we focus on sensory-evoked locomotion by examining how two different sensory modalities influence the activation RS cells in a basal vertebrate, the lamprey. Because lampreys share a common brain "bauplan" with jawed vertebrates, including mammals, knowledge gained from neural circuits and mechanisms in lampreys provides insight into fundamental principles of vertebrate brain organization and function (Grillner et al., 1998b; Robertson et al., 2014). This review article focuses on some recent work in the lamprey from our labs on the pathways and neural mechanisms involved in the transformation of cutaneous and olfactory inputs into motor output. These sensory modalities are of paramount importance for the survival and reproductive success of individuals as they drive feeding, reproductive, and escape behaviors. We will also discuss 5-hydroxytryptamine (5-HT) modulation of these sensorimotor pathways. Indeed, 5-HT modulates both the sensory inputs to the RS cells at the supraspinal level and the descending motor commands of the RS cells in the spinal cord (SC). The mechanisms by which 5-HT modulates synaptic transmission has been well described in lampreys (Takahashi et al., 2001; Alford et al., 2003; Gerachshenko et al., 2005; Schwartz et al., 2005; Photowala et al., 2006; Schwartz et al., 2007; Gerachshenko et al., 2009; Alpert and Alford, 2013).

After a brief review of the motor circuitry and neural mechanisms of locomotion, sensorimotor transformations will be addressed starting with the neural pathways, from the receptors to the neural centers, followed by the neural mechanisms. Sensorimotor transformations of cutaneous and olfactory inputs will be addressed similarly. We have recently shown that the neural connections for these two sensory modalities differ considerably, yet activate the same target cells in the lower brainstem, the RS cells (Viana Di Prisco et al., 2000; Derjean et al., 2010). The pathway for cutaneous-induced locomotor reactions is shorter and more direct to the RS cells than the pathway involved in olfactory-induced locomotion. The transformation mechanisms are also different. The RS cell responses to cutaneous inputs (mechanical stimulation) switch from subthreshold excitatory postsynaptic potentials (EPSPs) to large sustained depolarizations; the output being an all-or-none escape locomotor bout. The olfactory inputs likely provide a more finely controlled locomotor output by acting via the mesencephalic locomotor region (MLR), a specific brainstem region involved in controlling goal directed locomotion. This review article will compare these two sensorimotor systems at the levels of neuronal connectivity and cellular mechanisms. We will then examine how 5-HT acts on these systems. In the past we have shown that cutaneous sensory inputs to RS cells are modulated by 5-HT (Antri et al., 2008). Similarly, there is prominent 5-HT innervation in the olfactory system, from the olfactory epithelium to the olfactory bulb (OB; Zielinski et al., 2000). Finally, we will review 5-HT modulation at the SC level. It has been extensively documented in the past that it exerts presynaptic effects that modulate the transmission from descending RS axons on SC neurons (Schwartz et al., 2005, 2007). This modulation has powerful effects on locomotor behavior.

THE LAMPREY AS A VERTEBRATE MODEL OF SENSORIMOTOR INTEGRATION

There is large interest in understanding the neural basis of behavior. The use of lampreys as a model system has made it possible to bridge the gap between cellular mechanisms and behavior. Indeed, the lamprey nervous system is remarkably similar to the mammalian nervous system, but it contains considerably fewer neurons and is thus greatly simpler. Moreover, lamprey brainstem command cells are more easily accessible for electrophysiological studies, which can be readily combined with imaging techniques in controlled *in vitro* conditions with the entire locomotor circuitry intact. The supraspinal mechanisms responsible for initiating and controlling locomotion can be studied with an array of *in vitro* techniques, with the added benefit of including all relevant structures needed for locomotor control, and the ability to monitor ongoing swimming behavior in a semi-intact preparation consisting of the exposed brain and rostral SC with the rest of the body left intact. As such, the lamprey model has paved the way for important discoveries, including the first detailed characterization of a vertebrate CPG for locomotion (Buchanan and Grillner, 1987). Acquisition of detailed knowledge of its motor circuitry opened the way for rapid progress in understanding sensorimotor integration at the system and cellular levels. For instance, new information on sensory-evoked locomotion was provided by describing the cellular mechanisms underlying the transformation of cutaneous inputs into locomotor output at the supraspinal level (Viana Di Prisco et al., 1997, 2000; Antri et al., 2009). For the first time in any vertebrate species, the neural substrate responsible for the transformation of olfactory inputs into a locomotor output was identified using the lamprey model (Derjean et al., 2010). Overall, the lamprey nervous system is ideally suited for the mechanistic study of sensorimotor integration.

THE NEURAL CONTROL OF LOCOMOTION

As indicated above, the basic muscle synergies responsible for locomotor propulsion are generated by SC networks collectively known as CPGs (reviewed in Grillner et al., 2008). CPGs are also involved in generating respiration (reviewed in Del Negro et al., 2002) and mastication (reviewed in Westberg and Kolta, 2011). The neuronal activity is produced by integrating the intrinsic properties of the CPG neurons and the synaptic connectivity of the inextricably linked neural network (Marder and Thirumalai, 2002; Alford et al., 2003). Synaptic activity, whether mediating the release of fast acting neurotransmitters such as glutamate or

neuromodulators such as the monoamines, dopamine or 5-HT, modifies the intrinsic properties. The neural network of the lamprey locomotor CPG has been well characterized (Buchanan and Grillner, 1987). Ipsilateral glutamatergic excitation in conjunction with contralateral inhibition play a crucial role (Grillner and Wallén, 1980; Buchanan and Cohen, 1982; Brodin et al., 1985; Alford and Williams, 1989; Hellgren et al., 1992); They generate ventral root (VR) bursting that alternates across the SC (Grillner et al., 1995). The spinal locomotor circuit is activated by descending commands and in particular by glutamate release from brainstem RS cells (Buchanan et al., 1987; Ohta and Grillner, 1989). The intensity of input from RS axons regulates the frequency of these bursts of activity and therefore the speed of locomotion (Viana Di Prisco et al., 2000; Brocard and Dubuc, 2003), which may range from 0.1 to 10 Hz. Experimentally, locomotor CPG activity in the SC may be activated by stimulating the lamprey brainstem in semi-intact preparations which generate RS output (McClellan and Grillner, 1984; Sirota et al., 2000; Brocard and Dubuc, 2003; Le Ray et al., 2003) or alternatively by applying glutamate receptor agonists in isolated SCs (Cohen and Wallén, 1980; Grillner et al., 1981). The alternating pattern of VR bursting recorded under these experimental conditions is referred to as "fictive locomotion", and drives the coordinated contraction of muscles necessary for lamprey swimming.

The principal neurotransmitter that activates spinal CPGs is glutamate. Work in lampreys (Grillner et al., 1981; Buchanan et al., 1987; Marder, 1994), *Xenopus* tadpoles (Dale and Roberts, 1984; Roberts and Alford, 1986; Marder and Thirumalai, 2002; Alford et al., 2003), newborn rats (Armstrong, 1986; Kudo and Yamada, 1987) and cats (Shik et al., 1966; Douglas et al., 1993; Sirota et al., 2000) demonstrates that α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptor mediated transmission in the SC activates and maintains locomotion. These data are supported by direct recordings of EPSPs onto motoneurons and premotoneurons (Dale and Roberts, 1985; Brodin et al., 1988; Noga et al., 2003; Dubuc et al., 2008) and pharmacological manipulation of the resultant behaviors (Dale and Roberts, 1984; Brodin and Grillner, 1985; Cazalets et al., 1992; Chau et al., 2002; Rybak et al., 2006). Glutamatergic neurotransmission in the SC both directly excites neurons of the CPG, but may also activate either plateau properties of spinal cells as shown in the turtle (Hounsgaard and Kiehn, 1985; Guertin and Hounsgaard, 1998), or complex oscillatory properties in these neurons mediated by NMDA receptor voltage dependency and Ca^{2+} permeability. NMDA receptor-evoked neuronal oscillations were first shown in lamprey (Sigvardt et al., 1985; Wallén and Grillner, 1987), and were since identified in mammals (Hochman et al., 1994a,b; Wilson et al., 2005; Masino et al., 2012; for a review, see Schmidt et al., 1998).

The identity of the descending glutamatergic RS command neurons is well-defined in lampreys (**Figure 1A**; Dubuc et al., 2008). RS cells have been described anatomically and physiologically. They constitute about 90% of the neurones projecting to the SC (Swain et al., 1993; Bussières, 1994; Davis and McClellan, 1994a,b). RS cells are located in one mesencephalic reticular nucleus (MRN) and three rhombencephalic reticular nuclei, the anterior (ARRN), the middle (MRRN) and the posterior (PRRN; Nieuwenhuys, 1972, 1977; Brodin et al., 1988; Swain et al., 1993; Davis and McClellan, 1994a,b). There are about 1250 RS cells on each side and about 85% of these are located in the PRRN and MRRN (Bussières, 1994). Numerous attempts to establish homologies of these nuclei to reticular nuclei in other vertebrate species have been made in the past (Kimmel et al., 1982; Cruce and Newman, 1984; ten Donkelaar et al., 1987; Nieuwenhuys and Nicholson, 1998; Brocard and Dubuc, 2003; Butler and Hodos, 2005; Nieuwenhuys, 2011). Based on the cytoarchitecture, anatomical position and connections of the reticular nuclei, the following homologies were proposed: (i) the ARRN and MRRN, which contain large medially-projecting RS cells (Müller cells; Rovainen, 1967), are homologous to the superior and middle reticular nuclei of fish, amphibians and reptiles. These nuclei would be respectively homologous to the nuclei pontis oralis and caudalis of mammals; (ii) the PRRN, which contains laterally-projecting RS cells, is homologous to the inferior reticular nuclei of fish, amphibians and reptiles. This nucleus is comparable to the nuclei reticularis gigantocellularis, ventralis and magnocellularis of mammals.

It has been shown in lampreys that the axons of large RS cells make synaptic contacts with several classes of spinal neurons and some are involved in generating locomotion (Rovainen, 1974; Buchanan, 1982; Ohta and Grillner, 1989; for a review of RS pathways in mammals, see Perreault and Glover, 2013). Similarly, studies conducted in mice and zebrafish showed that genetically identified glutamatergic RS cells of the hindbrain are involved in controlling locomotion (Hägglund et al., 2010; Bretzner and Brownstone, 2013; Kimura et al., 2013). Bouvier et al. (2015) recently identified a new population of RS cells involved in stopping locomotion. These studies opened the way to the genetic dissection of RS pathways, thus leading to further examination of their evolutionary conservation. As will be outlined below, locomotor activity is produced by spinal CPGs that are activated and maintained by descending commands. During goal-directed locomotion, this activity originates in forebrain structures, including the basal ganglia (Armstrong, 1986), to activate a largely serial process in which these higher centers recruit locomotor centers including the MLR (Shik et al., 1966; Eidelberg et al., 1981; Skinner and Garcia-Rill, 1984; Bernau et al., 1991; Marlinsky and Voitenko, 1991; Sirota et al., 2000; Cabelguen et al., 2003; Musienko et al., 2008; Ryczko et al., 2016; for reviews, see Le Ray et al., 2011; Ryczko and Dubuc, 2013) and then the RS system (Orlovskiĭ, 1970a,b, 1972; Shefchyk et al., 1984; Steeves and Jordan, 1984; Garcia-Rill et al., 1986; Jordan, 1986; Garcia-Rill and Skinner, 1987a,b). The MLR exerts an excitatory influence on RS cells either directly via glutamatergic and cholinergic (nicotinic) projections to the RS cells (Brocard and Dubuc, 2003; Le Ray et al., 2003; Noga et al., 2003; Grillner et al., 2008; Brocard et al., 2010) or indirectly via a cholinergic projection to glutamatergic muscarinoceptive cells of the brainstem that project back to

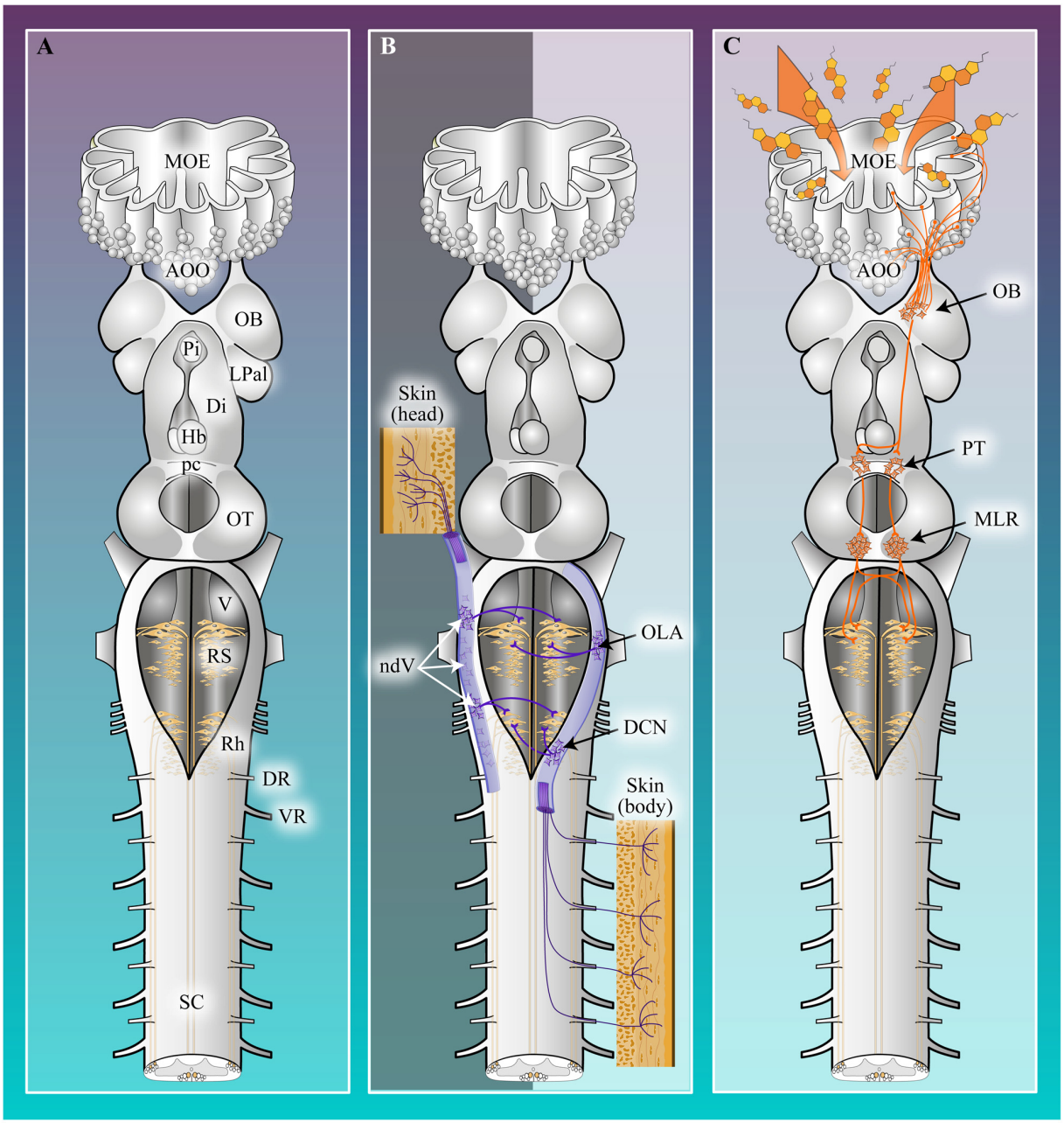

FIGURE 1 | Schematic representation of the brain and sensory-locomotor circuitry in lampreys. (A) The lamprey central nervous system (CNS). AOO, Accessory olfactory organ; Di, Diencephalon; DR, Dorsal root; Hb, Habenula; LPal, Lateral pallium; MOE, Main olfactory epithelium; OB, Olfactory bulb; OT, Optic tectum; pc, Posterior commissure; Pi, Pineal gland; Rh, Rhombencephalon; RS, Reticulospinal cells; SC, Spinal cord; V, Motor nucleus of the trigeminal nerve; VR, Ventral root. **(B)** The somato-locomotor pathway (purple) involves only a single relay, located in the alar plate, between the afferent sensory fibers and the RS cells. The inputs from the head region are relayed to RS cells by neurons located in the nucleus of the descending root of the trigeminal nerve (ndV), whereas inputs from the body are relayed to RS cells by neurons located in the dorsal column nucleus (DCN) or in the octavolateralis area (OLA). **(C)** The olfacto-locomotor pathway (orange) consists of a projection from the medial part of the OB to the mesencephalic locomotor region (MLR) via the posterior tuberculum (PT). The MLR controls locomotion in all vertebrate species through a direct projection to the command cells for locomotion, the RS cells (beige). The RS cells, in turn, project to the spinal central pattern generators (CPGs) that generate muscle synergies responsible for locomotion.

the RS cells (Smetana et al., 2007, 2010). The involvement of the cholinergic system in the control of locomotion is still subject to debate. Indeed, MLR cholinergic inputs have an excitatory influence on RS cells, but are not essential to induce locomotion (Brocard and Dubuc, 2003; Le Ray et al., 2003; Smetana et al., 2007, 2010; Jordan et al., 2014; Roseberry et al., 2016). As such,

it seems that cholinergic inputs cooperate with glutamatergic inputs to amplify and sustain the locomotor output. Further investigations of the respective roles of the glutamatergic and cholinergic systems in the control of locomotion are needed.

SENSORIMOTOR TRANSFORMATIONS OF CUTANEOUS INPUTS

Cutaneous inputs can induce, modulate and stop locomotion in vertebrates (Duysens, 1977; Viala et al., 1978; Clarke and Roberts, 1984; McClellan and Grillner, 1984; Boothby and Roberts, 1992; Frigon et al., 2012; reviewed in Grillner, 1985; Rossignol et al., 2006). The anatomical pathways and cellular mechanisms underlying these sensorimotor transformations were first characterized in the lamprey (Dubuc et al., 1993a,b; Viana Di Prisco et al., 1997, 2000, 2005; Antri et al., 2009; Le Ray et al., 2010) and more recently in *Xenopus* (Buhl et al., 2012).

The Neural Pathway: From Cutaneous Receptors to Brain Locomotor Centers

As in other vertebrates, different types of mechanoreceptors are located on the skin of lampreys (Lethbridge and Potter, 1982). These include Merkel cells (Fahrenholz, 1936; Whitear and Lane, 1981), free nerve endings, and neuromasts (Lethbridge and Potter, 1982). Neuromasts are mechanosensory organs associated with the lateral line system and will not be discussed further here (see Gelman et al., 2007). The Merkel cells are present all over the body and are particularly abundant in the epidermis of the mouth, gills and fins (Fahrenholz, 1936; Whitear and Lane, 1981; Lethbridge and Potter, 1982). As seen in other vertebrates, these microvillar cells are connected to the surrounding epidermal cells by desmosomes and granules are concentrated at the site of apparent synaptic junctions with afferent nerve fibers. However, in the lamprey, their association with a nerve fiber is unique by the presence of a spur on the neurite (Whitear and Lane, 1981). Nerve fibers conveying cutaneous input enter the CNS by the dorsal roots (DRs) of the SC (body inputs) and by the trigeminal nerve (head inputs) (**Figure 1B**; Martin and Wickelgren, 1971; Matthews and Wickelgren, 1978; Rovainen and Yan, 1985; Christenson et al., 1988). Afferent fibers of the DRs and trigeminal nerve have their cell bodies within the brain/SC (dorsal cells) or in the DR ganglia/trigeminal ganglia (Rovainen and Yan, 1985). Most DR afferent fibers carrying somatosensory information from the body ascend, via the dorsal column, to terminate in the dorsal column nucleus (DCN) in the brainstem (**Figure 1B**; Dubuc et al., 1993b). Some fibers, however, continue further rostrally and reach the octavolateralis area (OLA; **Figure 1B**; Ronan and Northcutt, 1990; Dubuc et al., 1993b). Alar plate neurons from the DCN and OLA then project to the RS cells thus providing a disynaptic pathway for cutaneous inputs to reach the RS cells (**Figure 1B**; Dubuc et al., 1993b; Pflieger and Dubuc, 2004). Similarly, there is a disynaptic pathway relaying head somatosensory information to the RS cells (**Figure 1B**). After entering the brain by the sensory root of the trigeminal nerve, trigeminal afferent fibers form the descending root of the trigeminal nerve (dV) that extends down to the rostral SC. There is no well-defined sensory nucleus of the descending root (ndV) in lampreys but neurons scattered among the dV fibers constitute a diffuse ndV (Northcutt, 1979; Viana Di Prisco et al., 2005). Interestingly, tract-tracing experiments showed that some of these neurons project to the RS cells and thus could constitute a trigeminal sensory relay to RS cells (Viana Di Prisco et al., 2005).

The Neural Mechanisms

Cutaneous primary sensory neurons (ganglion and dorsal cells) have been classified as touch, pressure, and possibly nociception, based on their response patterns to the skin stimulation. Touch cells are fast-adapting cells that respond to light mechanical stimulation of the skin with one or two spikes at the onset and offset of the stimulation (Martin and Wickelgren, 1971; Matthews and Wickelgren, 1978; Christenson et al., 1988). Pressure cells respond to mechanical stimulation of the skin by a slowly-adapting discharge with frequency related to the stimulus intensity. A third type of cell, the nociceptive cell, has been reported to respond to heavy pressure applied on the skin by a slowly-adapting discharge (Martin and Wickelgren, 1971; Matthews and Wickelgren, 1978; Rovainen and Yan, 1985). Activation of these primary sensory neurons by mechanical stimulation of the skin or electrical stimulation of the nerves (trigeminal or spinal DRs) induces post-synaptic potentials in intracellularly recorded RS cells (Viana Di Prisco et al., 1997, 2000, 2005). This finding is in accordance with behavioral observations showing that skin stimulation elicits escape swimming in intact animals (McClellan, 1988; Cardin et al., 1999). Excitatory and inhibitory amino acids are involved in the transmission of cutaneous inputs to RS cells (Dubuc et al., 1993a,b; Viana Di Prisco et al., 1995, 2005). The transmission from cutaneous (trigeminal and dorsal column) sensory afferent fibers to alar plate relay neurons was shown to be glutamatergic (Dubuc et al., 1993a,b; Viana Di Prisco et al., 1995, 2005). Cutaneous inputs are then relayed to RS cells by glycinergic and glutamatergic neurons of the relay nuclei (OLA and DCN for body inputs; dV for head inputs).

Further examination of the physiology of this disynaptic pathway led to a very interesting discovery on how RS cells transform a brief sensory input into a long-lasting motor output due to intrinsic plateau properties of RS cells (Viana Di Prisco et al., 1997). Indeed, the skin stimulation intensity—RS cell response intensity relationship is not strictly linear. At low intensities, skin stimulation elicits graded post-synaptic potentials in RS cells in a linear fashion. As the sensory stimulus intensity reaches a high level, the excitatory response in RS cells switches from sub-threshold to a large sustained depolarization that triggers escape locomotion in a semi-intact preparation. The sustaining depolarization is NMDA receptor-dependent and Ca^{2+} entry into the cell in turn activates a Ca^{2+}-activated non-selective cation current (I_{CAN}). The sustained depolarizations often last for a very long duration (up to minutes). It was found that synaptic

inputs could feed back onto intrinsic properties to temporally amplify the sustained depolarizations (Antri et al., 2009). Reversibly blocking SC inputs to RS cells markedly reduced the duration of the sustained depolarizations. In addition, pressure ejecting ionotropic glutamate receptor blockers on a recorded RS cell during the sustained depolarization reduced both their amplitude and duration. These findings indicate that excitatory synaptic inputs cooperate with intrinsic properties to prolong the sustained depolarizations (Antri et al., 2009). Whether similar mechanisms are involved in transforming sensory inputs from other sources into motor output remains to be determined.

SENSORIMOTOR TRANSFORMATIONS OF OLFACTORY INPUTS

Olfactory cues can induce locomotion in vertebrates (Hasler and Wisby, 1951; Fady et al., 1998; Varendi and Porter, 2001; Johnson et al., 2009; for a review, see Daghfous et al., 2012). However, the neural substrate underlying olfactory-activated locomotion has long eluded characterization. Pioneering work (Grimm, 1960; Døving and Selset, 1980) showed that electrical stimulation of the olfactory tracts in fishes induced stereotyped motor behaviors including locomotion. Moreover, recent investigations of neural circuits and cellular mechanisms in the sea lamprey have unraveled how olfactory inputs can initiate locomotion (Derjean et al., 2010).

The Neural Pathway: From Olfactory Sensory Neurons to Brain Locomotor Centers

In lampreys, a single nostril located along the midline on the dorsal surface of the head, anterior to the eyes opens into a single nasal cavity containing the peripheral olfactory organ. The walls of this cavity form folds or lamellae lined by an epithelium containing olfactory sensory neurons (OSNs). These lamellae house the main olfactory epithelium (MOE), and contain three ciliated OSN morphotypes: tall, intermediate and short OSNs (Laframboise et al., 2007). Their shapes and locations are similar to OSN morphotypes present in teleost fishes (Hansen and Zielinski, 2005). Diverticula (i.e., epithelial vesicles) of the MOE, mainly located in the caudoventral part of the olfactory organ, form the accessory olfactory organ (AOO) of lampreys (Scott, 1887; Leach, 1951; Hagelin and Johnels, 1955). The lumina of the AOO diverticula are linked to the lumen of the olfactory organ by tiny ducts (Hagelin and Johnels, 1955), and the cuboidal epithelium lining the AOO vesicles contains ciliated short OSNs with a broader surface than the short OSNs in the MOE (Ren et al., 2009; Chang et al., 2013). Axons extend from both MOE and AOO into the underlying lamina propria, where small axonal bundles gather to form the olfactory nerve. These OSN axons enter the OB, the primary olfactory center of the brain, where synaptic contacts are made onto the second order olfactory neurons, the OB projection neurons (the equivalent of the "mitral/tufted" cells of mammals). Axons from AOO OSNs terminate only in the medial part of the OB whereas axons from MOE OSNs terminate in non-medial parts of the OB and possibly sparsely in the medial part of the OB (Ren et al., 2009). Interestingly, OSN axons extending into medial and non-medial regions of the OB have distinct biochemical properties (Frontini et al., 2003). Moreover, projection neurons located in the medial and non-medial part of OB have non-overlapping receptive fields and exhibit differences in size and dendritic morphology (Green et al., 2013). The projection neurons send their projections to third order olfactory neurons in different parts of the brain. Structures receiving these secondary olfactory projections are located mainly in the telencephalon, but some secondary olfactory fibers extend to the mesodiencephalic boundary. Third order olfactory neurons are located in the septum, striatum, pallium, habenula, hypothalamus as well as the posterior tuberculum (PT), a ventrocaudal region of the diencephalon. Tract-tracing experiments revealed that the olfactory connection to the PT originates exclusively from the medial projection neuron population (**Figure 1C**), whereas connectivity to the other aforementioned areas arise from non-medial projection neurons. Conversely, the PT appears to be the only target of the projection neurons of the medial OB (**Figure 1C**; Figure 5 in Derjean et al., 2010; Green et al., 2013). This OB projection to the PT is of special interest as it was previously shown that the PT sends downward inputs to the MLR (**Figure 1C**; Ménard et al., 2007). The MLR is a crucial motor center located at the border between the mesencephalon and the pons. In all vertebrate species, it controls locomotion in a graded fashion, via projections to RS cells (for reviews, see Dubuc et al., 2008; Ryczko and Dubuc, 2013). Thus, the projection from the medial OB to the PT provides a way for olfactory inputs to influence locomotion in a very direct fashion (**Figure 1C**).

The Neural Mechanisms

The activation of OSNs by chemical stimuli constitutes the first step of any olfactory-mediated behavior. In lampreys, OSNs have been shown to respond to three major classes of chemical stimuli: amino acids, steroids, and bile salts (Li et al., 1995). Stimulation of the olfactory epithelium with some of these naturally occurring olfactory stimuli can induce sustained depolarizations with spiking activity in RS cells. The stimulatory molecules include the sex pheromones 3-keto petromyzonol sulfate and 3-keto allocholic acid as well as odors such as taurocholic acid and L-arginine (Figure 1 in Derjean et al., 2010). Similarly, electrical stimulation of the olfactory nerve elicits excitatory synaptic responses in intracellularly recorded RS cells (Wickelgren, 1977; Brodin et al., 1988; Figure 2 in Derjean et al., 2010). Responses occur on both sides with a latency of around 100 ms. Calcium imaging experiments confirmed this finding by showing that repetitive stimulation of the olfactory nerve increases intracellular calcium in many RS cells (Figure 2 in Derjean et al., 2010), a sign of long-lasting afterdischarges in these cells (Viana Di Prisco et al., 1997). Local injections of glutamate antagonists in the OB blocked the responses of RS cells to olfactory nerve stimulation, indicating that synaptic transmission between OSNs and projection neurons

relies on glutamate (Figure 2 in Derjean et al., 2010). The glutamatergic nature of this synapse was confirmed by showing that glutamate injection into the OB induces fictive locomotion (Figure 3 in Derjean et al., 2010). Examination of RS cell responses following the stimulation of different OB regions revealed that stimulating the medial region of the OB was more effective than stimulating non-medial regions (Figure 4 in Derjean et al., 2010). In summary, anatomical evidence and physiological experiments emphasise the role of the medial OB region in the fast relay of olfactory inputs to locomotor centers.

Because projection neurons from the medial OB region only project to the PT, the effect of PT stimulation on RS cell activity was investigated. Electrical stimulation of the PT elicited excitatory responses in RS cells with a latency of around 15 ms. Pharmacological stimulation of the PT with glutamate induced locomotor bouts in semi-intact preparations (Figure 6 in Derjean et al., 2010; Ryczko et al., 2013). Moreover, glutamate antagonist injections into the PT abolished RS cells responses to olfactory nerve stimulation, demonstrating that the OB projections to the PT are glutamatergic and that the PT is involved in olfacto-motor transformations (Figure 7 in Derjean et al., 2010). Because the MLR receives inputs from the PT (Ménard et al., 2007) and projects to RS cells (Sirota et al., 2000), it is an ideal candidate to relay PT olfactory inputs to RS cells. Physiological data support this hypothesis. Local injections of glutamate antagonists into the MLR block RS cells responses to olfactory nerve stimulation (Figure 7 in Derjean et al., 2010). In addition to driving the MLR via a glutamatergic projection, the PT also modulates its activity through a dopaminergic projection. It was shown recently that stimulation of the PT induces a dopamine release in the MLR, which increases the locomotor output by a D1 receptor-mediated mechanism (Ryczko et al., 2013). This dopaminergic drive seems to build on the glutamatergic drive to amplify the overall PT input onto the MLR. It remains to be shown, however, whether PT dopaminergic neurons are actually recruited by olfactory inputs from medial OB projection neurons. In turn, the MLR activates RS cells (Brocard and Dubuc, 2003; Le Ray et al., 2003; Brocard et al., 2010). As such, the MLR plays a key central role in initiating and controlling locomotion (Sirota et al., 2000; Le Ray et al., 2011; Ryczko and Dubuc, 2013) induced by olfactory inputs and finely tunes the power of the locomotor output.

COMPARISON OF THE TWO SENSORIMOTOR SYSTEMS

The activation of locomotion by the two sensory modalities, cutaneous mechanoreception and olfaction, relies on the activation of RS cells in the brainstem. Cutaneous inputs activate RS cells through relay cells located in the lateral part of the hindbrain or in the dorsal column nuclei. Olfactory inputs activate RS cells through the MLR. It is well documented that the MLR activates RS cells in a graded fashion and this results in a graded locomotor output. On the other hand, cutaneous inputs generate sustained depolarizations in RS cells in an all-or-none fashion. The sustained depolarizations were recently shown to rely on intrinsic properties (I_{CAN}) as well as glutamatergic synaptic transmission (Viana Di Prisco et al., 2000). Ca^{2+} imaging experiments indicate that the large RS cells are activated by both olfactory and cutaneous inputs (Viana Di Prisco et al., 1997, 2000; Derjean et al., 2010), indicating that these RS cells play a crucial role in activating locomotion induced by either sensory modality. The mechanism by which the same RS cell population could elicit graded vs. all-or-none locomotor responses have not been elucidated. It is tempting to propose that intrinsic plateau properties could be inhibited by the MLR, which is known to excite RS cells via two neurotransmitters, glutamate and acetylcholine (Le Ray et al., 2003). For instance, cholinergic inputs could suppress NMDA-induced sustained depolarizations in RS cells. There are many other possible mechanisms. The level of excitation from the MLR could be insufficient to activate the intrinsic plateau properties in RS cells, as shown in the cat preparation for sensory inputs (Brownstone et al., 1994). Another possibility would be that the intrinsic plateau properties are activated by an unidentified synaptic input originating from the cutaneous sensory inputs and not the MLR. Further work is needed to decipher between these different options.

5-HT MODULATION

The general organization of the serotoninergic system in lampreys is relatively well described (Pierre et al., 1992; Antri et al., 2006; Barreiro-Iglesias et al., 2009) and it is similar to that of mammals. 5-HT modulates sensory transmission at different levels in the nervous system (**Figure 2**, left). For instance, several studies have shown that 5-HT modulates sensory transmission in the SC. In mammals, sensory transmission to superficial and deep dorsal horn neurons is either depressed by 5-HT (cat: Headley et al., 1978; Anwyl, 1990; and rat: Lopez-Garcia and King, 1996; Lopez-Garcia, 1998; Garraway and Hochman, 2001) or, in a small proportion of cases, potentiated by 5-HT (rat: El-Yassir et al., 1988; Lopez-Garcia and King, 1996). In tadpoles (Sillar and Simmers, 1994) and lampreys, 5-HT decreases the amplitude of EPSPs recorded in large secondary sensory neurons (giant interneurons) in response to stimulation of primary afferents (El Manira et al., 1997). In frog motoneurons, 5-HT also depresses the EPSPs induced by DR stimulation (Ovsepian and Vesselkin, 2006). Less is known about 5-HT effects on sensory transmission at the supraspinal level. A study in guinea pigs suggested that 5-HT depresses glutamate release from trigeminal primary afferents through presynaptic inhibition (Travagli and Williams, 1996). In the lamprey, 5-HT modulation occurs at several locations along the sensorimotor pathways.

Serotoninergic Modulation of Cutaneous Inputs

Modulatory effects of 5-HT were also investigated on the transmission of sensory inputs in the brainstem of lampreys

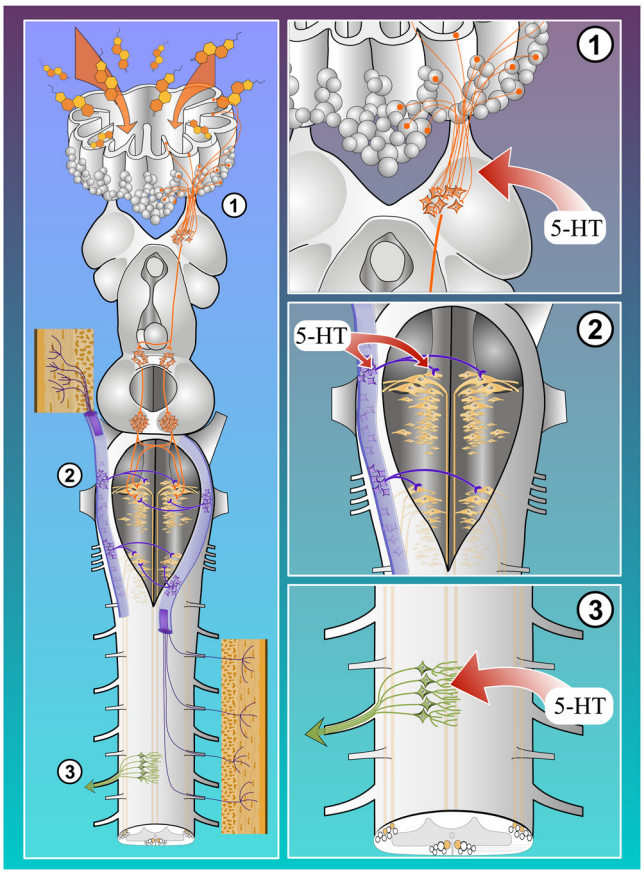

FIGURE 2 | 5-HT modulation of the sensorimotor circuitry. Both olfactory **(1)** and cutaneous **(2)** inputs to lamprey RS cells seem to be modulated by 5-HT (red arrows). 5-HT also acts at the spinal level by modulating the transmission from descending RS axons on spinal neurons **(3)**.

(**Figure 2-2**). The lamprey brainstem contains rich 5-HT innervation (Steinbusch et al., 1981). There are 5-HT fibers surrounding the cell bodies of some of the large RS cells (Viana Di Prisco et al., 1994). Moreover, there is abundant 5-HT innervation within the trigeminal descending tract (Pierre et al., 1992), where the trigeminal sensory relay cells are located (Viana Di Prisco et al., 2005). 5-HT modulation of trigeminal inputs to RS cells was investigated in the brainstem of lampreys (Antri et al., 2008). Bath application of 5-HT reduced disynaptic excitatory responses in RS cells elicited by trigeminal nerve stimulation. Similar effects were seen by local ejection of 5-HT either onto the RS cells or onto the relay cells in the lateral part of the brainstem (**Figure 2-2**). 5-HT also reduced the monosynaptic EPSPs elicited from stimulation of the relay cells that receive trigeminal inputs and project onto RS cells. 5-HT increased the threshold for eliciting sustained depolarizations in response to trigeminal nerve stimulation but did not prevent them. The 5-HT innervation on RS cells appeared to originate from 5-HT neurons in the isthmic region, and also from neurons located in the pretectum and caudal rhombencephalon (**Figure 2-2**). These results indicate that 5-HT strongly modulates sensory transmission to neural networks involved in the control of movements.

Serotoninergic Modulation of Olfactory Inputs

In the lamprey olfactory system, 5-HT fibers were particularly prominent in the lamina propria of the olfactory epithelium, as well as in the olfactory nerve and bulb (**Figure 2-1**; Zielinski et al., 2000; Frontini et al., 2003; Abalo et al., 2007). On the other hand, 5-HT cell bodies were restricted to the lamina propria underlying the olfactory epithelium (Figures 1–3 in Zielinski et al., 2000). The 5-HT fibers were prominent within the olfactory nerve, parallel to the axons of the OSNs, from the lamina propria to the OB. However, some 5-HT fibers were also seen adjacent to the olfactory nerve. Cross sections of the olfactory nerve revealed that the 5-HT fibers where distributed evenly among the primary olfactory afferent fibers forming the nerve. Analysis of the pathway of individual 5-HT fibers using confocal z-series showed that these fibers terminated either at the junction of the olfactory nerve and OB or in the outer OB layers (i.e., olfactory nerve layer and glomerular layer). Olfactory nerve lesions experiments showed that these 5-HT fibers originate from cell bodies located in the mucosa of the olfactory organ (Zielinski et al., 2000). On the other hand, the abundant 5-HT innervation observed in the OB inner layers (i.e., granular) was not altered after cutting the olfactory nerve, demonstrating that this innervation has a different, probably central, origin. In lampreys, 5-HT neurons are present from the diencephalon to the caudal rhombencephalon (Antri et al., 2006). The telencephalon is devoid of serotonergic cell bodies (Pierre et al., 1992). Most afferents to the OB come from the telencephalon. However, some neurons projecting to the OB are located in the diencephalon (preoptic area) and midbrain tegmentum (Northcutt and Puzdrowski, 1988). Both these regions contain 5-HT neurons (Pierre et al., 1992; Antri et al., 2006), making them prime candidates as the central source of the OB 5-HT innervation. Interestingly, the meso-rhombencephalic group of 5-HT neurons seems to be homologous to the superior raphe of mammals (Antri et al., 2006), which is known to project to the OB and gate the olfactory information flow (Petzold et al., 2009). The function of the 5-HT innervation of the lamprey olfactory system is not fully understood (Zielinski et al., 2000). However, based on ongoing work in our group (Boyes et al., 2014), on the location of the 5-HT fibers in the OB (Zielinski et al., 2000), on how 5-HT acts on other sensory systems (Antri et al., 2008), and on the role of 5-HT in olfactory processing in other vertebrates (Petzold et al., 2009), it probably acts on olfactory processes by gating the sensory inflow.

Serotoninergic Modulation of the Spinal Motor System

Several endogenous neurotransmitters have been shown to alter the output of locomotor CPGs and to modulate cellular and synaptic properties of the neurons involved (see for

instance: Barbeau and Rossignol, 1991; Schotland et al., 1996; MacLean et al., 1998; Parker and Grillner, 2000; Schmidt and Jordan, 2000; MacLean and Schmidt, 2001; Grillner and Wallén, 2002; Alford et al., 2003; Perrier et al., 2003; Svensson et al., 2003). Little is known about the mechanisms involved. However, the subject is broad and we will not review the different neurotransmitter systems in different vertebrate species.

In lampreys, 5-HT modulates the descending motor commands in the lamprey SC in addition to gating sensory inputs to RS cells. Paracrine release of 5-HT activates at least two distinct receptor subtypes at three distinct subcellular locations with transduction mechanisms converging on a single behavioral modification. It has been observed a while ago that the frequency of fictive locomotion is modulated by endogenous release of neurotransmitters within the SC (Harris-Warrick and Cohen, 1985; Christenson et al., 1989; Schotland et al., 1996; Parker and Grillner, 2000; Svensson et al., 2003). Of these modulatory neurotransmitters, 5-HT reduces the frequency of VR bursting during fictive locomotion (Harris-Warrick and Cohen, 1985). This modulation occurs if 5-HT is applied exogenously, but it is also clear that activity-dependent release of 5-HT from within the SC occurs and that this release similarly reduces the frequency of the CPG output (Christenson et al., 1989). This behavioral outcome of 5-HT is due, in part, to 5-HT-mediated inhibition of a postsynaptic Ca^{2+}-dependant K^+-current (KCa2) that underlies the late after-hyperpolorization of action potentials in neurons of the CPG (Wallén et al., 1989a; El Manira et al., 1994; Wikström et al., 1995; Parker and Grillner, 2000). Separately and associated directly with the synaptic activation of NMDA receptors, 5-HT mediated inhibition of a postsynaptic KCa2 is thought to play a role in prolonging fictive locomotion bursts through prolonging the plateau of NMDA tetrodotoxin (TTX) oscillations (Wallén and Grillner, 1987; Christenson et al., 1989; Wallén et al., 1989b; Schotland and Grillner, 1993; El Manira et al., 1994; Alpert and Alford, 2013; Nanou et al., 2013). Finally, presynaptic 5-HT receptor activation filters synaptic output from both descending RS command neurons (Buchanan and Grillner, 1991; Blackmer et al., 2001) and from intraspinal excitatory interneurons (Parker and Grillner, 1999; Schwartz et al., 2005). This form of presynaptic inhibition causes an augmenting synaptic response that is initially inhibited but enhanced during bursting behavior.

Serotonin Modulation of Ca^{2+} Dependent K^+ Conductances (KCa2) in Spinal Neurons

5-HT acts on most if not all neurons of the spinal CPG to inhibit the latter after hyperpolarization following action potentials (Wallén et al., 1989a). This effect may be mediated by 5-HT1A receptors to inhibit voltage gated Ca^{2+} channels (Hill et al., 2003). The consequent reduction in Ca^{2+} will inhibit the KCa2 channel activation that mediates the late after-hyperpolarization in these neurons (Wikström and El Manira, 1998). The late after-hyperpolarization strongly impacts the ability of spinal neurons to fire repetitively during bursting activity that some neurons of the CPG show and thus, 5-HT modulation of KCa2 channels is important in controlling the burst output of the CPG (Wallén et al., 1989a; Hill et al., 1992; Meer and Buchanan, 1992). Indeed, computer simulations of the lamprey CPG network that incorporate its connectivity and ionic intrinsic properties provide evidence that inhibition of KCa2 in neurons within the lamprey CPG prolongs fictive locomotion VR bursting (Hellgren et al., 1992; Lansner and Ekeberg, 1994; Grillner et al., 1998a). 5-HT also causes a prolongation of the depolarization recorded during NMDA-TTX driven intrinsic oscillations (Wallén et al., 1989a). These oscillations are mediated by intrinsic membrane properties of spinal neurons seen following NMDA receptor activation. The oscillations require Ca^{2+} permeation of the NMDA receptors and subsequent activation of a KCa2 channel. The prolongation of the depolarizing phase of these oscillations caused by 5-HT may be mediated by direct interaction of 5-HT receptors on KCa2 channels, or alternatively, via an indirect inhibition of N-methyl-D-aspartate receptors (NMDARs; Schotland and Grillner, 1993) or voltage-gated calcium channels (VGCCs; Wang et al., 2014) supplying Ca^{2+} for KCa2 channels responsible for the repolarization. More recent work ties the activation of NMDA receptors and Ca^{2+} permeation of these receptors directly to the activation of KCa2 channels, which are held in very close proximity (Alpert and Alford, 2013; Nanou et al., 2013). There is evidence that the effect of 5-HT on the NMDA mediated TTX resistant oscillations is also very important for the modulatory effects of 5-HT on the locomotor pattern. The effects of 5-HT are absent when the network is activated by kainate, which will not activate NMDARs directly. Thus, NMDAR-dependent Ca^{2+} entry contributes to burst termination (Alpert and Alford, 2013; Nanou et al., 2013). This effect is mediated by KCa2 activation, which is modified by 5-HT. These effects of 5-HT mediated through KCa2 have been shown principally in the isolated SC during fictive locomotion activated by the artificial application of NMDA. However, the effects can be readily reproduced in the SC following brainstem activation of fictive locomotion (**Figure 3**; Gerachshenko et al., 2009; Nanou et al., 2013).

Serotonin Modulates Glutamate Release in the Spinal Cord

In addition to activating a postsynaptic IK(Ca), 5-HT presynaptically inhibits synaptic transmission in the lamprey SC (**Figure 2-3**; Buchanan and Grillner, 1991; El Manira et al., 1994; Shupliakov et al., 1995; Blackmer et al., 2001; Takahashi et al., 2001). The inhibition of synaptic transmission by 5-HT has been observed in the CPGs of several jawed vertebrate as well. 5-HT presynaptically inhibits midcycle glycinergic inputs and prolongs VR bursting during *Xenopus* larval swimming (Sillar et al., 1998). In neonatal rat, activation of 5-HT receptors presynaptically decreases inspiratory modulated synaptic currents (Lindsay and Feldman, 1993; Di Pasquale et al., 1997; Hilaire et al., 1997) and suppresses descending glutamatergic responses (Skagerberg and Björklund, 1985). In mammalian locomotor descending command systems,

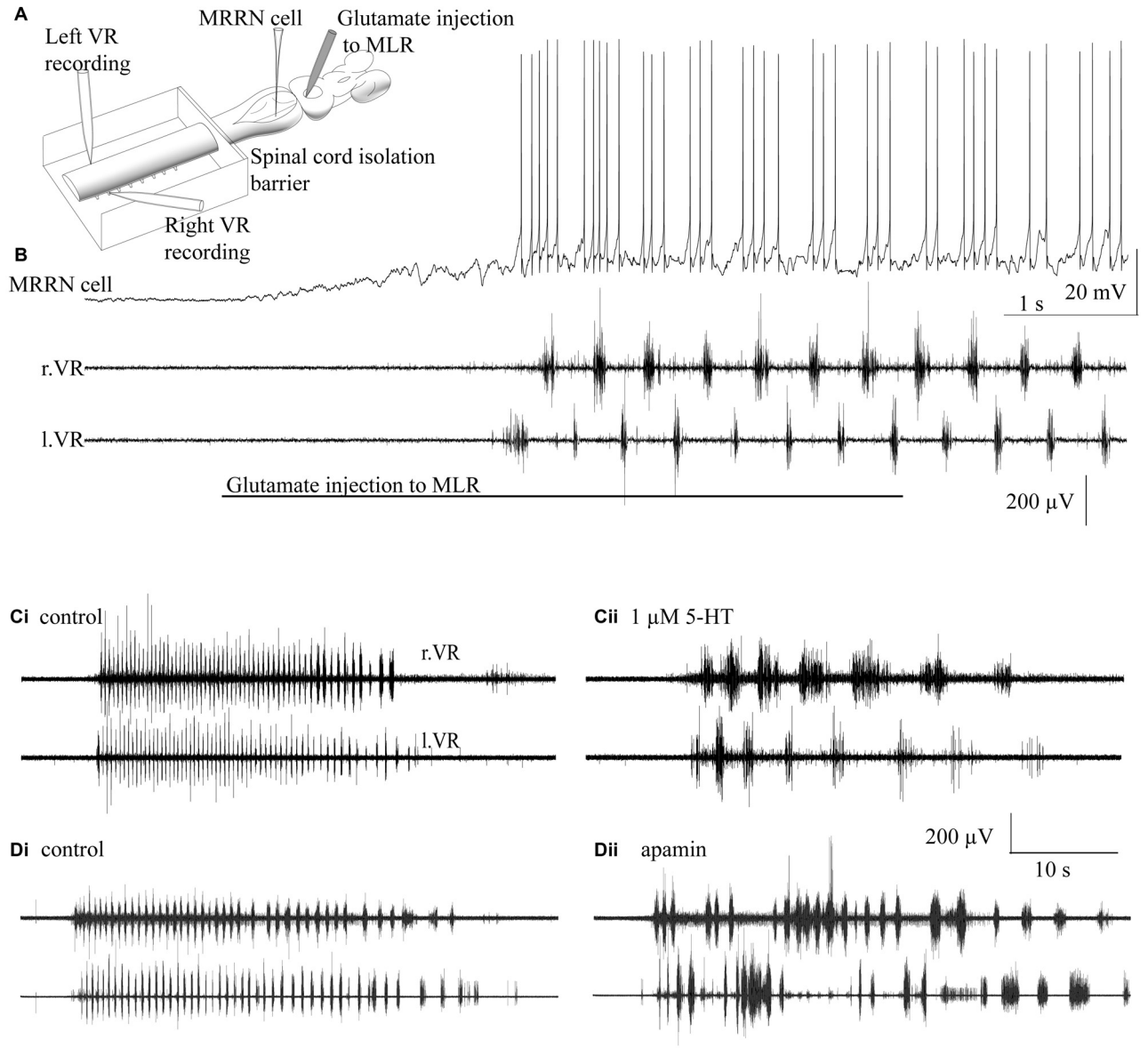

FIGURE 3 | 5-HT and apamin reduce the cycle frequency of brainstem-evoked locomotion. (A) Recording arrangement to evaluate effects of spinal agonist/antagonist application on brainstem evoked fictive locomotion. The SC was pharmacologically isolated from the brainstem with a barrier to superfusate flow at the 2nd to 5th spinal segment. Glutamate (1 mM) was microinjected into the MLR. MRRN neurons were recorded intracellularly and fictive locomotion with suction electrodes over left and right pairs of spinal VRs (l.VR, r.VR). **(B)** Injection of glutamate into the MLR evokes depolarization and rhythmic firing of the MRRN neuron and alternating VR activity. **(Ci)** Similar fictive locomotion to **(B)** recorded in a pair of VRs. **(Cii)** 5-HT (1 μM) superfused over the SC reduced fictive locomotion cycle frequencies. **(D)** Apamin—a blocker of KCa2 channels also slows MLR evoked fictive locomotion. **(Di)** Similar bout of locomotion to **(B)**. **(Dii)** Apamin (5 μM) superfused over the SC reduced the fictive locomotion frequencies. Adapted from Gerachshenko et al. (2009) and Nanou et al. (2013).

5-HT is a critical neurotransmitter. Bath applied 5-HT activates spinal rhythmic activity in rats (Cazalets et al., 1992) and refines locomotor-like activity (Pearlstein et al., 2005). 5-HT acting at 5-HT2A and 5-HT7 receptors to mediate excitatory effects (Liu and Jordan, 2005; Sławińska et al., 2014). However, 5-HT can also mediate inhibitory effects on spinal locomotor circuitry. Within descending locomotor command systems serotonin inhibits locomotor activity acting through either 5-HT1A or 5-HT1B/D receptor subtypes (Beato and Nistri, 1998; Dunbar et al., 2010), though the cellular and molecular sites of action remain unexplored. In the lamprey, the effect of presynaptic modulation of glutamatergic transmission converges on the same behavioral outcome (shown in **Figure 3**). This is true whether the CPG is activated by brainstem stimulation (Gerachshenko et al., 2009) or by bath application of glutamatergic agonists to

the SC (Schwartz et al., 2005). The mechanism by which presynaptic 5-HT receptors mediate presynaptic modulation provides an explanation of how this convergence can occur.

In lamprey RS axons, 5-HT acting at a 5-HT1B receptor (Schwartz et al., 2005) liberates presynaptic Gβγ from the G protein heterodimer, to compete with Ca^{2+}-dependent binding of the Ca^{2+} sensor for synaptic vesicle fusion, synaptotagmin, to the machinery for synaptic vesicle fusion—the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex (Blackmer et al., 2001, 2005; Takahashi et al., 2001; Gerachshenko et al., 2005, 2009). This competition is mediated principally by a small number of synaptosomal-associated protein 25 (SNAP-25) residues (Wells et al., 2012). Rather than causing a reduction in the probability of release at these synapses, this competitive interaction between Gβγ and synaptotagmin reduces synaptic cleft glutamate concentrations (Schwartz et al., 2007), by causing kiss-and-run fusion of the synaptic vesicles (Photowala et al., 2006). Lower synaptic glutamate concentrations cause AMPA receptor excitatory post synaptic currents (EPSCs) to be more profoundly inhibited than NMDA receptor EPSCs (Schwartz et al., 2007). Thus, 5-HT causes a differential inhibition with much stronger inhibition of AMPA mediated EPSCs. This effect is seen both at RS synapses and in synapses from intraspinal excitatory interneurons (Schwartz et al., 2005; Gerachshenko et al., 2009). Interestingly, fictive locomotion mediated by NMDA receptor activation is much slower than that mediated by AMPA or kainate application (Brodin et al., 1985; Brodin and Grillner, 1986). This is one means by which presynaptic 5-HT receptors slow fictive locomotion. However, the effect of Gβγ on modulating neurotransmitter release is dependent upon its competition with synaptotagmin in binding to the SNARE complex. Synaptotagmin binding to the SNARE complex is Ca^{2+}-dependent. Consequently, at high Ca^{2+} concentrations, preferential synaptotagmin binding to SNARE complexes occludes 5-HT receptor-mediated inhibition (Yoon et al., 2007) by displacing Gβγ from the SNARE complex. During bursting activity, Ca^{2+} concentrations in the presynaptic terminals summate. This rising Ca^{2+} concentration inactivates 5-HT mediated presynaptic inhibition within 3–5 action potentials of a 50 Hz burst (Gerachshenko et al., 2009). Computational simulations of the spinal circuitry for locomotion reveal that such an augmenting excitatory synaptic signal within the spinal CPG mediates a slower fictive locomotor activity (Hellgren et al., 1992; Parker and Grillner, 1999). In mammalian systems, the cellular mechanisms of action of 5-HT are less well understood, but a similarly complex series of effects has been associated with excitatory 5-HT2 and 5-HT7 receptors and inhibitory 5-HT1 receptors. Thus, 5-HT release within the mammalian SC both facilitates locomotion but also modulates rhythmic activity (Perrier and Cotel, 2015).

Summary of the Effects of 5-HT on the Spinal CPG

5-HT can be released within the SC (**Figure 2-3**), and its release causes a slowing of fictive locomotion, whether activated by exogenous agonists, or by brainstem stimulation. However, at least three functionally distinct receptors mediate effects that converge on this behavioral outcome. Two postsynaptic receptors cause a reduction in the activation of a KCa2 channel. One of these inhibits the action potential late after-hyperpolarization to sustain spiking during bursts, the other prevents burst termination by inhibiting an NMDA receptor dependent activation of a KCa2. Presynaptically, 5-HT1B receptors inhibit release by causing kiss and run fusion. This effect favors activation of postsynaptic NMDA receptors over AMPA, but also the inhibition is lost during bursts. Both of these presynaptic effects contribute to a slowing of the CPG frequency. Further studies are needed to establish whether similar mechanisms act at the sensory level and how 5-HT modulation in the SC impacts sensory-evoked locomotion.

GENERAL CONCLUSIONS

Sensory inputs from different modalities induce locomotion. Studies in lampreys have been extremely useful for gaining an understanding of the function and the regulation of these mechanisms. Both cutaneous and olfactory inputs impinge on RS cells that constitute the final common descending pathway for eliciting locomotion. Yet, the mechanisms by which RS cells are activated differ according to the sensory modality. Future research should indicate more precisely how graded vs. all-or-none locomotor output is elicited by olfactory vs. cutaneous inputs. Moreover, modulatory mechanisms also play a crucial role in gating the sensory inflow and determining the strength of the locomotor output. For instance, we have discussed how 5-HT acts at all levels of the sensorimotor pathways via different modulatory mechanisms.

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REFERENCES

Abalo, X. M., Villar-Ceda, B., Meléndez-Ferro, M., Pérez-Costas, E., Anadón, R., and Rodicio, M. C. (2007). Development of the serotonergic system in the central nervous system of the see lamprey. *J. Chem. Neuroanat.* 34, 29–46. doi: 10.1016/j.jchemneu.2007.03.010

Alford, S., Schwartz, E., and Viana di Prisco, G. (2003). The pharmacology of vertebrate spinal central pattern generators. *Neuroscientist* 9, 217–228. doi: 10.1177/1073858403009003014

Alford, S., and Williams, T. L. (1989). Endogenous activation of glycine and NMDA receptors in lamprey spinal cord during fictive locomotion. *J. Neurosci.* 9, 2792–2800.

Alpert, M. H., and Alford, S. (2013). Synaptic NMDA receptor-dependent Ca(2+) entry drives membrane potential and Ca(2+) oscillations in spinal ventral horn neurons. *PLoS One* 8:e63154. doi: 10.1371/journal.pone.0063154

Antri, M., Auclair, F., Albretch, J., Djeudjang, S., and Dubuc, R. (2008). Serotoninergic modulation of sensory transmission to brainstem reticulospinla cells. *Eur. J. Neurosci.* 28, 655–667. doi: 10.1111/j.1460-9568.2008.06368.x

Antri, M., Cyr, A., Auclair, F., and Dubuc, R. (2006). Ontogeny of 5-HT neurons in the brainstem of the lamprey, *Petromyzon marinus*. *J. Comp. Neurol.* 495, 788–800. doi: 10.1002/cne.20910

Antri, M., Fénelon, K., and Dubuc, R. (2009). The contribution of synaptic inputs to sustained depolarizations in reticulospinal neurons. *J. Neurosci.* 29, 1140–1151. doi: 10.1523/JNEUROSCI.3073-08.2009

Anwyl, R. (1990). Neurophysiological actions of 5-hydroxytryptamine in the vertebrate nervous system. *Prog. Neurobiol.* 35, 451–468. doi: 10.1016/0301-0082(90)90031-b

Armstrong, D. M. (1986). Supraspinal contributions to the initiation and control of locomotion in the cat. *Prog. Neurobiol.* 26, 273–361. doi: 10.1016/0301-0082(86)90021-3

Barbeau, H., and Rossignol, S. (1991). Initiation and modulation of the locomotor pattern in the adult chronic spinal cat by noradrenergic, serotonergic and dopaminergic drugs. *Brain Res.* 546, 250–260. doi: 10.1016/0006-8993(91)91489-n

Barreiro-Iglesias, A., Aldegunde, M., Anadón, R., and Rodicio, M. C. (2009). Extensive presence of serotonergic cells and fibers in the peripheral nervous system of lampreys. *J. Comp. Neurol.* 512, 478–499. doi: 10.1002/cne.21914

Beato, M., and Nistri, A. (1998). Serotonin-induced inhibition of locomotor rhythm of the rat isolated spinal cord is mediated by the 5-HT1 receptor class. *Proc. Biol. Sci.* 265, 2073–2080. doi: 10.1098/rspb.1998.0542

Bernau, N. A., Puzdrowski, R. L., and Leonard, R. B. (1991). Identification of the midbrain locomotor region and its relation to descending locomotor pathways in the Atlantic stingray, *Dasyatis sabina*. *Brain Res.* 557, 83–94. doi: 10.1016/0006-8993(91)90119-g

Blackmer, T., Larsen, E. C., Bartleson, C., Kowalchyk, J. A., Yoon, E.-J., Preininger, A. M., et al. (2005). G protein βγ directly regulates SNARE protein fusion machinery for secretory granule exocytosis. *Nat. Neurosci.* 8, 421–425. doi: 10.1038/nn1423

Blackmer, T., Larsen, E. C., Takahashi, M., Martin, T. F., Alford, S., and Hamm, H. E. (2001). G protein betagamma subunit-mediated presynaptic inhibition: regulation of exocytotic fusion downstream of Ca^{2+} entry. *Science* 292, 293–297. doi: 10.1126/science.1058803

Boothby, K. M., and Roberts, A. (1992). The stopping response of *Xenopus laevis* embryos: behaviour, development and physiology. *J. Comp. Physiol. A* 170, 171–180. doi: 10.1007/bf00196899

Bouvier, J., Caggiano, V., Leiras, R., Caldeira, V., Bellardita, C., Balueva, K., et al. (2015). Descending command neurons in the brainstem that halt locomotion. *Cell* 163, 1191–1203. doi: 10.1016/j.cell.2015.10.074

Boyes, K., Green, W. W., Aurangzeb, Z., Dubuc, R., and Zielinski, B. (2014). "Serotonergic modulation of odour-evoked neural activity in the olfactory bulb of the sea lamprey (Petromyzon marinus)," in *Electronic Theses and Dissertations*, Paper 5062, Department of Biological Sciences, University of Windsor: Windsor, ON.

Bretzner, F., and Brownstone, R. M. (2013). Lhx3-Chx10 reticulospinal neurons in locomotor circuits. *J. Neurosci.* 33, 14681–14692. doi: 10.1523/JNEUROSCI.5231-12.2013

Brocard, F., and Dubuc, R. (2003). Differential contribution of reticulospinal cells to the control of locomotion induced by the mesencephalic locomotor region. *J. Neurophysiol.* 90, 1714–1727. doi: 10.1152/jn.00202.2003

Brocard, F., Ryczko, D., Fénelon, K., Hatem, R., Gonzales, D., Auclair, F., et al. (2010). The transformation of a unilateral locomotor command into a symmetrical bilateral activation in the brainstem. *J. Neurosci.* 30, 523–533. doi: 10.1523/JNEUROSCI.3433-09.2010

Brodin, L., and Grillner, S. (1985). The role of putative excitatory amino acid neurotransmitters in the initiation of locomotion in the lamprey spinal cord. I. The effects of excitatory amino acid antagonists. *Brain Res.* 360, 139–148. doi: 10.1016/0006-8993(85)91229-6

Brodin, L., and Grillner, S. (1986). Effects of magnesium on fictive locomotion induced by activation of N-methyl-D-aspartate (NMDA) receptors in the lamprey spinal cord *in vitro*. *Brain Res.* 380, 244–252. doi: 10.1016/0006-8993(86)90219-2

Brodin, L., Grillner, S., Dubuc, R., Ohta, Y., Kasicki, S., and Hökfelt, T. (1988). Reticulospinal neurons in lamprey: transmitters, synaptic interactions and their role during locomotion. *Arch. Ital. Biol.* 126, 317–345.

Brodin, L., Grillner, S., and Rovainen, C. M. (1985). N-Methyl-D-aspartate (NMDA), kainate and quisqualate receptors and the generation of fictive locomotion in the lamprey spinal cord. *Brain Res.* 325, 302–306. doi: 10.1016/0006-8993(85)90328-2

Brownstone, R. M., Gossard, J. P., and Hultborn, H. (1994). Voltage-dependent excitation of motoneurones from spinal locomotor centres in the cat. *Exp. Brain Res.* 102, 34–44. doi: 10.1007/bf00232436

Buchanan, J. T. (1982). Identification of interneurons with colateral, caudal axons in the lamprey spinal cord: synaptic interactions and morphology. *J. Neurophysiol.* 47, 961–975.

Buchanan, J. T., Brodin, L., Dale, N., and Grillner, S. (1987). Reticulospinal neurones activate excitatory amino acid receptors. *Brain Res.* 408, 321–325. doi: 10.1016/0006-8993(87)90397-0

Buchanan, J. T., and Cohen, A. H. (1982). Activities of identified interneurons, motoneurons and muscle fibers during fictive swimming in the lamprey and effects of reticulospinal and dorsal cell stimulation. *J. Neurophysiol.* 47, 948–960.

Buchanan, J. T., and Grillner, S. (1987). Newly identified 'glutamate interneurons' and their role in locomotion in the lamprey spinal cord. *Science* 236, 312–314. doi: 10.1126/science.3563512

Buchanan, J. T., and Grillner, S. (1991). 5-hydroxytryptamine depresses reticulospinal excitatory post synaptic potentials in motoneurons of the lamprey. *Neurosci. Lett.* 122, 71–74. doi: 10.1016/0304-3940(91)90196-z

Buhl, E., Roberts, A., and Soffe, S. R. (2012). The role of a trigeminal sensory nucleus in the initiation of locomotion. *J. Physiol.* 590, 2453–2469. doi: 10.1113/jphysiol.2012.227934

Bussières, N. (1994). *Les Systèmes Descendants Chez la Lamproie. Étude Anatomique et Fonctionnelle*. Ph.D. thesis, Université de Montréal: Montréal, QC

Butler, A. B., and Hodos, W. (2005). "The reticular formation," in *Comparative Vertebrate Neuroanatomy*, 2nd Edn. eds A. B. Butler and W. Hodos (New York: Wiley-Liss), 164–179.

Cabelguen, J. M., Bourcier-Lucas, C., and Dubuc, R. (2003). Bimodal locomotion elicited by electrical stimulation of the midbrain in the salamander *Notophthalmus viridescens*. *J. Neurosci.* 23, 2434–2439.

Cardin, S., Le Ray, D., Robitaille, R., and Dubuc, R. (1999). Motor responses elicited by skin stimulation in lampreys. *Soc. Neurosci. Abstr.* 25: 1906.

Cazalets, J. R., Sqalli-Houssaini, Y., and Clarac, F. (1992). Activation of the central pattern generators for locomotion by serotonin and excitatory amino acids in neonatal rat. *J. Physiol.* 455, 187–204. doi: 10.1113/jphysiol.1992.sp019296

Chang, S., Chung-Davidson, Y. W., Libants, S. V., Nanlohy, K. G., Kiupel, M., Brown, C. T., et al. (2013). The sea lamprey has a primordial accessory olfactory system. *BMC Evol. Biol.* 13:172. doi: 10.1186/1471-2148-13-172

Chau, C., Giroux, N., Barbeau, H., Jordan, L., and Rossignol, S. (2002). Effects of intrathecal glutamatergic drugs on locomotion I. NMDA in short-term spinal cats. *J. Neurophysiol.* 88, 3032–3045. doi: 10.1152/jn.00138.2002

Christenson, J., Boman, A., Lagerbäck, P. A., and Grillner, S. (1988). The dorsal cell, one class of primary sensory neuron in the lamprey spinal cord. I. Touch,

pressure but nonociception—a physiological study. *Brain Res.* 440, 1–8. doi: 10.1016/0006-8993(88)91152-3

Christenson, J., Franck, J., and Grillner, S. (1989). Increase in endogenous 5-hydroxytryptamine levels modulates the central network underlying locomotion in the lamprey spinal cord. *Neurosci. Lett.* 100, 188–192. doi: 10.1016/0304-3940(89)90682-4

Clarke, J. D., and Roberts, A. (1984). Interneurones in the *Xenopus* embryo spinal cord: sensory excitation and activity during swimming. *J. Physiol.* 354, 345–362. doi: 10.1113/jphysiol.1984.sp015380

Cohen, A. H., and Wallén, P. (1980). The neuronal correlate of locomotion in fish. "Fictive swimming" induced in an *in vitro* preparation of the lamprey spinal cord. *Exp. Brain Res.* 41, 11–18. doi: 10.1007/bf00236674

Cruce, W. L. R., and Newman, D. B. (1984). Evolution of motor systems: the reticulospinal pathways. *Amer. Zool.* 24, 733–753. doi: 10.1093/icb/24.3.733

Daghfous, G., Green, W. W., Zielinski, B. S., and Dubuc, R. (2012). Chemosensory-induced motor behaviors in fish. *Curr. Opin. Neurobiol.* 22, 223–230. doi: 10.1016/j.conb.2011.10.009

Dale, N., and Roberts, A. (1984). Excitatory amino acid receptors in *Xenopus* embryo spinal cord and their role in the activation of swimming. *J. Physiol.* 348, 527–543. doi: 10.1113/jphysiol.1984.sp015123

Dale, N., and Roberts, A. (1985). Dual-component amino-acid-mediated synaptic potentials: excitatory drive for swimming in *Xenopus* embryos. *J. Physiol.* 363, 35–59. doi: 10.1113/jphysiol.1985.sp015694

Davis, G. R., Jr., and McClellan, A. D. (1994a). Long distance axonal regeneration of identified lamprey reticulospinal neurons. *Exp. Neurol.* 127, 94–105. doi: 10.1006/exnr.1994.1083

Davis, G. R., Jr., and McClellan, A. D. (1994b). Extent and time course of restoration of descending brainstem projections in spinal cord-transected lamprey. *J. Comp. Neurol.* 344, 65–82. doi: 10.1002/cne.903440106

Del Negro, C. A., Morgado-Valle, C., and Feldman, J. L. (2002). Respiratory rhythm: an emergent network property? *Neuron* 34, 821–830. doi: 10.1016/S0896-6273(02)00712-2

Derjean, D., Moussaddy, A., Atallah, E., St-Pierre, M., Auclair, F., Chang, S., et al. (2010). A novel neural substrate for the transformation of olfactory inputs into motor output. *PLoS Biol.* 8:e1000567. doi: 10.1371/journal.pbio.1000567

Di Pasquale, E., Lindsay, A., Feldman, J., Monteau, R., and Hilaire, G. (1997). Serotonergic inhibition of phrenic motoneuron activity: an *in vitro* study in neonatal rat. *Neurosci. Lett.* 230, 29–32. doi: 10.1016/s0304-3940(97)00469-2

Douglas, J. R., Noga, B. R., Dai, X., and Jordan, L. M. (1993). The effects of intrathecal administration of excitatory amino acid agonists and antagonists on the initiation of locomotion in the adult cat. *J. Neurosci.* 13, 990–1000.

Døving, K. B., and Selset, R. (1980). Behavior patterns in cod released by electrical stimulation of olfactory tract bundlets. *Science* 207, 559–560. doi: 10.1126/science.7352272

Dubuc, R., Bongianni, F., Ohta, Y., and Grillner, S. (1993a). Dorsal root and dorsal column mediated synaptic inputs to reticulospinal neurons in lampreys: involvement of glutamatergic, glycinergic, and GABAergic transmission. *J. Comp. Neurol.* 327, 251–259. doi: 10.1002/cne.903270207

Dubuc, R., Bongianni, F., Ohta, Y., and Grillner, S. (1993b). Anatomical and physiological study of brainstem nuclei relaying dorsal column inputs in lampreys. *J. Comp. Neurol.* 327, 260–270. doi: 10.1002/cne.903270208

Dubuc, R., Brocard, F., Antri, M., Fenelon, K., Gariépy, J.-F., Smetana, R., et al. (2008). Initiation of locomotion in lampreys. *Brain Res. Rev.* 57, 172–182. doi: 10.1016/j.brainresrev.2007.07.016

Dunbar, M. J., Tran, M. A., and Whelan, P. J. (2010). Endogenous extracellular serotonin modulates the spinal locomotor network of the neonatal mouse. *J. Physiol.* 588, 139–156. doi: 10.1113/jphysiol.2009.177378

Duysens, J. (1977). Reflex control of locomotion as revealed by stimulation ofcutaneous afferents in spontaneously walking premammillary cats. *J. Neurophysiol.* 40, 737–751.

Eidelberg, E., Walden, J. G., and Nguyen, L. H. (1981). Locomotor control in macaque monkeys. *Brain* 104, 647–663. doi: 10.1093/brain/104.4.647-a

El Manira, A., Pombal, M. A., and Grillner, S. (1997). Diencephalic projection to reticulospinal neurons involved in the initiation of locomotion in adult lampreys *Lampetra fluviatilis*. *J. Comp. Neurol.* 389, 603–616. doi: 10.1002/(sici)1096-9861(19971229)389:4<603::aid-cne5>3.3.co;2-f

El Manira, A., Tegnér, J., and Grillner, S. (1994). Calcium-dependent potassium channels play a critical role for burst termination in the locomotor network in lamprey. *J. Neurophysiol.* 72, 1852–1861.

El-Yassir, N., Fleetwood-Walker, S. M., and Mitchell, R. (1988). Heterogeneous effects of serotonin in the dorsal horn of rat: the involvement of 5-HT1 receptor subtypes. *Brain Res.* 456, 147–158. doi: 10.1016/0006-8993(88)90356-3

Fady, J. C., Jamon, M., and Clarac, F. (1998). Early olfactory-induced rhythmic limb activity in the newborn rat. *Brain Res. Dev. Brain Res.* 108, 111–123. doi: 10.1016/s0165-3806(98)00040-6

Fahrenholz, C. (1936). Die sensiblen Einrichtungen der Neunaugenhaut. *Z. Mikrosk Anat. Forsch.* 40, 323–380.

Frigon, A., Thibaudier, Y., Johnson, M. D., Heckman, C. J., and Hurteau, M. F. (2012). Cutaneous inputs from the back abolish locomotor-like activity and reduce spastic-like activity in the adult cat following complete spinal cord injury. *Exp. Neurol.* 235, 588–598. doi: 10.1016/j.expneurol.2012.03.013

Frontini, A., Zaidi, A. U., Hua, H., Wolak, T. P., Greer, C. A., Kafitz, K. W., et al. (2003). Glomerular territories in the olfactory bulb from the larval stage of the sea lamprey *Petromyzon marinus*. *J. Comp. Neurol.* 465, 27–37. doi: 10.1002/cne.10811

Garcia-Rill, E., and Skinner, R. D. (1987a). The mesencephalic locomotor region. I. Activation of a medullary projection site. *Brain Res.* 411, 1–12. doi: 10.1016/0006-8993(87)90675-5

Garcia-Rill, E., and Skinner, R. D. (1987b). The mesencephalic locomotor region. II. Projections to reticulospinal neurons. *Brain Res.* 411, 13–20. doi: 10.1016/0006-8993(87)90676-7

Garcia-Rill, E., Skinner, R. D., Conrad, C., Mosley, D., and Campbell, C. (1986). Projections of the mesencephalic locomotor region in the rat. *Brain Res. Bull.* 17, 33–40. doi: 10.1016/0361-9230(86)90158-9

Garraway, S. M., and Hochman, S. (2001). Modulatory actions of serotonin, norepinephrine, dopamine, and acetylcholine in spinal cord deep dorsal horn neurons. *J. Neurophysiol.* 86, 2183–2194.

Gelman, S., Ayali, A., Tytell, E. D., and Cohen, A. H. (2007). Larval lampreys possess a functional lateral line system. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 193, 271–277. doi: 10.1007/s00359-006-0183-9

Gerachshenko, T., Blackmer, T., Yoon, E.-J., Bartleson, C., Hamm, H. E., and Alford, S. (2005). Gbetagamma acts at the C terminus of SNAP-25 to mediate presynaptic inhibition. *Nat. Neurosci.* 8, 597–605. doi: 10.1038/nn1439

Gerachshenko, T., Schwartz, E., Bleckert, A., Photowala, H., Seymour, A., and Alford, S. (2009). Presynaptic G-protein-coupled receptors dynamically modify vesicle fusion, synaptic cleft glutamate concentrations and motor behavior. *J. Neurosci.* 29, 10221–10233. doi: 10.1523/JNEUROSCI.1404-09.2009

Green, W. W., Basilious, A., Dubuc, R., and Zielinski, B. S. (2013). The neuroanatomical organization of projection neurons associated with different olfactory bulb pathways in the sea lamprey, *Petromyzon marinus*. *PLoS One* 8:e69525. doi: 10.1371/journal.pone.0069525

Grillner, S. (1985). Neurobiological bases of rhythmic motor acts in vertebrates. *Science* 228, 143–149. doi: 10.1126/science.3975635

Grillner, S., Deliagina, T., Ekeberg, O., El Manira, A., Hill, R. H., Lansner, A., et al. (1995). Neural networks that co-ordinate locomotion and body orientation in lamprey. *Trends Neurosci.* 18, 270–279. doi: 10.1016/0166-2236(95)93914-j

Grillner, S., Ekeberg, O., El Manira, A., Lansner, A., Parker, D., Tegnér, J., et al. (1998a). Intrinsic function of a neuronal network—a vertebrate central pattern generator. *Brain Res. Brain Res. Rev.* 26, 184–197. doi: 10.1016/s0165-0173(98)00002-2

Grillner, S., Parker, D., and El Manira, A. (1998b). Vertebrate locomotion—a lamprey perspective. *Ann. N Y Acad. Sci.* 860, 1–18. doi: 10.1111/j.1749-6632.1998.tb09035.x

Grillner, S., McClellan, A., Sigvardt, K., Wallén, P., and Wilén, M. (1981). Activation of NMDA-receptors elicits "fictive locomotion" in lamprey spinal cord *in vitro*. *Acta Physiol. Scand.* 113, 549–551. doi: 10.1111/j.1748-1716.1981.tb06937.x

Grillner, S., and Wallén, P. (1980). Does the central pattern generation for locomotion in lamprey depend on glycine inhibition? *Acta Physiol. Scand.* 110, 103–105. doi: 10.1111/j.1748-1716.1980.tb06637.x

Grillner, S., and Wallén, P. (2002). Cellular bases of a vertebrate locomotor system-steering, intersegmental and segmental co-ordination and sensory control. *Brain Res. Brain Res. Rev.* 40, 92–106. doi: 10.1016/s0165-0173(02)00193-5

Grillner, S., Wallén, P., Saitoh, K., Kozlov, A., and Robertson, B. (2008). Neural bases of goal-directed locomotion in vertebrates-an overview. *Brain Res. Rev.* 57, 2–12. doi: 10.1016/j.brainresrev.2007.06.027

Grimm, R. J. (1960). Feeding behavior and electrical stimulation of the brain of *Carassius auratus*. *Science* 131, 162–163. doi: 10.1126/science.131.3394.162

Guertin, P. A., and Hounsgaard, J. (1998). NMDA-Induced intrinsic voltage oscillations depend on L-type calcium channels in spinal motoneurons of adult turtles. *J. Neurophysiol.* 80, 3380–3382.

Hagelin, L. O., and Johnels, A. G. (1955). On the structure and function of the accessory olfactory organ in lampreys. *Acta Zool.* 36, 113–125. doi: 10.1111/j.1463-6395.1955.tb00376.x

Hägglund, M., Borgius, L., Dougherty, K. J., and Kiehn, O. (2010). Activation of groups of excitatory neurons in the mammalian spinal cord or hindbrain evokes locomotion. *Nat. Neurosci.* 13, 246–252. doi: 10.1038/nn.2482

Hansen, A., and Zielinski, B. S. (2005). Diversity in the olfactory epithelium of bonyfishes: development, lamellar arrangement, sensory neuron cell types andtransduction component. *J. Neurocytol.* 34, 183–208. doi: 10.1007/s11068-005-8353-1

Harris-Warrick, R. M., and Cohen, A. H. (1985). Serotonin modulates the central pattern generator for locomotion in the isolated lamprey spinal cord. *J. Exp. Biol.* 116, 27–46.

Hasler, A. D., and Wisby, W. J. (1951). Discrimination of stream odors by fishes and its relation to parent stream behavior. *Am. Nat.* 135, 223–238. doi: 10.1086/281672

Headley, P. M., Duggan, A. W., and Griersmith, B. T. (1978). Selective reduction by noradrenaline and 5-hydroxytryptamine of nociceptive responses of cat dorsal horn neurones. *Brain Res.* 145, 185–189. doi: 10.1016/0006-8993(78)90809-0

Hellgren, J., Grillner, S., and Lansner, A. (1992). Computer simulation of the segmental neural network generating locomotion in lamprey by using populations of network interneurons. *Biol. Cybern.* 68, 1–13. doi: 10.1007/bf00203132

Hilaire, G., Bou, C., and Monteau, R. (1997). Serotonergic modulation of central respiratory activity in the neonatal mouse: an *in vitro* study. *Eur. J. Pharmacol.* 329, 115–120. doi: 10.1016/s0014-2999(97)89173-x

Hill, R., Matsushima, T., Schotland, J., and Grillner, S. (1992). Apamin blocks the slow AHP in lamprey and delays termination of locomotor bursts. *Neuroreport* 3, 943–945. doi: 10.1097/00001756-199210000-00032

Hill, R. H., Svensson, E., Dewael, Y., and Grillner, S. (2003). 5-HT inhibits N-type but not L-type Ca(2+) channels via 5-HT1A receptors in lamprey spinal neurons. *Eur. J. Neurosci.* 18, 2919–2924. doi: 10.1111/j.1460-9568.2003.03051.x

Hochman, S., Jordan, L. M., and MacDonald, J. F. (1994a). N-methyl-D-aspartate receptor-mediated voltage oscillations in neurons surrounding the central canal in slices of rat spinal cord. *J. Neurophysiol.* 72, 565–577.

Hochman, S., Jordan, L. M., and Schmidt, B. J. (1994b). TTX-resistant NMDA receptor-mediated voltage oscillations in mammalian lumbar motoneurons. *J. Neurophysiol.* 72, 2559–2562.

Hounsgaard, J., and Kiehn, O. (1985). Ca++ dependent bistability induced by serotonin in spinal motoneurons. *Exp. Brain Res.* 57, 422–425. doi: 10.1007/bf00236551

Johnson, N. S., Yun, S. S., Thompson, H. T., Brant, C. O., and Li, W. (2009). A synthetized pheromone induces upstream movement in female sea lamprey and summons them into traps. *Proc. Natl. Acad. Sci. U S A* 106, 1021–1026. doi: 10.1073/pnas.0808530106

Jordan, L. M. (1986). "Initiation of locomotion from the mammalian brainstem," in *Neurobiology of Vertebrate Locomotion*, eds S. Grillner, P. S. G. Stein, D. G. Stuart, H. Forssberg, and R. M. Herman (London: Macmillan Press), 21–37.

Jordan, L. M., McVagh, J. R., Noga, B. R., Cabaj, A. M., Majczyński, H., Sławińska, U., et al. (2014). Cholinergic mechanisms in spinal locomotion-potential target for rehabilitation approaches. *Front. Neural Circuits* 8:132. doi: 10.3389/fncir.2014.00132

Kimmel, C. B., Powell, S. L., and Metcalfe, W. K. (1982). Brain neurons which project to the spinal cord in young larvae of the zebrafish. *J. Comp. Neurol.* 205, 112–127. doi: 10.1002/cne.902050203

Kimura, Y., Satou, C., Fujioka, S., Shoji, W., Umeda, K., Ishizuka, T., et al. (2013). Hindbrain V2a neurons in the excitation of spinal locomotor circuits during zebrafish swimming. *Curr. Biol.* 23, 843–849. doi: 10.1016/j.cub.2013.03.066

Kudo, N., and Yamada, T. (1987). N-methyl-D, L-aspartate-induced locomotor activity in a spinal cord-hindlimb muscles preparation of the newborn rat studied *in vitro*. *Neurosci. Lett.* 75, 43–48. doi: 10.1016/0304-3940(87)90072-3

Laframboise, A. J., Ren, X., Chang, S., Dubuc, R., and Zielinski, B. S. (2007). Olfactory sensory neurons in the sea lamprey display polymorphisms. *Neurosci. Lett.* 414, 277–281. doi: 10.1016/j.neulet.2006.12.037

Lansner, A., and Ekeberg, O. (1994). Neuronal network models of motor generation and control. *Curr. Opin. Neurobiol.* 4, 903–908. doi: 10.1016/0959-4388(94)90140-6

Leach, J. (1951). The hypophysis of lampreys in relation to the nasal apparatus. *J. Morphol.* 89, 217–255. doi: 10.1002/jmor.1050890203

Le Ray, D., Brocard, F., Bourcier-Lucas, C., Auclair, F., Lafaille, P., and Dubuc, R. (2003). Nicotinic activation of reticulospinal cells involved in the control of swimming in lampreys. *Eur. J. Neurosci.* 17, 137–148. doi: 10.1046/j.1460-9568.2003.02417.x

Le Ray, D., Juvin, L., Boutin, T., Auclair, F., and Dubuc, R. (2010). A neuronal substrate for a state-dependent modulation of sensory inputs in the brainstem. *Eur. J. Neurosci.* 32, 53–59. doi: 10.1111/j.1460-9568.2010.07276.x

Le Ray, D., Juvin, L., Ryczko, D., and Dubuc, R. (2011). Supraspinal control of locomotion: the mesencephalic locomotor region. *Prog. Brain Res.* 188, 51–70. doi: 10.1016/B978-0-444-53825-3.00009-7

Lethbridge, R. C., and Potter, I. C. (1982). "The skin," in *The Biology of Lampreys* (Vol. III), eds M. S. Hardisty and I. C. Potter (New York: Academic Press), 377–448.

Li, W., Sorensen, P. W., and Gallaher, D. D. (1995). The olfactory system of migratory adult sea lamprey (*Petromyzon marinus*) is specifically and acutely sensitive to unique bile acids released by conspecific larvae. *J. Gen. Physiol.* 105, 569–587. doi: 10.1085/jgp.105.5.569

Lindsay, A. D., and Feldman, J. L. (1993). Modulation of respiratory activity of neonatal rat phrenic motoneurones by serotonin. *J. Physiol.* 461, 213–233. doi: 10.1113/jphysiol.1993.sp019510

Liu, J., and Jordan, L. M. (2005). Stimulation of the parapyramidal region of the neonatal rat brain stem produces locomotor-like activity involving spinal 5-HT7 and 5-HT2A receptors. *J. Neurophysiol.* 94, 1392–1404. doi: 10.1152/jn.00136.2005

Lopez-Garcia, J. A. (1998). Serotonergic modulation of the responses to excitatory amino acids of rat dorsal horn neurons *in vitro*: implications for somatosensory transmission. *Eur. J. Neurosci.* 10, 1341–1349. doi: 10.1046/j.1460-9568.1998.00153.x

Lopez-Garcia, J. A., and King, A. E. (1996). Pre- and post-synaptic actions of 5-hydroxytryptamine in the rat lumbar dorsal horn *in vitro*: implications for somatosensory transmission. *Eur. J. Neurosci.* 8, 2188–2197. doi: 10.1111/j.1460-9568.1996.tb00740.x

MacLean, J. N., Cowley, K. C., and Schmidt, B. J. (1998). NMDA receptor-mediated oscillatory activity in the neonatal rat spinal cord is serotonin dependent. *J. Neurophysiol.* 79, 2804–2808.

MacLean, J. N., and Schmidt, B. J. (2001). Voltage-sensitivity of motoneuron NMDA receptor channels is modulated by serotonin in the neonatal rat spinal cord. *J. Neurophysiol.* 86, 1131–1138.

Marder, E. (1994). Invertebrate neurobiology. Polymorphic neural networks. *Curr. Biol.* 4, 752–754. doi: 10.1016/s0960-9822(00)00169-x

Marder, E., and Thirumalai, V. (2002). Cellular, synaptic and network effects of neuromodulation. *Neural Netw.* 15, 479–493. doi: 10.1016/s0893-6080(02)00043-6

Marlinsky, V. V., and Voitenko, L. P. (1991). The effect of procaine injection into the medullary reticular formation on forelimb muscle activity evoked by mesencephalic locomotor region and vestibular stimulation in the decerebrated guinea-pig. *Neuroscience* 45, 753–759. doi: 10.1016/0306-4522(91)90287-x

Martin, A. R., and Wickelgren, W. O. (1971). Sensory cells in the spinal cord of the sea lamprey. *J. Physiol.* 212, 65–83. doi: 10.1113/jphysiol.1971.sp009310

Masino, M. A., Abbinanti, M. D., Eian, J., and Harris-Warrick, R. M. (2012). TTX-resistant NMDA receptor-mediated membrane potential oscillations in neonatal mouse Hb9 interneurons. *PLoS One* 7:e47940. doi: 10.1371/journal.pone.0047940

Matthews, G., and Wickelgren, W. O. (1978). Trigeminal sensory neurons of the sea lamprey. *J. Comp. Physiol.* 123, 329–333. doi: 10.1007/bf00656966

McClellan, A. D. (1988). Brainstem command systems for locomotion in the lamprey: localization of descending pathways in the spinal cord. *Brain Res.* 457, 338–349. doi: 10.1016/0006-8993(88)90704-4

McClellan, A. D., and Grillner, S. (1984). Activation of "fictive swimming" by electrical microstimulation of brainstem locomotor regions in an *in vitro* preparation of the lamprey central nervous system. *Brain Res.* 300, 357–361. doi: 10.1016/0006-8993(84)90846-1

Meer, D. P., and Buchanan, J. T. (1992). Apamin reduces the late afterhyperpolarization of lamprey spinal neurons, with little effect on fictive swimming. *Neurosci. Lett.* 143, 1–4. doi: 10.1016/0304-3940(92)90219-w

Ménard, A., Auclair, F., Bourcier-Lucas, C., Grillner, S., and Dubuc, R. (2007). GABAergic projections to the mesencephalic locomotor region in the lamprey *Petromyzon marinus*. *J. Comp. Neurol.* 501, 260–273. doi: 10.1002/cne.21258

Musienko, P. E., Zelenin, P. V., Lyalka, V. F., Orlovsky, G. N., and Deliagina, T. G. (2008). Postural performance in decerebrated rabbit. *Behav. Brain Res.* 190, 124–134. doi: 10.1016/j.bbr.2008.02.011

Nanou, E., Alpert, M. H., Alford, S., and El Manira, A. (2013). Differential regulation of synaptic transmission by pre- and postsynaptic SK channels in the spinal locomotor network. *J. Neurophysiol.* 109, 3051–3059. doi: 10.1152/jn.00067.2013

Nieuwenhuys, R. (1972). Topological analysis of the brain stem of the lamprey *Lampetra fluviatilis*. *J. Comp. Neurol.* 145, 165–177. doi: 10.1002/cne.901450204

Nieuwenhuys, R. (1977). The brain of the lamprey in a comparative perspective. *Ann. N Y Acad. Sci.* 299, 97–145. doi: 10.1111/j.1749-6632.1977.tb41902.x

Nieuwenhuys, R. (2011). The structural, functional and molecular organization of the brainstem. *Front. Neuroanat.* 5:33. doi: 10.3389/fnana.2011.00033

Nieuwenhuys, R., and Nicholson, C. (1998). "Lampreys, petromyzontidae," in *The Central Nervous System of Vertebrates* (Vol. 1), eds R. Nieuwenhuys, H. J. ten Donkelaar, and C. Nicholson (Berlin: Springer), 397–496.

Noga, B. R., Kriellaars, D. J., Brownstone, R. M., and Jordan, L. M. (2003). Mechanism for activation of locomotor centers in the spinal cord by stimulation of the mesencephalic locomotor region. *J. Neurophysiol.* 90, 1464–1478. doi: 10.1152/jn.00034.2003

Northcutt, R. G. (1979). Experimental determination of the primary trigeminal projections in lampreys. *Brain Res.* 163, 323–327. doi: 10.1016/0006-8993(79)90360-3

Northcutt, R. G., and Puzdrowski, R. L. (1988). Projections of the olfactory bulb and nervus terminalis in the silver lamprey. *Brain Behav. Evol.* 32, 96–107. doi: 10.1159/000116537

Ohta, Y., and Grillner, S. (1989). Monosynaptic excitatory amino acid transmission from the posterior rhombencephalic reticular nucleus to spinal neurons involved in the control of locomotion in lamprey. *J. Neurophysiol.* 62, 1079–1089.

Orlovskiĭ, G. N. (1970a). [Relations between reticulo-spinal neurons and locomotor regions of the brain stem]. *Biofizika* 15, 171–178.

Orlovskiĭ, G. N. (1970b). [Work of reticulo-spinal neurons during locomotion]. *Biofizika* 15, 728–737.

Orlovskiĭ, G. N. (1972). The effect of different descending systems on flexor and extensor activity during locomotion. *Brain Res.* 40, 359–371. doi: 10.1016/0006-8993(72)90139-4

Ovsepian, S. V., and Vesselkin, N. P. (2006). Serotonergic modulation of synaptictransmission and action potential firing in frog motoneurons. *Brain Res.* 1102, 71–77. doi: 10.1016/j.brainres.2006.04.035

Parker, D., and Grillner, S. (1999). Activity-dependent metaplasticity of inhibitory and excitatory synaptic transmission in the lamprey spinal cord locomotor network. *J. Neurosci.* 19, 1647–1656.

Parker, D., and Grillner, S. (2000). Neuronal mechanisms of synaptic and network plasticity in the lamprey spinal cord. *Prog. Brain Res.* 125, 381–398. doi: 10.1016/s0079-6123(00)25027-9

Pearlstein, E., Ben Mabrouk, F., Pflieger, J. F., and Vinay, L. (2005). Serotonin refines the locomotor-related alternations in the *in vitro* neonatal rat spinal cord. *Eur. J. Neurosci.* 21, 1338–1346. doi: 10.1111/j.1460-9568.2005.03971.x

Perreault, M. C., and Glover, J. C. (2013). Glutamatergic reticulospinal neurons in the mouse: developmental origins, axon projections and functional connectivity. *Ann. N Y Acad. Sci.* 1279, 80–89. doi: 10.1111/nyas.12054

Perrier, J. F., Alaburda, A., and Hounsgaard, J. (2003). 5-HT1A receptors increase excitability of spinal motoneurons by inhibiting a TASK-1-like K+ current in the adult turtle. *J. Physiol.* 548, 485–492. doi: 10.1111/j.1469-7793.2003.00485.x

Perrier, J. F., and Cotel, F. (2015). Serotonergic modulation of spinal motor control. *Curr. Opin. Neurobiol.* 33, 1–7. doi: 10.1016/j.conb.2014.12.008

Petzold, G. C., Hagiwara, A., and Murthy, V. N. (2009). Serotonergic modulation of odor input to the mammalian olfactory bulb. *Nat. Neurosci.* 12, 784–791. doi: 10.1038/nn.2335

Pflieger, J. F., and Dubuc, R. (2004). Vestibulo-reticular projections in adult lamprey: their role in locomotion. *Neuroscience* 129, 817–829. doi: 10.1016/j.neuroscience.2004.08.025

Photowala, H., Blackmer, T., Schwartz, E., Hamm, H. E., and Alford, S. (2006). G protein betagamma-subunits activated by serotonin mediate presynaptic inhibition by regulating vesicle fusion properties. *Proc. Natl. Acad. Sci. U S A* 103, 4281–4286. doi: 10.1073/pnas.0600509103

Pierre, J., Reperant, J., Ward, R., Vesselkin, N. P., Rio, J. P., Miceli, D., et al. (1992). The serotoninergic system of the brain of the lamprey, *Lampetra fluviatilis*: an evolutionary perspective. *J. Chem. Neuroanat.* 5, 195–219. doi: 10.1016/0891-0618(92)90046-s

Ren, X., Chang, S., Laframboise, A., Green, W., Dubuc, R., and Zielinski, B. (2009). Projections from the accessory olfactory organ into the medial region of the olfactory bulb in the sea lamprey (*Petromyzon marinus*): a novel vertebrate sensory structure? *J. Comp. Neurol.* 516, 105–116. doi: 10.1002/cne.22100

Roberts, A., and Alford, S. (1986). Descending projections and excitation during fictive swimming in *Xenopus* embryos: neuroanatomy and lesion experiments. *J. Comp. Neurol.* 250, 253–261. doi: 10.1002/cne.902500212

Robertson, B., Kardamakis, A., Capantini, L., Pérez-Fernández, J., Suryanarayana, S. M., Wallén, P., et al. (2014). The lamprey blueprint of the mammalian nervous system. *Prog. Brain Res.* 212, 337–349. doi: 10.1016/b978-0-444-63488-7.00016-1

Ronan, M., and Northcutt, R. G. (1990). Projections ascending from the spinal cord to the brain in petromyzontid and myxinoid agnathans. *J. Comp. Neurol.* 291, 491–508. doi: 10.1002/cne.902910402

Roseberry, T. K., Lee, A. M., Lalive, A. L., Wilbrecht, L., Bonci, A., and Kreitzer, A. C. (2016). Cell-type-specific control of brainstem locomotor circuits by basal ganglia. *Cell* 164, 526–537. doi: 10.1016/j.cell.2015.12.037

Rossignol, S., Dubuc, R., and Gossard, J. P. (2006). Dynamic sensorimotor interactions in locomotion. *Physiol. Rev.* 86, 89–154. doi: 10.1152/physrev.00028.2005

Rovainen, C. M. (1967). Physiological and anatomical studies on large neurons of central nervous system of the sea lamprey (*Petromyzon marinus*) I. Müller and Mauthner cells. *J. Neurophysiol.* 30, 1000–1023.

Rovainen, C. M. (1974). Synaptic interactions of reticulospinal neurons and nerve cells in the spinal cord of the sea lamprey. *J. Comp. Neurol.* 154, 207–224. doi: 10.1002/cne.901540207

Rovainen, C. M., and Yan, Q. (1985). Sensory responses of dorsal cells in the lamprey brain. *J. Comp. Physiol. A.* 156, 181–183. doi: 10.1007/bf00610859

Rybak, I. A., Shevtsova, N. A., Lafreniere-Roula, M., and McCrea, D. A. (2006). Modelling spinal circuitry involved in locomotor pattern generation: insights from deletions during fictive locomotion. *J. Physiol.* 577, 617–639. doi: 10.1113/jphysiol.2006.118703

Ryczko, D., Auclair, F., Cabelguen, J. M., and Dubuc, R. (2016). The mesencephalic locomotor region sends a bilateral glutamatergic drive to hindbrain reticulospinal neurons in a tetrapod. *J. Comp. Neurol.* 524, 1361–1383. doi: 10.1002/cne.23911

Ryczko, D., and Dubuc, R. (2013). The multifunctional mesencephalic locomotor region. *Curr. Pharm. Des.* 19, 4448–4470. doi: 10.2174/13816128113192 40011

Ryczko, D., Grätsch, S., Auclair, F., Dubé, C., Bergeron, S., Alpert, M. H., et al. (2013). Forebrain dopamine neurons project down to a brainstem region controlling locomotion. *Proc. Natl. Acad. Sci. U S A* 110, E3235–E3242. doi: 10.1073/pnas.1301125110

Schmidt, B. J., Hochman, S., and MacLean, J. N. (1998). NMDA receptor-mediated oscillatory properties: potential role in rhythm generation in the mammalian spinal cord. *Ann. N Y Acad. Sci.* 860, 189–202. doi: 10.1111/j.1749-6632.1998.tb09049.x

Schmidt, B. J., and Jordan, L. M. (2000). The role of serotonin in reflex modulation and locomotor rhythm production in the mammalian spinal cord. *Brain Res. Bull.* 53, 689–710. doi: 10.1016/s0361-9230(00)00402-0

Schotland, J. L., and Grillner, S. (1993). Effects of serotonin on fictive locomotion coordinated by a neural network deprived of NMDA receptor-mediated cellular properties. *Exp. Brain Res.* 93, 391–398. doi: 10.1007/bf00229355

Schotland, J. L., Shupliakov, O., Grillner, S., and Brodin, L. (1996). Synaptic and nonsynaptic monoaminergic neuron systems in the lamprey spinal cord. *J. Comp. Neurol.* 372, 229–244. doi: 10.1002/(sici)1096-9861(19960819)372:2<229::aid-cne6>3.0.co;2-5

Schwartz, E. J., Blackmer, T., Gerachshenko, T., and Alford, S. (2007). Presynaptic G-protein-coupled receptors regulate synaptic cleft glutamate via transient vesicle fusion. *J. Neurosci.* 27, 5857–5868. doi: 10.1523/jneurosci.1160-07.2007

Schwartz, E. J., Gerachshenko, T., and Alford, S. (2005). 5-HT prolongs ventral root bursting via presynaptic inhibition of synaptic activity during fictive locomotion in lamprey. *J. Neurophysiol.* 93, 980–988. doi: 10.1152/jn.00669.2004

Scott, W. B. (1887). Notes on the development of *Petromyzon*. *J. Morphol.* 1, 253–310. doi: 10.1002/jmor.1050010203

Shefchyk, S. J., Jell, R. M., and Jordan, L. M. (1984). Reversible cooling of the brainstem reveals areas required for mesencephalic locomotor region evoked treadmill locomotion. *Exp. Brain Res.* 56, 257–262. doi: 10.1007/bf00236281

Shik, M. L., Severin, F. V., and Orlovskiĭ , G. N. (1966). [Control of walking and running by means of electric stimulation of the midbrain]. *Biofizika* 11, 659–666.

Shupliakov, O., Pieribone, V. A., Gad, H., and Brodin, L. (1995). Synaptic vesicle depletion in reticulospinal axons is reduced by 5-hydroxytryptamine: direct evidence for presynaptic modulation of glutamatergic transmission. *Eur. J. Neurosci.* 7, 1111–1116. doi: 10.1111/j.1460-9568.1995.tb01099.x

Sigvardt, K. A., Grillner, S., Wallén, P., and Van Dongen, P. A. (1985). Activation of NMDA receptors elicits fictive locomotion and bistable membrane properties in the lamprey spinal cord. *Brain Res.* 336, 390–395. doi: 10.1016/0006-8993(85)90676-6

Sillar, K. T., Reith, C. A., and McDearmid, J. R. (1998). Development and aminergic neuromodulation of a spinal locomotor network controlling swimming in *Xenopus* larvae. *Ann. N Y Acad. Sci.* 860, 318–332. doi: 10.1111/j.1749-6632.1998.tb09059.x

Sillar, K. T., and Simmers, A. J. (1994). Presynaptic inhibition of primary afferent transmitter release by 5-hydroxytryptamine at a mechanosensory synapse in the vertebrate spinal cord. *J. Neurosci.* 14, 2636–2647.

Sirota, M. G., Viana Di Prisco, G., and Dubuc, R. (2000). Stimulation of the mesencephalic locomotor region elicits controlled swimming in semi-intact lampreys. *Eur. J. Neurosci.* 12, 4081–4092. doi: 10.1046/j.1460-9568.2000.00301.x

Skagerberg, G., and Björklund, A. (1985). Topographic principles in the spinal projections of serotonergic and non-serotonergic brainstem neurons in the rat. *Neuroscience* 15, 445–480. doi: 10.1016/0306-4522(85)90225-8

Skinner, R. D., and Garcia-Rill, E. (1984). The mesencephalic locomotor region (MLR) in the rat. *Brain Res.* 323, 385–389. doi: 10.1016/0006-8993(84)90319-6

Sławińska, U., Miazga, K., and Jordan, L. M. (2014). 5-HT_2 and 5-HT_7 receptor agonists facilitate plantar stepping in chronic spinal rats through actions on different populations of spinal neurons. *Front. Neural Circuits* 8:95. doi: 10.3389/fncir.2014.00095

Smetana, R., Alford, S., and Dubuc, R. (2007). Muscarinic receptor activation elicits sustained, recurring depolarizations in reticulospinal neurons. *J. Neurophysiol.* 97, 3181–3192. doi: 10.1152/jn.00954.2006

Smetana, R., Juvin, L., Dubuc, R., and Alford, S. (2010). A parallel cholinergic brainstem pathway for enhancing locomotor drive. *Nat. Neurosci.* 13, 731–738. doi: 10.1038/nn.2548

Steeves, J. D., and Jordan, L. M. (1984). Autoradiographic demonstration of the projections from the mesencephalic locomotor region. *Brain Res.* 307, 263–276. doi: 10.1016/0006-8993(84)90480-3

Steinbusch, H. W., Verhofstad, A. A., Penke, B., Vlarga, J., and Joosten, H. W. (1981). Immunohistochemical characterization of monoamine-containing neurons in the central nervous system by antibodies to serotonin and noradrenalin. A study in the rat and the lamprey (*Lampetra fluviatilis*). *Acta Histochem. Suppl.* 24, 107–122.

Svensson, E., Wikström, M. A., Hill, R. H., and Grillner, S. (2003). Endogenous and exogenous dopamine presynaptically inhibits glutamatergic reticulospinal transmission via an action of D2-receptors on N-type Ca2+ channels. *Eur. J. Neurosci.* 17, 447–454. doi: 10.1046/j.1460-9568.2003.02466.x

Swain, G. P., Snedeker, J. A., Ayers, J., and Selzer, M. E. (1993). Cytoarchitecture of spinal-projecting neurons in the brain of the larval sea lamprey. *J. Comp. Neurol.* 336, 194–210. doi: 10.1002/cne.903360204

Takahashi, M., Freed, R., Blackmer, T., and Alford, S. (2001). Calcium influx-independent depression of transmitter release by 5-HT at lamprey spinal cord synapses. *J. Physiol.* 532, 323–336. doi: 10.1111/j.1469-7793.2001.0323f.x

ten Donkelaar, H. J., Bangma, G. C., Barbas-Henry, H. A., de Boer-van Huizen, R., and Wolters, J. G. (1987). The brain stem in a lizard, *Varanus exanthematicus*. *Adv. Anat. Embryol. Cell. Biol.* 107, 1–168.

Travagli, R. A., and Williams, J. T. (1996). Endogenous monoamines inhibit glutamate transmission in the spinal trigeminal nucleus of the guinea-pig. *J. Physiol.* 491, 177–185. doi: 10.1113/jphysiol.1996.sp021205

Varendi, H., and Porter, R. H. (2001). Breast odour as the only maternal stimulus elicits crawling towards the odour source. *Acta Paediatr.* 90, 372–375. doi: 10.1080/08035250119715

Viala, G., Orsal, D., and Buser, P. (1978). Cutaneous fiber groups involved in the inhibition of fictive locomotion in the rabbit. *Exp. Brain Res.* 33, 257–267. doi: 10.1007/bf00238064

Viana Di Prisco, G., Boutin, T., Petropoulos, D., Brocard, F., and Dubuc, R. (2005). The trigeminal sensory relay to reticulospinal neurones in lampreys. *Neuroscience* 131, 535–546. doi: 10.1016/j.neuroscience.2004.11.003

Viana Di Prisco, G., Dubuc, R., and Grillner, S. (1994). 5-HT innervation of reticulospinal neurons and other brainstem structures in lamprey. *J. Comp. Neurol.* 342, 23–34. doi: 10.1002/cne.903420104

Viana Di Prisco, G., Ohta, Y., Bongianni, F., Grillner, S., and Dubuc, R. (1995). Trigeminal inputs to reticulospinal neurones in lampreys are mediated by excitatory and inhibitory amino acids. *Brain Res.* 695, 76–80. doi: 10.1016/0006-8993(95)00936-k

Viana Di Prisco, G., Pearlstein, É., Le Ray, D., Robitaille, R., and Dubuc, R. (2000). A cellular mechanism for the transformation of a sensory input into a motor command. *J. Neurosci.* 20, 8169–8176.

Viana Di Prisco, G., Pearlstein, É., Robitaille, R., and Dubuc, R. (1997). Sensory-evoked NMDA plateau potentials and their role in the initiation of locomotion. *Science* 278, 1122–1125. doi: 10.1126/Science278.5340.1122

Wallén, P., Buchanan, J. T., Grillner, S., Hill, R. H., Christenson, J., and Hökfelt, T. (1989a). Effects of 5-hydroxytryptamine on the afterhyperpolarization, spike frequency regulation and oscillatory membrane properties in lamprey spinal cord neurons. *J. Neurophysiol.* 61, 759–768.

Wallén, P., Christenson, J., Brodin, L., Hill, R., Lansner, A., and Grillner, S. (1989b). Mechanisms underlying the serotonergic modulation of the spinal circuitry for locomotion in lamprey. *Prog. Brain Res.* 80, 321–327; discussion 315–319. doi: 10.1016/s0079-6123(08)62227-x

Wallén, P., and Grillner, S. (1987). N-methyl-D-aspartate receptor-induced, inherent oscillatory activity in neurons active during fictive locomotion in the lamprey. *J. Neurosci.* 7, 2745–2755.

Wang, D., Grillner, S., and Wallén, P. (2014). Endogenous release of 5-HT modulates the plateau phase of NMDA-induced membrane potential oscillations in lamprey spinal neurons. *J. Neurophysiol.* 112, 30–38. doi: 10.1152/jn.00582.2013

Wells, C. A., Zurawski, Z., Betke, K. M., Yim, Y. Y., Hyde, K., Rodriguez, S., et al. (2012). Gβγ inhibits exocytosis via interaction with critical residues on soluble N-ethylmaleimide-sensitive factor attachment protein-25. *Mol. Pharmacol.* 82, 1136–1149. doi: 10.1124/mol.112.080507

Westberg, K. G., and Kolta, A. (2011). The trigeminal circuits responsible for chewing. *Int. Rev. Neurobiol.* 97, 77–98. doi: 10.1016/B978-0-12-385198-7.00004-7

Whitear, M., and Lane, E. B. (1981). Fine structure of Merkel cells in lampreys. *Cell Tissue Res.* 220, 139–151. doi: 10.1007/bf00209973

Wickelgren, W. O. (1977). Physiological and anatomical characteristics of reticulospinalneurones in lamprey. *J. Physiol.* 270, 89–114. doi: 10.1113/jphysiol.1977.sp011940

Wikström, M. A., and El Manira, A. (1998). Calcium influx through N- and P/Q-type channels activate apamin-sensitive calcium-dependent potassium

channels generating the late afterhyperpolarization in lamprey spinal neurons. *Eur. J. Neurosci.* 10, 1528–1532. doi: 10.1046/j.1460-9568.1998.00194.x

Wikström, M., Hil, R., Hellgren, J., and Grillner, S. (1995). The action of 5-HT on calcium-dependent potassium channels and on the spinal locomotor network in lamprey is mediated by 5-HT1A-like receptors. *Brain Res.* 678, 191–199. doi: 10.1016/0006-8993(95)00183-q

Wilson, J. M., Hartley, R., Maxwell, D. J., Todd, A. J., Lieberam, I., Kaltschmidt, J. A., et al. (2005). Conditional rhythmicity of ventral spinal interneurons defined by expression of the Hb9 homeodomain protein. *J. Neurosci.* 25, 5710–5719. doi: 10.1523/jneurosci.0274-05.2005

Yoon, E. J., Gerachshenko, T., Spiegelberg, B. D., Alford, S., and Hamm, H. E. (2007). Gbetagamma interferes with Ca^{2+}-dependent binding of synaptotagmin to the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex. *Mol. Pharmacol.* 72, 1210–1219. doi: 10.1124/mol.107.039446

Zielinski, B. S., Moretti, N., Hua, H. N., Zaidi, A. U., and Bisaillon, A. D. (2000). Serotonergic nerve fibers in the primary olfactory pathway of the larval sea lamprey, *Petromyzon marinus*. *J. Comp. Neurol.* 420, 324–334. doi: 10.1002/(sici)1096-9861(20000508)420:3<324::aid-cne4>3.0.co;2-6

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Monoamine Release in the Cat Lumbar Spinal Cord during Fictive Locomotion Evoked by the Mesencephalic Locomotor Region

Brian R. Noga, Riza P. Turkson, Songtao Xie, Annette Taberner, Alberto Pinzon and Ian D. Hentall*

The Miami Project to Cure Paralysis, Department of Neurological Surgery, University of Miami Miller School of Medicine, Miami, FL, United States

Spinal cord neurons active during locomotion are innervated by descending axons that release the monoamines serotonin (5-HT) and norepinephrine (NE) and these neurons express monoaminergic receptor subtypes implicated in the control of locomotion. The timing, level and spinal locations of release of these two substances during centrally-generated locomotor activity should therefore be critical to this control. These variables were measured in real time by fast-cyclic voltammetry in the decerebrate cat's lumbar spinal cord during fictive locomotion, which was evoked by electrical stimulation of the mesencephalic locomotor region (MLR) and registered as integrated activity in bilateral peripheral nerves to hindlimb muscles. Monoamine release was observed in dorsal horn (DH), intermediate zone/ventral horn (IZ/VH) and adjacent white matter (WM) during evoked locomotion. Extracellular peak levels (all sites) increased above baseline by 138 ± 232.5 nM and 35.6 ± 94.4 nM (mean ± SD) for NE and 5-HT, respectively. For both substances, release usually began prior to the onset of locomotion typically earliest in the IZ/VH and peaks were positively correlated with net activity in peripheral nerves. Monoamine levels gradually returned to baseline levels or below at the end of stimulation in most trials. Monoamine oxidase and uptake inhibitors increased the release magnitude, time-to-peak (TTP) and decline-to-baseline. These results demonstrate that spinal monoamine release is modulated on a timescale of seconds, in tandem with centrally-generated locomotion and indicate that MLR-evoked locomotor activity involves concurrent activation of descending monoaminergic and reticulospinal pathways. These gradual changes in space and time of monoamine concentrations high enough to strongly activate various receptors subtypes on locomotor activated neurons further suggest that during MLR-evoked locomotion, monoamine action is, in part, mediated by extrasynaptic neurotransmission in the spinal cord.

Keywords: mesencephalic locomotor region, fictive locomotion, spinal cord, raphespinal, ceruleospinal, monoamine, fast cyclic voltammetry, volume transmission

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Ronald M. Harris-Warrick, Cornell University, United States

****Correspondence:***
Brian R. Noga
bnoga@miami.edu

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INTRODUCTION

The mesencephalic locomotor region (MLR; Shik et al., 1966, 1967) or MLR, a major relay center for the control of locomotion (Garcia-Rill, 1986), activates spinal locomotor generating neurons (Jordan, 1991; Noga et al., 1995a, 2003) via pathways originating in the medial reticular formation (Orlovskii, 1970; Steeves and Jordan, 1984; Garcia-Rill and Skinner, 1987; Noga et al., 1991, 2003) and descending through the ventral funiculus (Steeves and Jordan, 1980; Noga et al., 1991, 2003). The release of glutamate is thought to play a major role in the activation of these spinal neurons, both in mammals (Douglas et al., 1993; Hägglund et al., 2010; Bretzner and Brownstone, 2013) and in lower vertebrates (Buchanan et al., 1987; Brodin et al., 1989; Ohta and Grillner, 1989).

Monoamines have also been implicated in the activation of spinal locomotor networks. Intravenous injection of L-DOPA, the precursor of dopamine (DA), followed by norepinephrine (NE) produces long-latency, long-duration reflex discharges in acute spinal cats that are qualitatively similar to locomotor movements (Jankowska et al., 1967; Viala et al., 1974). A similar effect, produced by intravenous administration of the serotonin (5-HT) precursor 5-hydroxytryptophan, has been observed in rabbits (Viala and Buser, 1969) and, less reliably, in high spinal cats (Miller et al., 1975). Other monoaminergic drugs likewise evoke or modulate locomotion in spinally injured cats (Barbeau and Rossignol, 1991; Kiehn et al., 1992; Marcoux and Rossignol, 2000) and rats (Feraboli-Lohnherr et al., 1999; Antri et al., 2002) and in *in vitro* neonatal rats (Cazalets et al., 1992; Kiehn and Kjærulff, 1996; Sqalli-Houssaini and Cazalets, 2000) and mice (Christie and Whelan, 2005). Monoamines can be expected to influence locomotion, since terminals of serotonergic and noradrenergic fibers appose spinal locomotor-activated neurons that express a number of monoaminergic receptors implicated in the control of locomotion (Noga et al., 2009, 2011).

Because stimulating the MLR electrically is similar in effect to stimulating the spinal cord with L-DOPA, Grillner and Shik (1973) postulated that the MLR activates a noradrenergic descending pathway, which controls spinal mechanisms for generating locomotion. This idea gained further plausibility when catecholamine-containing cells were found in the vicinity of the MLR (Steeves et al., 1976) and when descending projections from the MLR were found to include the noradrenergic and serotonergic nuclei (Edwards, 1975; Steeves and Jordan, 1984; Sotnichenko, 1985). However, monoamine release is apparently not obligatory since depletion of spinal NE or 5-HT does not abolish the MLR's ability to evoke locomotion (Steeves et al., 1980).

Nevertheless, there is evidence that monoaminergic pathways are activated during spontaneous or voluntary locomotion. In the cat, the activity of raphespinal and ceruleospinal neurons increases during walking (Fornal et al., 1985, 2006; Rasmussen et al., 1986; Jacobs and Fornal, 1995, 1999; Veasey et al., 1995). A complex pattern of monoamine release has also been observed in the spinal cord of freely moving rats using microdialysis and high performance liquid chromatography (Gerin et al., 1994, 1995, 2008, 2011; Gerin and Privat, 1998).

Based on these findings we hypothesized that MLR stimulation would increase the spinal release of monoamines during evoked locomotion, raising their levels above those observed in resting (basal or steady-state) conditions (Noga et al., 2004). The aim of this study was therefore to determine the extent to which monoamines are released within the spinal cord during MLR-evoked fictive locomotion, to identify the location of this release and its temporal relationship to MLR stimulation and locomotion. The fictive locomotion preparation, in which animals are paralyzed by neuromuscular blockade and locomotor activity is monitored by electroneurogram (ENG) recordings from peripheral nerves, was chosen as the experimental model. This allows investigation of the central drive for induction of locomotion in the absence of peripheral afferent input that by itself can increase spinal release of monoamines (Tyce and Yaksh, 1981; Men et al., 1996). Measurements were made in the gray matter of middle-to-low lumbar segments of the cat where relatively large numbers of serotonergically and noradrenergically innervated locomotor-activated neurons are located (Huang et al., 2000; Dai et al., 2005; Noga et al., 2009, 2011). Measurements were also made in white matter (WM) for comparison to previously obtained microdialysis measurements from the WM of rats subject to treadmill exercise (Gerin et al., 1995). We used fast cyclic voltammetry (FCV; Armstrong-James and Millar, 1984; Stamford et al., 1992) to assess spinal monoamine release by measuring the oxidation of monoamines on the surface of single carbon fiber microelectrodes (CFMEs) during a voltage scan. Individual monoaminergic components of the signal were resolved by Principle Component Regression (PCR; Heien et al., 2004; Keithley et al., 2009). The temporal resolution afforded by this technique contrasts with other extractive methods of measurement, such as microdialysis combined with HPLC, which have temporal resolutions of several minutes and require prolonged conditioning stimulation. The small size of the CFME (33 μm carbon fiber diameter) also allows for higher spatial resolution and is less damaging than the larger microdialysis probes. As the technique measures release relative to a baseline resting state, our experiments were conducted on mesencephalic decerebrate animals in which no spontaneous locomotor activity was observed. Preliminary results have been presented (Noga et al., 2006, 2007).

MATERIALS AND METHODS

Animal Preparation

Experimental procedures were approved by the University of Miami IACUC committee in accordance with National Institute of Health guidelines (NIH Publications No. 80-23; revised 1996). The number of animals used, and their pain and distress, were minimized. Six adult cats (2.8–3.3 kg) were anesthetized with 1%–3% halothane. The trachea was intubated for direct administration of the anesthetic and cannulas inserted into the common carotid artery and jugular vein for blood pressure monitoring and administration of fluids, respectively. Animals were given 2–4 mg of dexamethasone

(Hexadrol phosphate, Organon) intravenously to reduce tissue swelling. A bicarbonate solution (100 mM $NaHCO_3$ with 5% glucose) was infused at 3–5 ml/h to replace fluid loss and help maintain a normal blood pH. Hindlimb nerves dissected free bilaterally and placed in tunnel electrodes included: semimembranosus/anterior biceps (SMAB), posterior biceps/semitendinosus (PBST), quadriceps (QUAD) and sartorius anterior/medialis (SA). Following a lumbar (L3–6) laminectomy, each animal was placed in a Transvertex headframe and suspended with all limbs pendant. A pool, formed by back muscle and skin flaps, was sealed with a thin layer of Reprosil (Dentsply Caulk, Milford, DE, USA) and filled with warm filtered saline, regularly replaced to prevent accumulation of blood and tissue fluids. Bath and core temperatures were maintained at 37°C using feedback-controlled heating lamps controlled by bath and rectal thermistors. Following a craniotomy, the anesthetic was discontinued and before the animal could awaken a precollicular-postmammillary (mesencephalic) decerebration was performed. After a brief recovery period, the animals were paralyzed with pancuronium bromide (Astra, Westborough, MA, USA: 0.1–0.2 mg/kg as needed—usually every 1–2 h) and artificially ventilated. Ringers solution was sometimes administered intravenously to maintain blood pressure >80 mmHg. Expired CO_2, O_2 and tissue oxygenation (SpO_2) was monitored throughout the experiment using a Datex/Engström Oscaroxy Multigas Monitor and Pulse Oximeter and the end tidal CO_2 maintained between 3.5% and 4.5%.

Stimulation and Recording

The experimental setup is illustrated in **Figure 1**. Animals were allowed to recover for 1.5–2 h following decerebration before stimulation was commenced. Bouts of locomotion were evoked by electrical stimulation of the MLR (typically 1.0 ms square wave pulses, 15–20 Hz, 30–200 μA) using monopolar stimulating electrodes (SNE-300; David Kopf Instruments, Tujunga, CA, USA). Electrode were stereotaxically inserted into the mesopontine tegmentum at an area bounded by posterior (P) 1–3 and lateral (L) 2.5–5.0 and included the cuneiform nucleus, subcuneiform area, brachium conjunctivum and pedunculopontine nucleus. Electrodes were advanced slowly while stimulating until the optimal response was obtained. If no response or stimulation strength is high, the electrode was repositioned and the procedure repeated. Final position was selected based upon best response and lowest threshold at the specified frequency (15–20 Hz) and pulse width (1.0 ms square wave pulses). Optimal responses were observed with stereotaxic coordinates within the cuneiform and subcuneiform area dorsal to the brachium conjunctivum. Rhythmic activity observed from ENG recordings from hindlimb peripheral nerves, captured digitally as continuous waveforms, was used as an indicator of "fictive" locomotion. ENG signals were amplified with AC-coupled amplifiers (bandwidth 300 Hz–10 kHz), rectified and low-pass filtered (30 ms time constant) and digitized directly through a 1 MHz, 16 channel analog-to-digital converter (12 bit) at 2 kHz, using customized software (Spinal Cord Research Centre, University of Manitoba, Canada).

Fast Cyclic Voltammetry

Microelectrodes were constructed from single carbon fibers, 33-μm in diameter (Textron Systems, Lowell, MA, USA) inserted into pulled borosilicate capillary tubes (ID 1.12 mm, OD 2.0 mm, WPI, Sarasota, FL, USA) according to procedures described previously (Hentall et al., 2003, 2006; Noga et al., 2004; Brumley et al., 2007). The electrodes were beveled at a 30° or 45° angle from horizontal (Model BV-10 Micropipette Beveller, Sutter Instrument Co., Novato, CA, USA) to produce a sensing elliptical surface defined by the cross section of the carbon fiber, so that the carbon fiber surface was flush with the tip (**Figure 1**, inset). The electrodes were cleaned in ethyl alcohol and rinsed with de-ionized water. Just prior to use, the electrodes were electrochemically pretreated in phosphate-buffered saline (PBS: 10 ml, pH 7.4) to increase their sensitivity (Stamford et al., 1992) by applying an offset 71 Hz triangular waveform for three consecutive 10-s periods: at −0.6 to +3.0 V, −0.6 to +2.0 V and −0.6 to +1.0 V (Hentall et al., 2003; Noga et al., 2004). In experiments with prolonged scanning and application of metabolic and uptake inhibitors, electrodes were coated with 5% Nafion (Aldrich, St. Louis, MO, USA; dipped five times, then placed in an 80°C oven for 10 min) to improve selectivity for the primary amines and reduce sensitivity to monoaminergic metabolites and ascorbic acid (Brazell et al., 1987).

FCV scans were generated using a three-lead voltage-clamp amplifier (Millar Voltammeter, P.D. Systems International Ltd., UK). The leads were connected to the carbon-fiber working electrode, to a carbon-based reference electrode internally filled with KCl (Dri-Ref: WPI, Sarasota, FL, USA) and to an Ag-AgCl auxiliary electrode that applied the clamping current. In a PBS-filled beaker, a triangular voltage waveform (scan) of 14.2 ms duration was applied to the CFME every 0.25 s, sweeping at 480 V/s, from 0 V to −1 V, then to +1.4 V, to −1 V and then back to 0 V (**Figure 2**, top trace). The amplifier's output signal, or voltammogram, is proportional to the current flowing through the microelectrode; it was captured digitally at 5 or 10 kHz and calibrated using a 10 or 100 nA current pulse. This output constitutes the so-called "full" signal, which includes the electrode background charging current generated in PBS (or when *in vivo*, in the extracellular fluid of the spinal cord) as well as the much smaller redox current or Faradic current resulting from the oxidation and reduction of any analytes in solution (Brumley et al., 2007). To monitor monoamine concentrations during each experiment, the output voltammogram for each electrode obtained during the first scan in the test sequence was subtracted from subsequent ouput voltammograms to yield a "subtracted voltammogram" of changes in the redox current (**Figure 2**).

Electrode Calibration

Electrodes were calibrated after each experiment, in varying concentrations of NE and 5-HT dissolved in PBS at pH 7.4 and at various pH levels. Electrodes are able to measure changes in monoamines from one scan to the next and detection times *in vitro* are likely related to the time required for the solute to reach the electrode (mixing time) with the addition

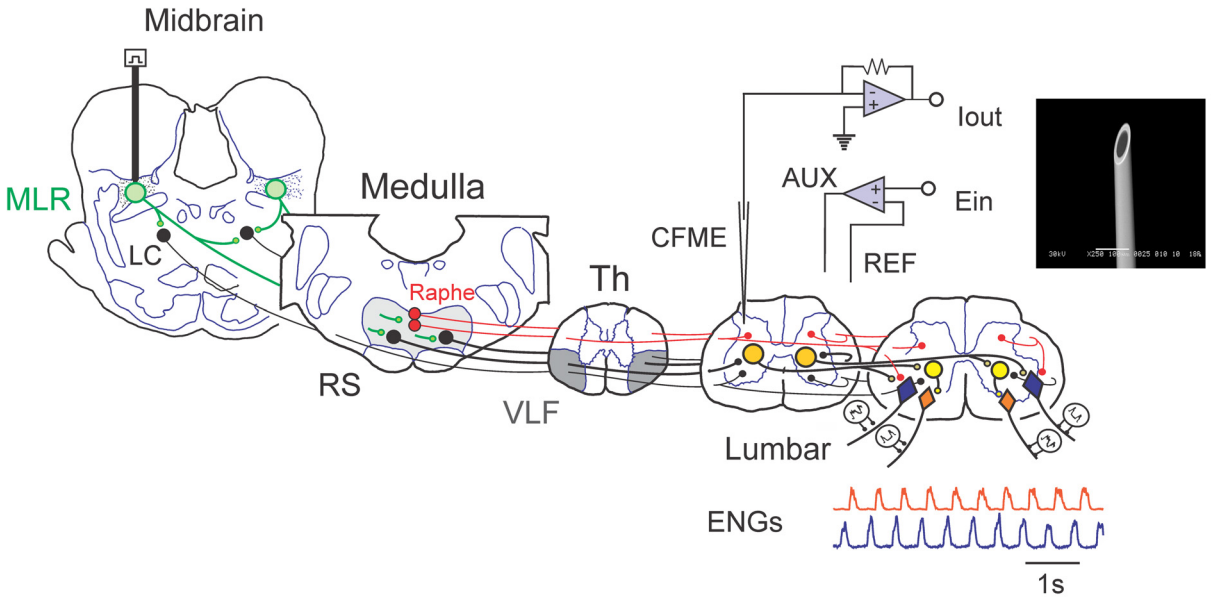

FIGURE 1 | Schematic view of experimental setup used to examine spinal monoamine release during mesencephalic locomotor region (MLR)-evoked fictive locomotion. Known neuronal projections from the MLR include the area of the noradrenergic locus ceruleus (LC), the serotonergic raphe nuclei in addition to the glutamatergic reticulospinal (RS) neurons within the medial reticular formation (Edwards, 1975; Steeves and Jordan, 1984; Sotnichenko, 1985). Axons of RS neurons descend via the ventrolateral funiculus (VLF) to innervate lumbar spinal locomotor interneurons comprising the central pattern generator (CPG; Steeves and Jordan, 1980; Noga et al., 2003). Fast cyclic voltammetry (FCV) scans within the lumbar spinal cord were applied throughout bouts of MLR evoked locomotion. All potentials (Ein) applied to the working carbon fiber microelectrode (CFME) are defined with respect to the reference (REF) electrode. If the potential applied to the CFME is different than the desired potential, then current is provided via the auxiliary (AUX) electrode (Ag/AgCl wire) to maintain the appropriate potential. Fictive locomotor activity is monitored by electroneurogram (ENG) recordings from hindlimb peripheral nerves. *Inset*: scanning electron micrograph of a CFME. The uninsulated carbon fiber (33 μm diameter) is visible at the beveled electrode tip.

of monoamines to the PBS. Arepresentative calibration set (training set) is illustrated in **Figure 2**. It is made up of 34 total concentration steps (9 NE, 9 5-HT and 17 for pH; each set done separately), ranging between 250 nM and 4.0 μM for NE, 125–2000 nM for 5-HT and ∼7.3–6.1 pH units, respectively. In the presence of monoamines, oxidation currents were generated during the first ascending phase of the triangular input waveform. Oxidation peaks of NE and 5-HT differed only slightly and were maximal when the applied input waveform was near 842 and 827 mV, respectively. The monoamines could be distinguished from each other, however, by the shape of their post-oxidation reduction current(s) produced during the second descending slope of the applied waveform. These smaller currents reflect reduction of the electrode oxidation reaction products that remain nearby (Stamford, 1989). As seen in **Figure 2**, 5-HT showed double reduction peaks, one near −80 mV and the other near −632 mV, whereas NE showed a single reduction peak near −345 mV. Changes in pH, expected to occur with stimulation *in vivo* (Syková and Svoboda, 1990), induced redox currents that peaked around 450 mV and −300 mV as acid levels increased. Current responses to changes in concentration of monoamines were linear over the range observed *in vivo*. Current responses to changes in pH in the range examined were curvi-linear.

The amplitude of the peak oxidation current was quantified by subtracting a reference level current at one or two points in the subtracted voltammogram where the sign of its slope changed (Hentall et al., 2003, 2006). Concentration changes *in vivo* could then be estimated from peak oxidation currents obtained during electrode calibrations. The mean sensitivity (± SD) of the microelectrodes was 15.2 ± 8.9 (n = 6) and 7.0 ± 4.7 (n = 6) nM/nA for NE and 5-HT, respectively. Since recording noise for individual scans was ∼0.5 nA *in vivo*, detection thresholds were ∼1.0 nA, which translates to about 5–10 nM for 5-HT and 10–20 nM for NE (see Hentall et al., 2006).

In Vivo Voltammetry

Monoamine levels were determined at points along microelectrode trajectories within the L4–7 lumbar spinal segments during MLR evoked fictive locomotion. CFMEs were typically inserted into the cord at a 10° (tip rostral) angle. Measurements were usually made every 250 μm up to a depth of ∼4500 μm, as measured by the digital readout of the stepping motor that controlled the micromanipulator. Scans were performed at intervals of 250 ms, prior to, during and following 20–60 s trials of MLR stimulation. Monitoring continued for approximately 20–80 s from termination of stimulation. To monitor release during the experiment, subtracted voltammograms were obtained by subtracting the full signal of a pre-locomotion scan, as described for *ex vivo* calibration, above. Subtracted voltammograms obtained *in vivo* are often associated with a linear amplitude change across the

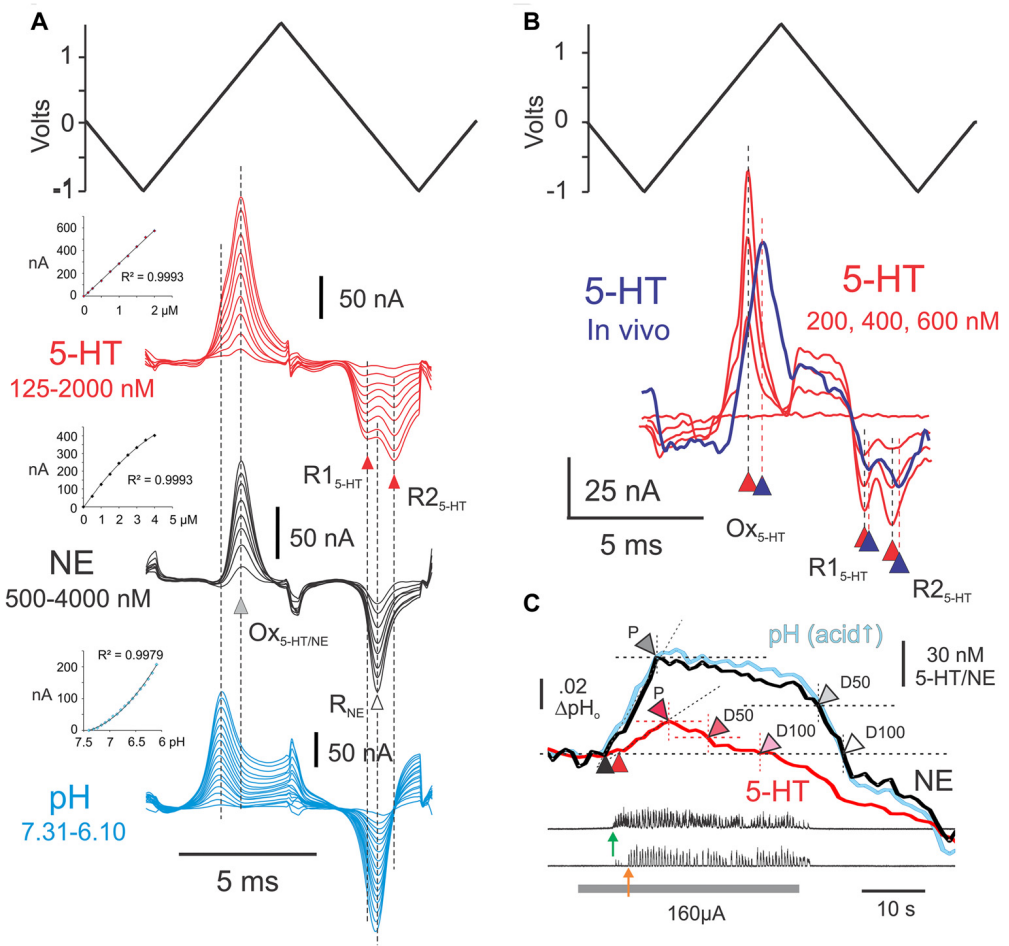

FIGURE 2 | Experimental procedures for detection and estimation of transmitter levels. **(A)** Post-experimental *ex vivo* calibration of CFMEs. Results of *ex vivo* calibrations in different concentrations of 5-HT and norepinephrine (NE) in phosphate buffered saline, pH 7.4 (5-HT: 125, 250, 500, 750, 1000, 1250, 1500, 1750 and 2000 nM; NE: 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 µM). Subtracted voltammograms are averages of 40 scans. Also shown is the triangular input waveform (*top*). Peak oxidation currents for 5-HT and NE ($Ox_{5-HT/NE}$) occur at approximately the same voltage *ex vivo* (*gray arrowhead)*. Double ($R1_{5-HT}$ and $R2_{5-HT}$) and single (R_{NE}) reduction peaks are observed for 5-HT and NE (*black and white arrowheads*, respectively). The single noradrenergic reduction peak occurs approximately mid-way between the double serotonergic reduction peaks. Subtracted voltammograms illustrating pH effects over the range 7.31–6.1, generated by the addition of concentrated hydrochloric acid (17 steps), are shown beneath the monoamine calibrations. Insets: calibration curves for 5-HT, NE and pH. **(B)** Subtracted voltammogram (*purple line*) obtained from the L6 spinal segment (lamina VII) following microinjection of 100 µM 5-HT near the microelectrode. The voltammogram is an average of 20 scans obtained at the peak of the 5-HT concentration wave observed after injection. It is overlayed with the voltammograms (*red lines*: 20 scans each) obtained during *ex vivo* calibration in concentrations of 5-HT that are within the range of the *in vivo* signal. Note slight delay in peak oxidation and reduction currents. **(C)** Measurements made from principle component regression (PCR) derived *in vivo* signals during MLR evoked locomotor activity. Onset of bursting and locomotor activity are indicated with *green* and *orange arrows*, respectively. *Black* and *red arrowheads* indicate onset of release of NE and 5-HT relative to baseline (pre-locomotor) levels. Other arrowheads indicate peak levels (P), and 50% (D50) and and 100% (D100) decline-to-baseline. MLR stimulation indicated by *gray bar*.

full signal (Phillips et al., 2002; Brumley et al., 2007) likely due to changes in tissue impedance during strong neuronal activity. These changes were mostly removed in subsequent offline analysis as described previously (Brumley et al., 2007). To improve the signal-to-noise ratio, single scans obtained at 4 Hz were subsequently averaged to generate an overall 1 Hz sampling frequency prior to processing the data with PCR (Keithley et al., 2009). PCR analysis of the data (Heien et al., 2004) was used to resolve individual components of the voltammograms obtained *in vivo*. To determine concentrations within spinal tissue of three predicted components (NE, 5-HT and pH), a calibration set was generated after each experiment (see *Ex vivo* calibration, above). The number of principal components needed to describe the dataset was between 4 and 6, with the scan range used for this analysis: −1.0 to 1.4 to −1.0 V, as determined using singular value decomposition and a log Scree graph (Keithley et al., 2009). A data matrix was constructed and a regression matrix relating the data projections to concentrations was calculated. PCR accuracy was examined using mixed analyte samples following individual calibrations and was found to have less than 5% error for NE and a 20% error with 5-HT. The shape and value

of peak oxidation or reduction potentials depend theoretically on many factors, such as diffusion rates, pH, temperature and redox environment, which can be expected to differ *in vivo* and *ex vivo* (Stamford, 1986; Palij and Stamford, 1994; O'Neill et al., 1998). Peak currents are known to be delayed (shift toward higher scan voltages) with more acidic conditions (Kawagoe et al., 1993), which are found in the spinal gray matter at rest and during periods of electrical stimulation (Syková and Svoboda, 1990; see also Chesler and Kaila, 1992; Chesler, 2003). This expected delay was confirmed by either spinal microinjection or superfusion of NE and 5-HT. These experiments revealed slight delays in redox peaks *in vivo* for 5-HT (see **Figure 2B**). While such delays may also be due to adsorption of 5-HT to the electrode surface (e.g., Dankoski and Wightman, 2013), electrode responses during experiments were stable and repeatable. Although alternative scanning waveforms are available to reduce such effects, they are too short to reveal double reduction peaks for 5-HT which allow differentiation of 5-HT from catecholamine signals in tissues with mixed monoamine terminal fields. Calibration set peak values were therefore adjusted to compensate for these delays according to subtracted traces from each experiment prior to PCR analysis of *in vivo* data. Residual analysis sometimes revealed additional signals in the dataset that could not be accounted for by the use of three principal components. However, this residual signal fell well outside of the monoaminergic or pH subtracted voltammograms.

Locomotion trials were performed with at least 2 min between trials to allow for clearance and to re-establish monoamine baseline levels when electrodes were advanced to new depths. Repeated trials were done in some locations where large signals were observed, to verify the reproducibility of the measured release and to examine the relationship between monoamine levels and quality of locomotion. The MLR stimulation was not synchronized to the voltammetric scans and any scans containing stimulus artifacts were excluded from the analysis.

In three animals, FCV scans during evoked locomotion were made after the microinjection of the monoamine oxidase inhibitor pargyline and the monoamine uptake inhibitors imipramine, desipramine and buproprion (Rivot et al., 1983; Martin et al., 1988) mixed together in aCSF (2.5–5 μL total volume; in 1 or 2 tracks). This was done to test the effects on monoamine release *in vivo* by blocking the clearance of monamines by uptake mechanisms and metabolism. Microinjections were made using a Neurophore BH-2 pressure ejection system (Medical Systems Corp., Greenvale, NY, USA) through pulled glass micropipettes of ∼2–5 μm tip diameter placed 2–3 mm away from the recording electrodes. Micropipettes were initially lowered to the base of the ventral horn (VH; deepest point ∼5 mm), and the drugs injected while withdrawing the electrodes toward the surface. Drugs were injected at a rate of 0.5 μL/min and a volume of 0.5–1.0 μL/mm depth (total injection of 5 uL over a ∼5 min period). Trials were also conducted during the nearby dialysis of 0.5 mM pargyline and 0.1 mM desipramine in aCSF, from two dialysis probes placed 9–15 mm apart. Electrodes were placed between the probes (4–9 mm distant) and scans conducted during the dialysis.

Histology

At the end of each experiment, the spinal cord was removed and immersion fixed in 10% formalin. Frozen transverse sections of the cord (100 μm thick) were counterstained with Toluidine blue or Cresyl Violet. Recording sites along reconstructed tracks were determined from microelectrode depth readings (from surface of spinal cord), and taking into account a shrinkage factor for processed tissue (indicated by decreased distances between parallel tracks) and a small correction factor (2%) to account for the 10° (tip-pointing rostral) electrode insertion angle.

Data Analysis

Locomotor quality was graded using a 0–2 point system for each hindlimb (maximum 4 for bilateral locomotion): 0, no locomotion or tonic activity only; 1, rhythmic excitatory bursts in flexor or extensor nerves without reciprocal rhythmic activity in antagonists; 2, locomotion—full reciprocal alternation between flexors and extensors. Detailed analyses of locomotor activity in some trials included the onset and offset of each step cycle in the recorded ENG bursts, as determined using a threshold detection algorithm with trigger hysteresis. The locomotor frequency, burst durations, duty cycle (proportion of cycle period in which the nerve is active during the step cycle) and burst areas were calculated. Changes in 5-HT and NE concentration and pH during and following MLR stimulation were plotted relative to baseline (pre-stimulation) values. A number of measurements were made from these plots (**Figure 2C**) and included: (1) onset and offset of bursting and/or locomotor activity relative to stimulation onset; (2) onset and offset latencies of 5-HT/NE concentration and pH changes; and (3) the time-to-peak (TTP) and the time for 50% and 100% decay to baseline (D_{50} and D_{100}). To determine onset of stimulated release we visually determined the point where the slopes of the signal at baseline and during stimulation intersected. Profiles of release were categorized. For repeated trials in the same location, cycle statistics for the best continuous bout of locomotion included the frequency and area of the averaged step cycle. Circular statistics (Liu and Jordan, 2005), were used to determine coordination of flexor and extensor nerve activity from ENG recordings on the same and opposite sides. Cycle onsets and offsets were first marked for each nerve. Phase values (Φ) for each step cycle were calculated by dividing the latency between the onsets of paired nerve cycles by the step cycle period. These were then displayed graphically as data points on a polar plot where a phase values of 180° represents out-of-phase activity (alternation) and values of 0° or 360° are in-phase and equivalent to each other. The mean phase value is indicated by the direction of a vector, with length *r* (Zar, 1974). The length of the vector ranged from 0 to 1, and is a measure of the concentration of phase lags around the mean phase value. Values of *r* greater than the critical Rayleigh's circular statistical test value (Zar, 1974) indicated by dotted circles inside

of the polar plot ($p < 0.05$ and < 0.01) were considered phase-related.

Statistical Analysis

Measurement sites were assigned to cytoarchitectural laminae (Rexed, 1954) and various measured parameters were averaged across tracks and subjects. Statistical significance was assessed by independent samples *t*-tests and paired samples *t*-tests to test various hypotheses. Non-parametric tests were used to calculate differences between dichotomous variables. The extent to which various factors would predict quality of locomotion was assessed with linear regression models. All statistical analyses were performed with SPSS 22 for Windows. A level of $p \leq 0.05$ was considered statistically significant. Mean and SD are reported throughout, unless otherwise indicated.

RESULTS

Locomotor Responses to MLR Stimulation

A total of 132 trials of MLR stimulation were conducted for general data analysis of monoamine release from four animals and data from all animals are grouped together unless otherwise stated. Rhythmic excitatory or full locomotor activity was induced in 125 of these trials, with the remaining trials showing only increased tonic nerve activity, bilaterally. Bilateral rhythmicity/locomotion was induced in 120 of 125 trials, with the remaining five trials showing only one-sided locomotion: four with locomotion ipsilateral to the CFME recording electrode and 1 with contralateral locomotion. Latencies from the start of stimulation to the onset of ipsilateral and contralateral locomotion were 16.1 ± 11.8 and 16.8 ± 13.1 s (mean ± SD), respectively, with a range of 2.7–52.5 s. Longer locomotor onset latencies were observed toward the end of the experiments in two animals and in one animal from the beginning. Locomotion could be preceded by the appearance of tonic bursting activity as is seen in other studies of MLR-evoked fictive locomotion (e.g., Noga et al., 1995a; MacDonell et al., 2015).

Spatial Patterns of Spinal Monoamine Release with MLR Stimulation

Changes in extracellular concentrations of monoamines were measured at various locations throughout gray and WM in L4–L6 lumbar segments during MLR evoked fictive locomotion (**Figures 3**, **4**). Levels were measured relative to baseline levels which reflect the dynamic equilibrium between release and uptake during non-locomotor steady state resting conditions. Transmitter profiles most typically observed within the dorsal horn (DH), intermediate zone (IZ)/VH and adjacent WM during evoked locomotion are illustrated in **Figure 3**. The first major response to MLR stimulation was most commonly an increase in release. This profile was observed in 89.4% and 78.1% of all trials (all locations) for NE and 5-HT, respectively. The MLR-evoked increase in NE or 5-HT levels gradually declined once stimulation was terminated, or before, if locomotor activity diminished during stimulation. In remaining trials, monoamine levels decreased below resting levels with stimulation. Such profiles of release were highly localized since neighboring areas mostly showed increases in transmitter release in subsequent trials. The profile of release (relative incidence of increased or decreased release with stimulation) differed significantly between NE and 5-HT (McNemar test; $p = 0.035$). An initial release of NE occurred in 90.0%, 89.3% and 88.9% of trials in DH, IZ/VH and adjacent WM, with no significant difference between locations (**Figure 5A**). The relative frequency of increased release of 5-HT in DH, IZ/VH and adjacent WM was 82.5%, 85.7% and 63.9%, respectively; these locations differed significantly ($p = 0.035$; Pearson Chi-Square test).

The extracellular levels of NE and 5-HT could vary in a complex way relative to each other during MLR stimulation (**Figure 4**), however, and especially if the induction of locomotor activity was somewhat delayed. For example, in lamina IX, the (delayed) onset of locomotor activity was associated with an offset of NE release and the onset of 5-HT release in lamina IX. This pattern of 5-HT release within lamina IX observed in this example indicates that 5-HT release covaries with motoneuron depolarization. This was further corroborated in other recordings from lamina IX. The timing of 5-HT release in lamina IX was related to onset of motoneuron discharge (bursting or locomotion) in all of the nine recorded trials with stable baselines (five examples are shown in **Figures 3**, **4**). Although there are differences in the total amount released from trial to trial, this seems to be related to the length of time for locomotor activity to be initiated. The increase was largest (45–200 nM) in trials with delayed locomotion (>14 s; 6/9 trials) and smallest (16–18 nM) in trials with faster initiated locomotion (≤10 s; 3/9 trials). In contrast, in the lateral portion of lamina VII, the onset of locomotor activity was associated with stabilization and ultimately with dramatic increases in both NE and 5-HT when locomotion was finally observed. Other complex waveforms are illustrated in **Figure 4**. In lamina X, the initial decrease in extracellular 5-HT with MLR stimulation was reversed with the appearance of ipsilateral locomotor activity. In the same trial, NE release occurred shortly after commencement of MLR stimulation and prior to the onset of ipsilateral locomotor activity. In another example, NE increased after MLR stimulation and dropped to a lower and eventually more stable level as 5-HT levels increased in the DH (laminae V/VI).

Temporal Pattern of Spinal Monoamine Release with MLR Stimulation

Onset latencies for initial changes in NE or 5-HT level were similar for trials showing increased or decreased release and were therefore grouped together.

Latency Relative to Onset of MLR Stimulation

Overall, changes in the extracellular concentration of NE ($NEonset_{MLR}$) and 5-HT ($5\text{-}HTonset_{MLR}$) began 8.3 ± 7.3 s and 10.1 ± 9.1 s (mean ± SD; unless otherwise noted)

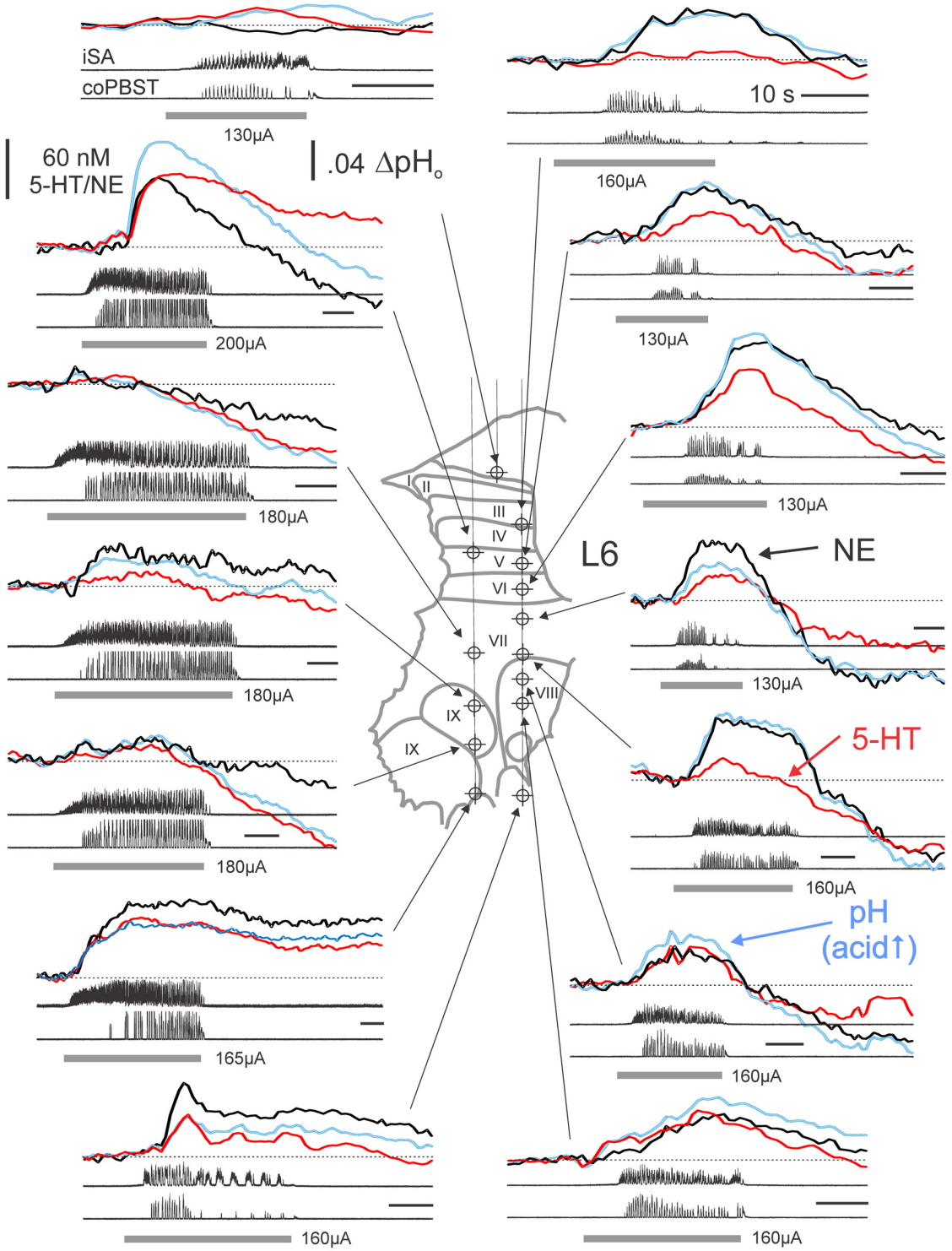

FIGURE 3 | Monoamine release profiles generated at various locations within the L6 spinal segment from a single experiment. Monoamine release profiles determined from PCR analysis of subtracted voltammograms are plotted with respect to the time of occurrence during separate trials of MLR-evoked fictive locomotion. Monoamine concentrations and pH measures reflect relative changes from baseline, non-locomotor (at rest) levels obtained at each site immediately prior to each trial. NE, 5-HT and pH signals are indicated by thick *black*, *red* and *blue* lines, respectively. Recording sites indicated on histological reconstruction of the electrode tracks observed in spinal sections create a "map" of monoamine release. Locomotor activity is observed in ENG recordings from single ipsilateral and contralateral nerves for each trial of MLR-evoked fictive locomotion. *Gray bars* indicate the period of MLR stimulation (ipsilateral to FCV recording; 130–200 μA, 15 Hz, 1 ms duration; strength indicated for each trial). Calibration bars: 10 s. SA: sartorius; PBST: posterior biceps/semitendinosus; i: ipsilateral; co: contralateral.

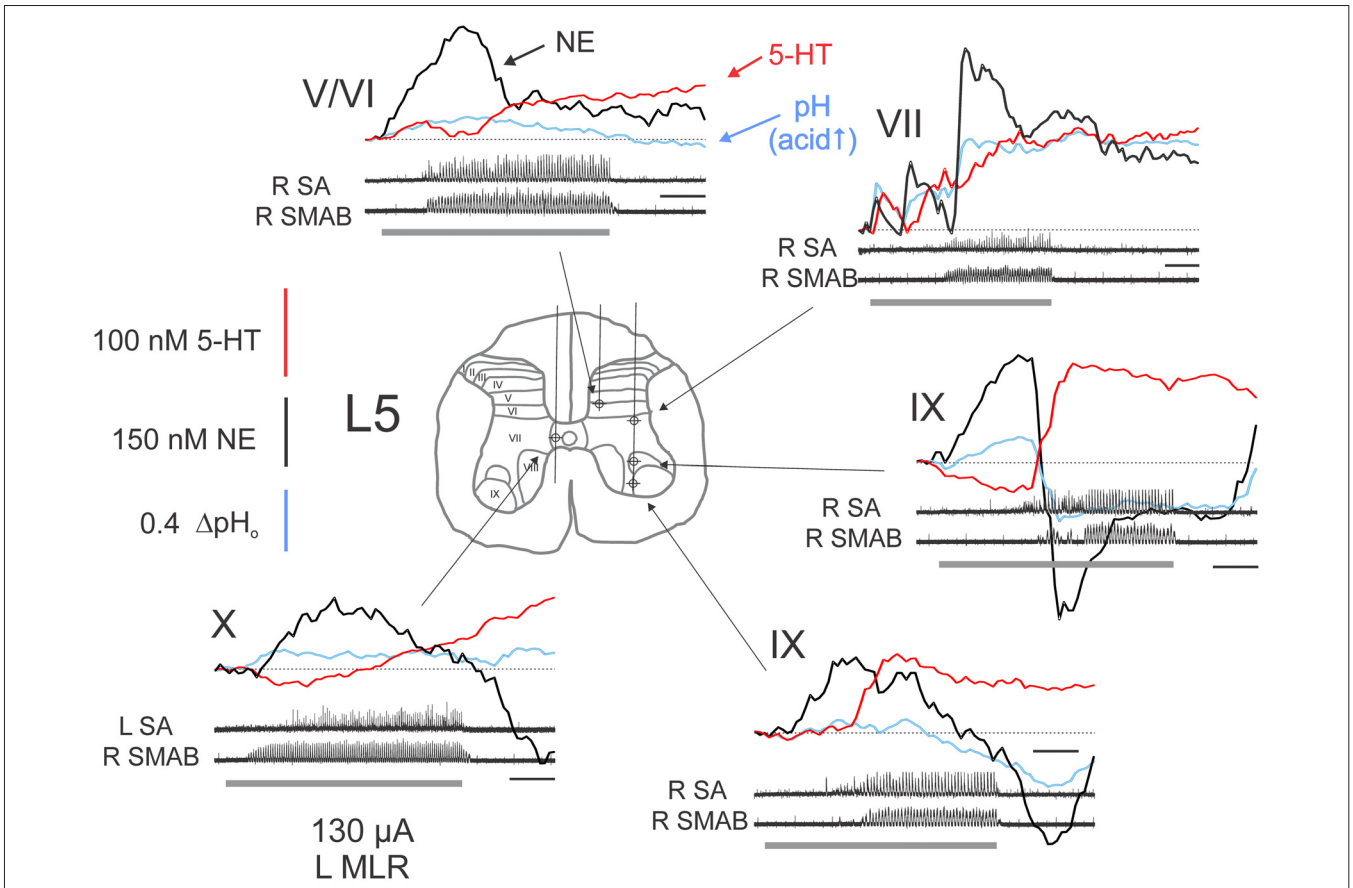

FIGURE 4 | Complex monoamine release profiles observed during trials with delayed MLR-evoked locomotion. Profiles recorded at various locations within the L5 spinal segment from a single experiment. *Gray bars* indicate the period of MLR stimulation (left side, 130 µA throughout, 20 Hz, 1 ms duration). Locomotor activity is indicated by ENG recordings from right (R) and left (L) nerves. Calibration bars: 10 s. SMAB: semimembranosus/anterior biceps. Figure format and abbreviations as in **Figure 3**.

after the start of MLR stimulation. There was a significant difference between locations in DH, IZ/VH and adjacent WM for NEonset$_{MLR}$ latencies (one-way analysis of variance (ANOVA); $F = 3.544$, $p = 0.032$). *Post hoc* testing showed NEonset$_{MLR}$ latency to be significantly shorter ($p = 0.05$, Tukey test) in the IZ/VH (6.3 ± 3.3 s, $n = 56$) than in the DH (9.9 ± 10.3 s, $n = 40$; **Figure 5B**) but not in the WM (9.5 ± 7.5 s, $n = 36$). 5-HTonset$_{MLR}$ latencies in the DH, IZ/VH and WM (9.9 ± 8.5 s, 9.8 ± 9.9 s, 11.0 ± 8.7 s, respectively) did not show significant differences by ANOVA. Since we observed delays in the onset of 5-HT release in some locations within the IZ/VH (e.g., lamina IX—**Figure 4**), we did a paired samples *t*-test comparing NEonset$_{MLR}$ latency and 5-HTonset$_{MLR}$ latency in the IZ/VH. The IZ/VH onset latency for NE was significantly shorter ($p = 0.010$, $t = -2.665$).

Latency Relative to Onset of Bursting Activity

MLR stimulation could evoke tonic activity prior to the appearance of locomotion in the ENGs (**Figure 3**). In this analysis, we measured the latency of release relative to the appearance of any kind of ENG activity (tonic or phasic). Overall, MLR-evoked changes in the extracellular concentrations of NE and 5-HT over baseline levels began before the onset of bursting activity (NEonset$_{burst}$ and 5-HTonset$_{burst}$) in 36.4% and 32.6% of trials, respectively. The frequency of occurrence did not statistically differ for recording trials obtained within the DH, IZ/VH and adjacent WM for either NE or 5-HT (**Figure 5C**) and there were no statistical differences in the frequency of occurrence between NE and 5-HT. However, NEonset$_{burst}$ latencies observed in DH, IZ/VH and WM, revealed an overall significant difference between locations (one-way ANOVA; $F = 3.156$, $p = 0.046$). With *post hoc* testing (Tukey), this was attributable to a significant difference ($p = 0.043$) in the NEonset$_{burst}$ latency in the IZ/VH (−2.9 ± 11.7 s; $n = 56$) compared to the DH, indicating that NE release in the IZ/VH precedes the onset of burst activity whereas in the DH, NE release was observed to occur after the onset of burst activity (2.8 ± 12.8 s; $n = 40$; **Figure 5D**). No significant difference was noted with *post hoc* testing for NEonset$_{burst}$ latency in the IZ/VH or DH compared to the adjacent WM (0.8 ± 8.0 s; $n = 36$). The overall ANOVA did not show significant differences in 5-HTonset$_{burst}$ latencies in the DH, IZ/VH and adjacent WM (2.8 ± 10.2 s, 0.6 ± 13.0 s

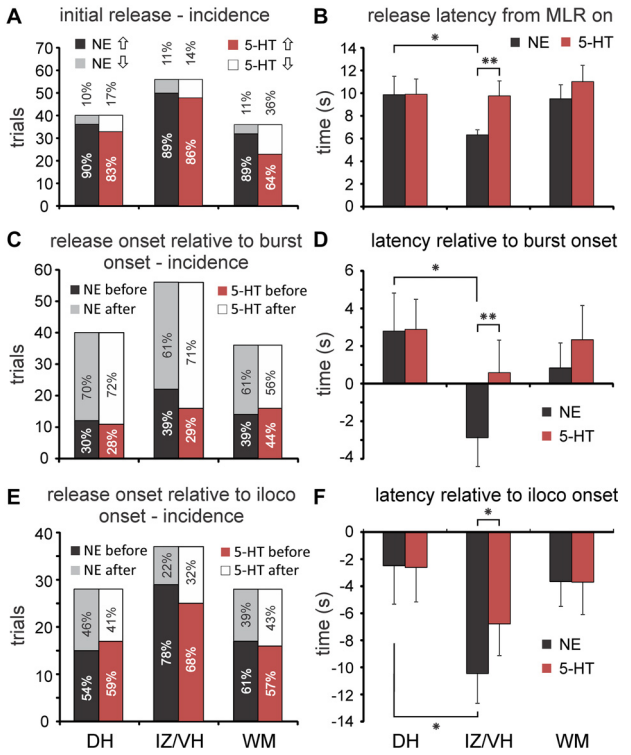

FIGURE 5 | Incidence and latencies of monoamine transmitter release during MLR-evoked fictive locomotion. **(A)** Profiles of initial changes in 5-HT and NE concentrations (incidence) in dorsal horn (DH), intermediate zone/ventral horn (IZ/VH) and in the adjacent white matter (WM) of middle and caudal lumbar segments with stimulation of the MLR. **(B)** Onset latencies of monoamine release (mean and SEM in **B**,**D**,**F**) relative to onset of MLR stimulation. NE onset latencies in the IZ/VH were significantly shorter compared to those observed in the DH and for those observed for 5-HT in the IZ/VH. **(C)** Timing of changes in release in DH, IZ/VH, and WM relative to the onset of burst activity in hindlimb ENGs. Percentages of occurrence (incidences) are given. **(D)** Onset latencies of monoamine release relative to onset of burst activity. NE onset latencies in the IZ/VH were significantly earlier compared to those observed in the DH and for those observed for 5-HT in the IZ/VH. **(E)** Timing of changes in release in DH, IZ/VH, and WM relative to the onset of ipsilateral locomotion. Percentages of occurrence (incidences) are given. Note that in all locations, changes in monoamine release usually occurred before the onset of locomotion. **(F)** Onset latencies of monoamine release relative to onset of ipsilateral locomotion. NE onset latencies in the IZ/VH were significantly earlier compared to those observed in the DH and for those observed for 5-HT in the IZ/VH. $^{*}p < 0.05$, $^{**}p < 0.01$.

and 2.3 ± 10.9 s, respectively). However, a paired samples *t*-test comparing $\text{NEonset}_{\text{burst}}$ and $\text{5-HTonset}_{\text{burst}}$ latency in the IZ/VH revealed that NE release measured relative to the burst onset, was significantly earlier than 5-HT ($p = 0.010$, $t = -2.665$).

Latency Relative to Onset of Ipsilateral Locomotion

Overall, MLR-evoked changes in the extracellular concentrations of NE and 5-HT over baseline levels began before the onset of ipsilateral locomotion ($\text{NEonset}_{\text{iloco}}$ and $\text{5-HTonset}_{\text{iloco}}$) in the majority of trials (65.6% and 62.4% of trials for NE and 5-HT, respectively). The frequency of occurrence (before or after onset of locomotion) did not statistically differ for recording trials obtained within the DH, IZ/VH and adjacent WM for either NE or 5-HT (**Figure 5E**) and there were no statistical differences in the frequency of occurrence between NE and 5-HT. Overall, for all trials and sites, the onset of NE and 5-HT release preceded the onset of ipsilateral locomotion: −6.0 ± 13.4 s and −4.6 ± 13.6 s for NE and 5-HT, respectively. $\text{NEonset}_{\text{iloco}}$ latencies observed in DH, IZ/VH and WM for trials with best locomotor responses to MLR stimulation, revealed an overall significant difference between locations (one-way ANOVA; $F = 3.641$, $p = 0.030$). *Post hoc* testing showed that $\text{NEonset}_{\text{iloco}}$ was significantly shorter ($p = 0.04$, Tukey) in the IZ/VH (−10.5 ± 13.5 s, $n = 37$) than in the DH (−2.5 ± 15.1 s, $n = 28$; **Figure 5F**), but not in the adjacent WM (−3.6 ± 9.8 s, $n = 28$). ANOVA showed no significant differences in $\text{5-HTonset}_{\text{iloco}}$ latencies in the DH, IZ/VH and adjacent WM (−2.6 ± 13.5 s, −6.8 ± 14.3 s, −3.7 ± 12.7 s, respectively). As before, a paired samples *t*-test showed that $\text{NEonset}_{\text{iloco}}$ latency was significantly shorter than $\text{5-HTonset}_{\text{iloco}}$ latency in the IZ/VH ($p = 0.013$, $t = -2.599$). Such differences in the latencies of release of NE and 5-HT within the IZ/VH were most noticeable in cases where locomotor activity is delayed relative to the onset of MLR stimulation (**Figure 4**).

Time-To-Peak (TTP)

Monoamine levels in gray and WM reached their peak or nadir values (referred to as the TTP) before termination of MLR stimulation in the majority of trials (78.0% and 65.9% of trials for NE and 5-HT, respectively; **Figures 3**, **4**). This often occurred when locomotor activity waned. Interestingly, NE levels peaked before the end of MLR stimulation significantly more often than 5-HT (McNemar test; $p = 0.018$). Of remaining trials, transmitter levels usually peaked soon after termination of MLR stimulation (12.9% and 16.7% of trials for NE and 5-HT, respectively) and less frequently, continued to increase (or decrease) past the monitoring period (see below). The percentage of trials with continuing release or uptake of NE amounted to 9.1% of the total number of trials (7.6% with increasing levels and 1.5% with decreasing levels). For 5-HT, this amounted to 17.4% of the total number of trials (12.9% with increasing levels and 4.5% with decreasing levels). Such trials were observed in both gray and WM. These results indicate that monoamine levels typically reach their peak (or nadir) in relation to stimulus-evoked locomotor activity and that continued increases in release/uptake past the monitoring period following termination of stimulation and locomotion are relatively rare.

Time-To-Decline-to-Baseline (D_{50} and D_{100})

Transmitter levels were continuously monitored for periods of 20–80 s of the termination of MLR stimulation. Periods of altered release usually outlasted the locomotor bouts and the decay of signal could be tens of seconds. By the end of the monitoring period, NE concentrations returned to baseline levels (D_{100}) in 90 of 132 trials (68.2%) and at least 50% of baseline levels (D_{50}) in 15 more (11.4%). On average, the D_{50} and D_{100} in these trials was 12.9 s ± 9.8 ($n = 105$) and 23.7 s ± 17.6 s ($n = 90$), respectively and did not significantly differ whether NE levels increased or decreased with stimulation. In an additional 15 trials (11.4%), NE levels

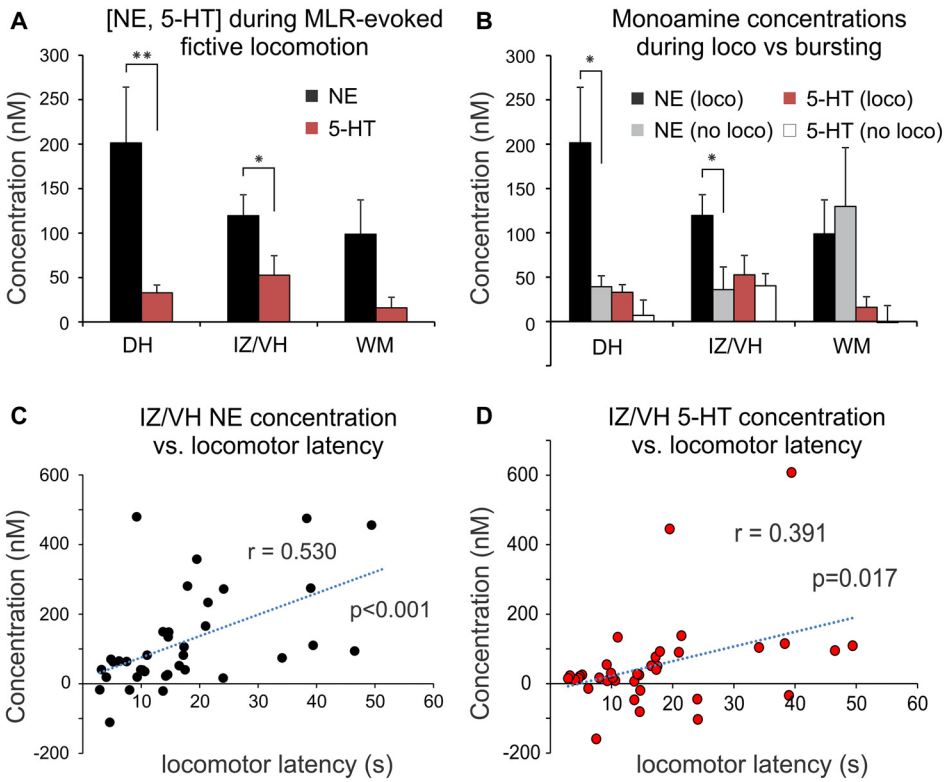

FIGURE 6 | Change in monoaminergic extracellular concentration during MLR-evoked fictive locomotion. **(A)** Peak NE and 5-HT release during MLR-evoked fictive locomotion in DH, IZ/VH and adjacent WM (mean ± SEM). Note that NE release was significantly greater than 5-HT in the gray matter. **(B)** Comparison of peak monoamine concentrations observed with MLR evoked locomotion (loco) vs. no locomotion (bursting) in DH, IZ/VH and adjacent WM. Note that in the DH and IZ/VH, a significantly greater release of NE over baseline was observed in trials with locomotor activity. **(C,D)** Release of NE and 5-HT within the IZ/VH was significantly correlated to the time required to elicit locomotion with MLR stimulation. $^*p < 0.05$, $^{**}p < 0.01$.

were returning to baseline levels but had not yet reached D_{50}. The remaining 9.1% of trials showed increasing or decreasing NE levels [see "Time-To-Peak (TTP)" Section, above]. For 5-HT, extracellular concentrations had returned to baseline levels by the end of the monitoring period in 73 of 132 trials (55.3%) and to at least 50% of baseline in 10 more (7.6%). On average, the D_{50} and D_{100} in these trials was 11.7 s ± 11.6 (n = 83) and 18.2 s ± 14.1 s (n = 73), respectively and did not significantly differ whether 5-HT levels increased or decreased with stimulation. In an additional 26 trials (19.7%), 5-HT levels were returning to baseline but had not yet reached D_{50}. The remaining 17.4% of trials showed increasing or decreasing 5-HT levels [see "Time-To-Peak (TTP)" Section, above].

Spinal NE and 5-HT Concentration Changes during MLR Evoked Fictive Locomotion

As described above (see "Spatial Patterns of Spinal Monoamine Release with MLR Stimulation" Section), spinal extracellular monoamine concentrations increased above baseline levels more often than decreased in response to MLR stimulation (**Figure 5A**) and transmitter levels usually peaked during evoked locomotion or shortly after. Overall, for all trials and sites (n = 93), NE and 5-HT transmitter concentrations increased above baseline levels during MLR-evoked fictive locomotion by 138.0 ± 232.5 (range: −558.0 to 1271 nM) and 35.6 ± 94.4 nM (range: −159.6 to 607.6 nM), respectively. Since direct stimulation of the locus ceruleus (LC) and raphe magnus in the rat (Hentall et al., 2003, 2006) results in a significantly higher average spinal release of NE than 5-HT, we compared concentrations of spinal 5-HT and NE release during MLR-evoked locomotion. The peak NE and 5-HT concentrations were highly significantly different from each other ($p < 0.001$, t = 4.097). NE concentrations observed in DH, IZ/VH and adjacent WM (**Figure 6A**) were 201.5 ± 331.2, 119.6 ± 142.4 and 98.8 ± 232.5 nM, respectively, and were not significantly different (oneway ANOVA). Similarly, there was no overall differences in 5-HT concentration in the DH, IZ/VH and adjacent WM (32.8 ± 46.1, 52.7 ± 132.5, 15.9 ± 63.3 nM, respectively). However, paired samples T-test statistics revealed significant differences between NE and 5-HT in the DH (p = 0.010, t = 2.787) and IZ/VH (p = 0.020, t = 2.425) but not in the adjacent WM. Comparing trials with increased release only, NE concentrations averaged 218.8 ± 337.8 (n = 26), 139.1 ± 137.8 (n = 33) and 136.0 ± 159.2 (n = 25) nM

for DH, IZ/VH and adjacent WM, respectively. Likewise, in trials with increased 5-HT release, concentrations averaged 45.2 ± 35.6 (n = 24), 84.6 ± 130.8 (n = 29) and 50.4 ± 54.3 (n = 17) nM for DH, IZ/VH and adjacent WM, respectively. No overall differences (oneway ANOVA) were observed for NE in the DH, IZ/VH and adjacent WM concentration and no differences were observed in these locations for 5-HT concentration.

We also examined whether spinal monoamine concentrations differed with the type of MLR evoked activity (**Figure 6B**). In trials recording from the DH, NE concentration increases were significantly greater (p = 0.017, t = −2.543) during locomotor (201.5 ± 331.2 nM; n = 28) compared to non-locomotor (tonic or phasic excitation lacking reciprocal bursting in antagonists) activity (39.3 ± 42.8 nM; n = 12). Similarly, in the IZ/VH, a significantly greater concentration increase of NE was observed (p = 0.020, t = −2.414) during locomotor (119.6 ± 142.4 nM; n = 37) compared to non-locomotor activity (36.0 ± 111.3 nM; n = 19). In contrast, although the mean 5-HT concentration increases were greater during locomotion than with non-locomotor activity in both DH [32.7 ± 46.1 nM (n = 28) vs. 6.8 ± 60.6 nM (n = 12)] and IZ/VH [52.7 ± 132.5 nM (n = 37) vs. 40.2 ± 59.4 nM (n = 19)], they were not statistically significant. Furthermore, no statistical differences in trials of locomotor (n = 28) or non-locomotor activity (n = 8) were observed for NE nor for 5-HT release in the adjacent WM. Lastly, in trials recording from the IZ/VH, the concentration of released NE and 5-HT was significantly correlated (Pearson correlation: r = 0.530, p < 0.001 and r = 0.391, p = 0.017, respectively) to the time required to elicit locomotion (**Figures 6C,D**) suggesting that delays in the initiation of locomotion may, in part, be related to the time required to increase monoamine concentrations to appropriate levels. A similar correlation was also noted for NE in the DH (r = 0.577, p = 0.001) but not for 5-HT. No such correlations were observed for NE and 5-HT release measured in the adjacent WM.

Reproducibility of Trials and Relationship of Release to Locomotor Intensity/Amplitude

Repeated measurements were made at various spinal locations (n = 6) in two animals to assess the reproducibility of transmitter release during trials of MLR evoked locomotion. This is illustrated for two such examples in **Figure 7**. In **Figure 7A**, the release of both NE and 5-HT above baseline values in laminae VI increased in tandem with an increased intensity of the ipsilateral flexor ENG as measured by burst area. In this example, the interlimb coordination was also altered. A similar modulation was observed in two additional tested sites. In lamina V, NE release was reduced (from 55.3 nM to 44.8 nM) and 5-HT unchanged (24.2–23.9 nM) in a repeated trial in which the burst area of the ipsilateral extensor was decreased. Additionally, in a trial from lamina VI, 5-HT release was dcreased (from 51.1 nM to 33.8 nM) and NE was relatively unchanged (44.9–43.3 nM) in a repeated trial in which locomotor activity illustrated a reduction in the burst intensity of the ipsilateral flexor. In tests where repeated trials showed comparable locomotor quality and interlimb coordination, the quantity of release was also comparable. This is shown for one such trial from lamina VII (**Figure 7B**) but was also observed in two additional trials in laminae VII and VIII.

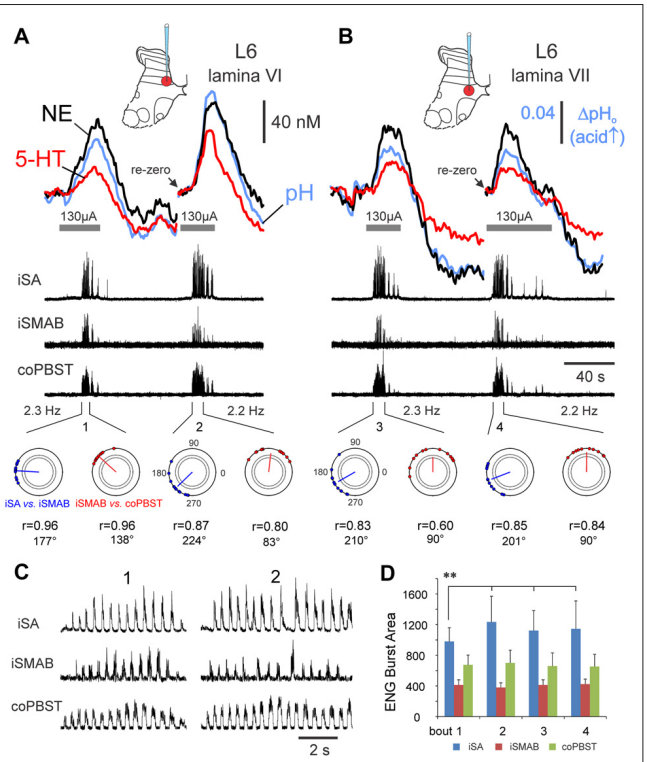

FIGURE 7 | Monoamine release within the spinal cord is modulated in tandem with locomotor activity. Changes in monoamine transmitter and pH levels during repeated trials of MLR-evoked fictive locomotion recorded in medial lamina VI **(A)** and VII **(B)** of the L6 lumbar segment. Release is reproducible when locomotor quality is similar in repeated trials at the same location, as gauged by locomotor frequency, ENG burst area and timing profiles (polar plots—bottom row in **(A,B)**): see similarity in peak concentrations and release profiles for repeated trials in lamina VII **(B)**. In contrast, when locomotor quality differs in repeated trials as in **(A)**, the peak concentrations of both NE and 5-HT show positive correlation to the burst area of the ipsilateral flexor nerve **(D)**, rather than to locomotor frequency, which does not significantly differ throughout trials (values listed at *bottom* in **(A,B)**). Locomotion is indicated by the recorded ENG activity in *middle traces in* **(A,B)** from various peripheral nerves and in expanded form in **(C)** for bouts 1 and 2. **(D)** ENG locomotor burst area (mean and ± SD, in arbitrary units) for each nerve from bouts 1–4 (n = 12, 18, 20 and 18 steps for bouts 1–4, respectively). Note that the averaged locomotor burst area of the iSA in bout 1 was significantly different (**p < 0.01) than for all other bouts. *Horizontal lines* beneath the oxidation current waveform show the period of MLR stimulation (130 μA, 15 Hz, 1 ms duration). Polar plots (*bottom* in **A**,**B**) showing the circular distribution of phase values of 9–12 random steps (*filled circles*) from each bout of locomotion (1–4) and the mean phase values indicated by the angle of the vector originating from the center of the circle. The position on the polar plot indicates the latency of onset of activity for iSA vs. the iSMAB ENGs (*blue*) and for iSMAB vs. contralateral coPBSt ENGs (red). The length of the vector (r) ranged from 0.6 to 0.96 and indicated that all nerves were phase-related since the r values were greater than the critical Rayleigh's values (dotted circles inside of the polar plot with p values of <0.05 and <0.01). A slight shift in phase value occurred between bouts 1 and 2.

Extracellular pH Changes during Evoked Locomotion

Local acid changes occur in response to neuronal activity, related to respiratory demands (Chesler and Kaila, 1992; Chesler, 2003) and have been observed in the spinal gray matter during periods of electrical stimulation (Syková and Svoboda, 1990). Similarly, stimulation of the MLR produced a variety of pH changes within the spinal cord, beginning at the same time or slightly earlier to any detectable changes in extracellular monoamine transmitter levels. MLR stimulation produced an initial large acid shift in 102 (77.3%) of 132 trials (**Figure 3**) which typically lasted the duration of the stimulus. A small number of these trials ($n = 12$) were preceded by a smaller transient base shift (Syková and Svoboda, 1990). In the remaining 30 trials (22.7%), the first response to MLR stimulation was a large base shift. The mean onset latency of acidic changes (with no initial base transient) was 6.3 ± 6.4 s, with a TTP (from onset) of 19.4 ± 18.0 s and a peak of 0.04 ± 0.04 pH units. Small base transients had an onset latency of 5.0 ± 3.2 s, and a TTP of 2.5 ± 1.3 s and a peak of 0.007 ± 0.004 pH units. Consequently, in trials with base transients, acid shifts were delayed with an onset latency of 11.6 ± 6.3 s, a TTP of 43.7 ± 34.4 s and a peak change of 0.03 ± 0.03 pH units. The mean onset latency of large base shifts in trials without preceding acid changes was 8.7 ± 8.2 s, with a TTP of 34.4 ± 26.6 s and a peak change of 0.04 ± 0.05 pH units. Such large initial base changes could often be observed in WM rather than gray. Post-stimulation alkaline shifts were also commonly observed (in 59% of trials) after the initial acidic shifts (Syková and Svoboda, 1990). In contrast, secondary acid shifts were observed in only 30% of trials with initial large base shifts.

Effect of Monoamine Oxidase and Uptake Inhibitors

The effect of monoamine oxidase and uptake inhibitors on MLR-evoked monoamine release was examined in three animals. This is illustrated in **Figure 8** for a trial conducted during dialysis of a mixture of 0.5 mM pargyline and 0.1 mM desipramine using two probes separated by 15 mm, with the CFME recording site located between the probes within lamina VIII (L5 segment). In this trial, monoamine release was maintained over the entire stimulation period lasting 600 s (illustrated is a 240 s period occurring between 270 s and 510 s). NE and 5-HT levels continued to increase following the termination of MLR stimulation to peak ∼2.5 and 1.5 min post stimulation, respectively. By 22 min, concentrations of both transmitters had declined to ∼50% of peak values. Peak concentrations were 685 and 304 nM for NE and 5-HT, respectively. During periods of stable locomotion, NE and 5-HT levels were reasonably steady. However, NE and 5-HT release increased at a much higher rate just prior to a period of variable locomotion which was characterized by shifts in locomotor frequency and changes in extensor/flexor burst area and burst duration (duty cycle). Monoamine levels later steadied once locomotor parameters stabilized. Repeated trials at the same location produced similarly high levels of NE (590–650 nM), 5-HT (∼470 nM) and a prolonged TTP (∼2 min post stimulation) with D_{50} values between 10 and 20 min. A similar trial conducted in lamina VIII of the L4 segment resulted in comparably lower levels of NE and 5-HT release (375 and 68 nM, respectively), but a prolonged TTP (∼13 min post stimulation) was observed (D_{50} and D_{100} not measured). In two other animals, mixtures of the monoamine oxidase inhibitor pargyline (1.3 mM) and the re-uptake inhibitors imipramine (1.5 mM), desipramine (1.6 mM) and buproprion (1.8 mM) were slowly microinjected into the spinal gray matter along each length of one or two adjacent electrode tracks (0.5 mm apart in rostral/caudal direction). Fifteen minutes after microinjection, MLR stimulation trials were commenced. An increase in the extracellular levels of NE (540–900 nM) and 5-HT (up to 250 nM) was observed at sites 1–3 mm distant to the injection sites (laminae VII and VIII of the L4 segment and lamina VIII of the L5 segment; see "Materials and Methods" Section). Levels increased after termination of the MLR stimulus and peaked ∼9 min later. In general, these results indicate that monoamine release is enhanced and prolonged by inhibition of monoamine uptake and metabolism.

DISCUSSION

General Observations

Changes in the extracellular concentrations of NE and 5-HT were measured at various locations throughout middle and lower lumbar spinal segments during MLR evoked fictive locomotion in the mesencephalic decerebrate cat. Levels were measured relative to the non-locomotor steady state. An increase in the extracellular concentration of NE and 5-HT was most commonly observed during evoked locomotion. Release was widespread: in the IZ/VH, in areas with centrally-activated locomotor neurons (Dai et al., 2005; Noga et al., 2009, 2011); in the DH, where additional neurons are activated by sensory feedback during over-ground locomotion (Dai et al., 2005); and in the WM, likely reflecting, through the process of diffusion, changes in the dynamic equilibrium between release and uptake in adjacent gray matter. Release was rapid on the timescale of seconds and usually began prior to the onset of locomotion, being earliest in the IZ/VH. Transmitter levels were sufficiently high to activate most monoaminergic receptor subtypes expressed by locomotor-activated neurons (Noga et al., 2009, 2011) supporting the idea that extrasynaptic neurotransmission in the spinal cord (Ridet et al., 1993) may be involved in spinal locomotor processes. Highest levels were usually observed during the most vigorous period of locomotion, covaried with net activity in peripheral nerves and were correlated with delays in the initiation of locomotion. NE release was significantly greater in the presence, than absence, of locomotion. Serotonin release within lamina IX covaried with the appearance of ENG locomotor activity (motoneuron firing). Monoamine oxidase and uptake inhibitors also increased the release magnitude, time to peak and time of decline to baseline, implying dynamic control of reuptake. These results demonstrate that spinal monoamine release is modulated

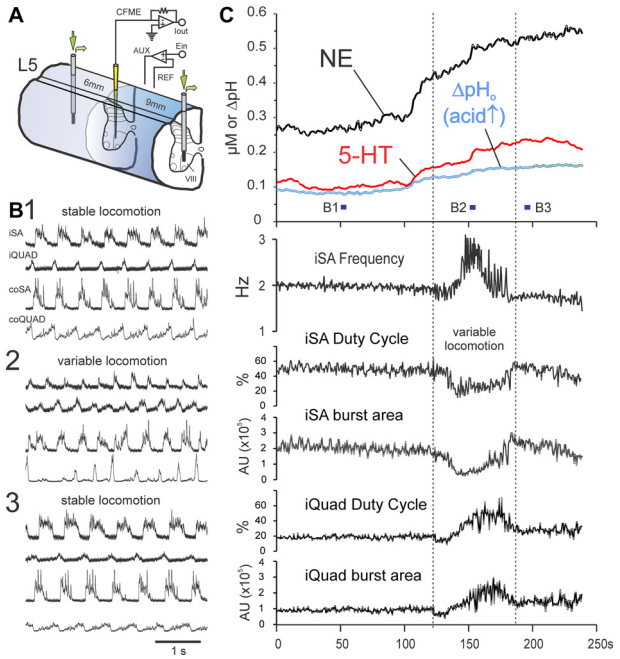

FIGURE 8 | MLR-evoked spinal monoamine release is enhanced by monoamine metabolic and uptake inhibitors and shows modulation during locomotor transition periods. Release of NE and 5-HT in lamina VIII of the L5 segment **(C)** during dialysis of metabolic and uptake inhibitors pargyline (0.5 mM) and desipramine (0.1 mM) through dialysis probes placed 6 and 9 mm distant to the voltammetric electrode (experimental setup illustrated in **(A)**). Trial illustrated shows a 4 min portion, between 4.5 min and 8.5 min, of a 10 min locomotion trial. Locomotor activity for indicated periods in **(C)** are shown in **(B,B1,2,3)**. At beginning of the illustrated sequence (as represented by locomotor period **B1**), extracellular levels of NE and 5-HT are already high (~275 and 100 nM, respectively) but are reasonably steady while locomotion parameters (frequency, duty cycle and burst area) remain stable **(C)**. Transmitter levels dramatically change just prior to a period of variable locomotion (represented in **B2**) with changes in locomotor frequency and extensor/flexor burst area and burst duration (duty cycle). Following this period, monoamine levels regain a steady-state and locomotion is stabilized **(B3)**. AU: arbitrary units. MLR stimulation parameters: 16 Hz, 160 µA, 1 ms duration pulses.

on a timescale of seconds, in tandem with centrally-generated locomotion and indicate that MLR-evoked locomotor activity involves concurrent activation of descending monoaminergic and reticulospinal pathways.

Methodological Considerations/Interpretation of *In Vivo* Voltammetric Findings

By removing background currents that mask faradic (redox) currents (Phillips et al., 2002; Brumley et al., 2007) and applying PCR analysis (Heien et al., 2004; Keithley et al., 2009), we were able to determine changes in the extracellular levels of 5-HT and NE despite the fact that monoaminergic terminal fields are mostly mixed in the spinal cord (Fleetwood-Walker and Coote, 1981; Huisman et al., 1981; Fuxe et al., 1990; Clark and Proudfit, 1991a,b; Doyle and Maxwell, 1991; Fields et al., 1995; Alvarez et al., 1998). Faradic signals were unlikely due to transmitter metabolites and other contaminants since our electrodes were relatively insensitive to them (Hentall et al., 2003). The rapid appearance of the redox signal during evoked locomotion and its usually rapid removal following termination of stimulation (**Figures 3**, **7**) and the fact that the release is enhanced and prolonged by inhibition of monoamine uptake and metabolism (**Figure 8**) indicates that the signal is due to monoamine overflow rather than their metabolites which take minutes to appear (Michael et al., 1985; Michael and Wightman, 1999). Nafion coating also improves selectivity for the primary amines by reducing sensitivity to monoaminergic metabolites and ascorbic acid (Kuhr and Wightman, 1986; Brazell et al., 1987; Jackson et al., 1995).

Although catecholamines are difficult to differentiate from each other using FCV (Phillips et al., 2002), we maintain that the major component detected in the present experiments is NE, rather than DA: (1) most spinal laminae are densely innervated by descending noradrenergic fibers (Clark and Proudfit, 1991a,b; Grzanna and Fritschy, 1991; Rajaofetra et al., 1992). This is in contrast to their dopaminergic innervation (Skagerberg et al., 1982; Ridet et al., 1992; Holstege et al., 1996). Furthermore, the concentration of DA in the spinal cord is much lower than that of NE (Basbaum et al., 1987); (2) the A11 cell group, the principle source of descending dopaminergic pathways to the spinal cord (Björklund and Skagerberg, 1979; Skagerberg et al., 1982), is absent in the precollicular, postmammillary decerebrate preparation, which spares the A5, A6 (LC) and A7 (Kölliker-Fuse) nuclear groups supplying the noradrenergic innervation of the spinal cord (Westlund et al., 1982); (3) the short stimulus pulse (1 ms) width utilized in the present experiments is more effective for the activation of NE, than DA release (Park et al., 2011); and (4) there are no dopaminergic cell bodies in the lumbar spinal cord (Mouchet et al., 1986; Ridet et al., 1992; Holstege et al., 1996).

Sites of Release Relative to Location of Locomotor Activated Neurons

In the present series of experiments, monoamine release was examined in mid-to-caudal lumbar segments, where centrally-activated, hindlimb locomotor neurons are most numerous (Dai et al., 2005; Noga et al., 2009, 2011). Stimulation of the MLR evokes the largest dorsal surface potentials in these segments (Noga et al., 1995a), reflecting the depolarization of centrally-activated spinal neurons in laminae VI-X (Jankowska and Noga, 1990; Carr et al., 1994, 1995; Noga et al., 1995a; Huang et al., 2000; Matsuyama et al., 2004). Application of noradrenergic drugs induces walking in spinally injured cats when applied to this area (Chau et al., 1998; Marcoux and Rossignol, 2000; Giroux et al., 2001), indicating that this area contains elements important for the initiation of locomotion. Monoamine release was detected in all areas of the spinal gray matter: DH, IZ and VH. Release in the IZ and VH is likely important in modulating the activity of neurons responsible for the central generation of locomotion. Locomotor activated neurons in these laminae are innervated by descending monoaminergic nerve fibers and possess both serotonergic and noradrenergic receptors implicated in the

production of locomotion (Noga et al., 2009, 2011). Release in the DH is likely important for the modulation of additional control elements activated by reflex inputs during the step cycle as seen in treadmill locomotion studies (Dai et al., 2005). Extracellular levels of monoamines are thus dynamically regulated during transitions from non-locomotor to locomotor states and throughout locomotor activity so as to affect the activation of specific subpopulations that produce locomotion and suppress those irrelevant or deleterious to the movement (Harris-Warrick, 1988).

Comparisons to Previous Studies of Release during Voluntary Locomotion

Spinal monoamine release during treadmill locomotion in the rat has been measured previously by extraction techniques (microdialysis). The temporal resolution in these studies (15 min/sample) necessitates long duration locomotor episodes, resulting in spatial resolutions greater than a millimeter in the diffusional environment of the CNS and increased likelihood of uptake saturation (Lu et al., 1998; Peters and Michael, 1998). This latter consideration makes quantitative interpretation difficult if, at that site, there is an imbalance in the uptake rates of the different transmitters (Lu et al., 1998). Additionally, short-term variations in transmitter release during locomotion (which may still affect second messenger mediated transduction mechanisms) will go undetected with prolonged sampling. Nevertheless, the results from microdialysis measurements of the rat lumbar spinal cord during treadmill locomotion are generally similar to those obtained in the present study, with some important differences. Levels of 5-HT and their metabolites are increased in DH and ventral funiculus WM but decreased in the VH (Gerin et al., 1994, 1995, 2008; Gerin and Privat, 1998). Catecholamine levels are decreased in the DH but are increased in the VH and ventral funiculus WM during locomotion (Gerin et al., 1995, 2011; Gerin and Privat, 1998). Discrepancies may be due to central fatigue under conditions of prolonged activation (Fornal et al., 2006), species differences in uptake kinetics, diffusion rates and the spatial distributions of release sites, differences in baseline levels of monoamines which depend upon the behavioral state (Noga et al., 2004) and differences in the quality of locomotor activity.

Patterns of Release Relative to Locomotor Activity

Increased Monoamine Levels during Stimulation-Evoked Locomotion

To determine whether monamines are released within the spinal cord during MLR-evoked fictive locomotion and to characterize this release with high spatial and temporal resolution, we used FCV methods. Since this method measures release relative to a baseline resting state, our experiments were conducted on mesencephalic decerebrate animals lacking spontaneous locomotor activity with presumably low baseline transmitter levels. The stimulation frequencies used in this study (15–20 Hz) were also within the natural discharge frequencies of raphespinal (Fornal et al., 2006) and ceruleospinal (Aston-Jones et al., 1980) neurons and thus were expected to approximate the release occurring naturally when activated. An increase in monoamine release over background levels was most commonly observed as the first major response to MLR stimulation in DH, IZ and VH. During MLR evoked fictive locomotion, transmitter levels in areas of the IZ/VH where locomotor activated neurons are located (Noga et al., 2009, 2011) increased approximately 150 and 50 nM over baseline, for NE and 5-HT, respectively. These transmitter levels were at concentrations that are physiologically relevant (see "Discussion" Section, below). Interestingly, NE and 5-HT release was correlated to the time required to generate locomotor activity with MLR stimulation. This observation suggests that preparations with slow onset of locomotion had much lower baseline amine levels or were much less excitable (possibly interrelated) and needed higher amine levels to reach sufficient excitation to activate the locomotor network. Additional experiments are warranted to confirm this idea.

Decreased Monoamine Levels during and Following Evoked Locomotion

Transmitter levels decreased below steady state levels shortly after the onset of MLR stimulation in a small percentage (∼10%–20%) of all trials for NE and 5-HT, respectively. Similar responses to stimulation of monoaminergic nuclei have been observed in the spinal cord previously and appear to be highly localized (Hentall et al., 2003, 2006). Additionally, in some locations, following an initial increase in extracellular levels of monoamines at the onset of locomotion, a decrease to baseline or below was observed often as locomotion diminished. Similar findings are observed for some amines sampled from the ventral and DH (see above) of adult rats during prolonged periods of treadmill exercise (Gerin and Privat, 1998; Gerin et al., 2011). Such rapid decreases in transmitter concentration with continuing stimulation likely reflect inhibition of active monoaminergic neurons and removal of transmitters by uptake and clearance mechanisms (Wightman et al., 1988; Jackson et al., 1995) rather than post-translational alterations in rates of uptake or clearance which may take minutes to occur (Frazer et al., 1999). For NE, some LC neurons may not be able to conduct long trains (>20 s of impulses at applied frequencies of 20 Hz (Aston-Jones et al., 1980; see also Hickey et al., 2014) which could account for a failure to maintain extracellular NE levels in some trials of fictive locomotion. For 5-HT, this increase and/or decrease release pattern likely reflects the activity of presumptive serotonergic neurons in the raphe obscurus and pallidus, which increases at or immediately before the onset of treadmill locomotion (Veasey et al., 1995) and which progressively decreases with prolonged locomotion (Fornal et al., 2006). It has been suggested that this is due to the mechanism of "central fatigue" and indicates the strong connection between the firing rate of these neurons and the maintenance of motor performance (Jacobs et al., 2002; Fornal et al., 2006). We favor this as an explanation for the observed profiles of release, since simple modeling of the time-course of extracellular 5-HT indicates that for constantly emitting sources of boutons, there is no spatial arrangement (even with

the addition of continuous sinks) that can produce a decline in concentration as that observed here (Hentall et al., 2006). The only alternative explanations would be a diminishment of transmitter release due to vesicle depletion, which is unlikely at the stimulation frequencies used (Hentall et al., 2006), or inhibition of release by auto-receptors or heteroreceptors on terminals of monoaminergic fibers (e.g., Matsumoto et al., 1990; Roberts et al., 1997) by released transmitters. This latter mechanism may be of importance during acute stimulation experiments, considering that high levels of monoamines are observed in highly localized areas of the spinal gray in steady state conditions (Noga et al., 2004).

Extracellular Monoamines and the Generation of Locomotion—The Role of Volume Transmission in Behavioral State Changes

As observed in the present study, the extracellular concentrations of 5-HT and NE within the lumbar gray matter can increase above baseline to levels that can affect several serotonergic and noradrenergic receptor subtypes (Allgaier et al., 1992; Zoli et al., 1998; Hochman et al., 2001) implicated in the control of locomotion (Marcoux and Rossignol, 2000; Schmidt and Jordan, 2000; Giroux et al., 2001; Hochman et al., 2001; Antri et al., 2003; Liu and Jordan, 2005) and found on locomotor activated spinal neurons (Noga et al., 2009, 2011). Depending on the levels achieved, volume transmission effects would be most selective for higher affinity receptors such as NEα_2, 5-HT_{1A} and 5-HT_7 and less so for lower affinity receptors such as NAα_{1A} and 5-HT_{2A} (Hochman et al., 2001; Alexander et al., 2015), which are therefore more likely to be involved in synaptic transmission. Electrical stimulation of the raphe and LC (Hentall et al., 2003, 2006) at rates that exceed those observed at rest and match or exceed those observed during locomotion (Aston-Jones et al., 1980; Veasey et al., 1995; Fornal et al., 2006) also results in a rapid (seconds) increase in extracellular levels of 5-HT and NE within the spinal cord. These spatiotemporal patterns of release are probably paralleled by rapid, local monoaminergic effects on neuronal firing within the spinal cord (e.g., Bras et al., 1990). That the extracellular levels of monoamines are dynamically regulated in widespread regions of the spinal cord at concentrations that are pharmacologically relevant indicates that extrasynaptic or volume neurotransmission (Agnati et al., 1995; Zoli et al., 1998; Fuxe et al., 2007) plays a significant role in the generation and/or modulation of spinal locomotor activity induced by stimulation of the MLR. Alterations in the level of activity within descending monoaminergic pathways and ultimately the extracellular levels of monoamines may be one of the principal mechanisms that mediate changes in "physiological state" (see also, Burke, 1999) so that the effect of descending commands on neural excitability within the spinal cord is fine tuned. In this way, changes in the behavioral state may be the result of neurochemical "resetting" or "remodeling" of interneuronal reflex pathways by volume transmission. By activating the descending monoaminergic system, the MLR can modulate the responsiveness (excitability) of spinal interneurons to input from the reticulospinal pathway (e.g., Jankowska and Noga, 1990; Hammar et al., 2004, 2007) to enable locomotion.

The increase in the concentration of monoamines in the extracellular space is the result of transmitter diffusion out from the synaptic cleft ("spillover") as well as the direct release into the extracellular space from boutons without opposing synaptic specializations. In the DH, many monoaminergic terminals exhibit little specialization (Maxwell et al., 1983; Marlier et al., 1991; Rajaofetra et al., 1992; Ridet et al., 1992, 1993, 1994; Jankowska et al., 1995, 1997) and a significant proportion of 5-HT receptors and membrane 5-HT transporters are found located remotely from release sites (Ridet et al., 1994; Zhou et al., 1998; Doly et al., 2004). In the IZ/VH, synaptic contacts predominate (Poulat et al., 1992; Ridet et al., 1994; Alvarez et al., 1998), although extrasynaptically-located 5-HT receptors have also been described (Doly et al., 2004).

Extracellular concentrations depend upon many factors related to diffusion, tortuosity and tissue volume fraction and the time-course for changes in extracellular transmitter levels are prolonged in comparison to that for synapses which require diffusion of transmitter over short distances to bind to postsynaptic receptors. On slower time-scales, extracellular volume transmission could account for the tonic depression of monosynaptic group II field potentials in mid-lumbar segments during fictive locomotion (Perreault et al., 1999). For more rapid, differential modulatory effects on group II muscle afferent-evoked field potentials during the flexion and extension phases of locomotion (Perreault et al., 1999), synaptic transmission is likely involved. Both MLR (Garcia-Rill et al., 1983a; Goetz et al., 2016) and raphe neurons (Rasmussen et al., 1986; Jacobs and Fornal, 1995, 1999; Veasey et al., 1995; Fornal et al., 2006) show rhythmically and tonically active neurons during spontaneous locomotion which strengthens the possibility that both modes of transmission from monoaminergic pathways may be utilized at the spinal level.

Timing of Release Relative to Initiation of Locomotion

Stimulation of the MLR altered spinal NE and 5-HT monoamine release in approximately 8–10 s, respectively. Latencies were most likely related to physiological effects and not a function of the assay method since FCV methods may detect changes in concentrations of monoamines from one scan to the next (<1 s). Release in the IZ/VH occurred at or prior to the onset of bursting and locomotor activity as observed in hindlimb ENGs. NE release was quickest in the IZ/VH and was significantly earlier relative to the onset of bursting or locomotor activity in this location in comparison to the DH or when compared to 5-HT. Latencies for NE or 5-HT release measured in the same locations were often closely related to each other when locomotion was initiated relatively quickly (**Figure 3**). However, when locomotion was delayed, 5-HT release could be delayed as well (**Figure 4**). These observations likely account for the significant differences in latencies between NE and 5-HT in this area. They also suggest

that the timing of 5-HT release in the VH is more closely tied to motoneuron discharge (as measured with ENG activity) than is NE. Since we did not record interneuronal activity or the activity of motoneurons intracellularly (e.g., locomotor drive potentials—Noga et al., 2003), we cannot determine the timing of the activation of the locomotor network *per se* in relation to the onset of monoamine release. The timing results, however, indicate that 5-HT release in some areas is influenced by factors other than stimulation. Theoretically, 5-HT release could also be affected at the soma or terminal level by autoreceptor or heteroreceptor inhibition (Matsumoto et al., 1990; Roberts et al., 1997), or by inhibitory inputs at the brainstem level. Additionally, 5-HT release may influence the appearance of locomotion (motoneuron discharge) by their action on motoneurons (plateau potentials—see "Discussion" Section, below) or alternatively by actions on reticular neurons (Green and Carpenter, 1985; Di Prisco et al., 1994).

In the rat, monoamine detection following onset of electrical stimulation of the LC or nucleus raphe magnus occurs with a mean latency of approximately 4–4.5 s (Hentall et al., 2003, 2006). Differences in latency between these studies and the present one are likely related to the additional time required for synaptic activation of monoaminergic neurons by MLR projections (as opposed to direct stimulation of the monoaminergic nuclei), the additional axonal conduction time required to reach the spinal cord (Wessendorf et al., 1981; Nakazato, 1987) due to the cat's larger size, and the differences in stimulation parameters (trains of 50 Hz vs. 20 Hz continuous, 0.2–0.5 ms vs. 1 ms duration for rat and cat, respectively) which affects the time-course/amount of release (Hentall et al., 2003, 2006).

Functional Aspects of Spinal Monoamine Release during MLR-Evoked Locomotion

It is hypothesized that locomotor pattern and/or frequency is controlled by the activation of descending monoaminergic pathways in parallel with the reticulospinal pathway. By changing the pattern/amount of spinal monoamine release, properties and firing patterns of functionally specific neuron populations including pattern generating circuits to segmental, propriospinal and descending inputs (Bras et al., 1990; Skoog and Noga, 1991; Noga et al., 1992, 1995b; El Manira et al., 1998; Hammar et al., 2004, 2007) may be modulated. Clues as to the functional importance of release at any particular site may be obtained by examining changes in the release profile during repeated locomotor trials that show alterations in locomotor pattern or frequency. This would be predicted on the basis of the effects of monoaminergic drugs on the frequency and pattern of locomotor bursts when administered to the spinal cord (e.g., Barbeau and Rossignol, 1990, 1991; Chau et al., 1998; Brustein and Rossignol, 1999; reviewed by Miles and Sillar, 2011). For example, we observed modulated release of 5-HT and NE over baseline levels near medial gray areas in locomotor trials when flexor and extensor burst amplitudes (pattern) were significantly changed and the timing between sides was modulated, but without concomitant changes in locomotor frequency (**Figure 7A**). Commissural interneurons in this and adjacent areas are involved in the control of right-left alternation during locomotion (Díaz-Ríos et al., 2007) and could be influenced by monoamines during locomotor activity, since they are innervated by descending reticulospinal neurons (Matsuyama et al., 2004; Szokol et al., 2011) and monoaminergic fibers (Hammar et al., 2004; Noga et al., 2009, 2011). Furthermore, reticulospinal inputs are facilitated by both monoamines, whereas segmental inputs from group II muscle afferents are facilitated or inhibited by 5-HT or NE, respectively (Hammar et al., 2004). This contrasts to that observed in lamina IX where the appearance of locomotor activity was precisely timed to increased levels of 5-HT with or without concomitant changes in the level of NE (**Figure 4**). Whether this is the result of the induction and/or modulation of plateau potentials in motoneurons (Conway et al., 1988; Hounsgaard et al., 1988) and the subsequent generation of action potentials requires further investigation. MacDonell et al. (2015) have shown that extensor motoneurons become more responsive (primed) during the tonic period of firing immediately prior to the onset of MLR-evoked fictive locomotion. Based on the present results, this neuromodulation could be accomplished by monoamines, which may have significantly more potent neuromodulatory effects on motoneuron excitability than ionotropic actions (Heckman et al., 2009). Additional measurements are needed to determine overall expected segmental and laminar differences (Marcoux and Rossignol, 2000; Liu and Jordan, 2005; Delivet-Mongrain et al., 2008) and the variations between NE and 5-HT release during perturbations that affect not only burst intensity but also the frequency of locomotion. This type of analysis is only possible with the high temporal resolution afforded by FCV.

As discussed elsewhere (Noga et al., 2009, 2011), monoaminergic effects will depend on the cell type and membrane properties, the location and distribution of receptors on individual neurons relative to the site(s) of release, the types/subtypes of receptors and G-proteins expressed in the target cells, the concentrations at synaptic and extrasynaptic sites and therefore the number and type of bound receptors, etc. Since monoamine release is highly dynamic, neuromodulatory effects may also vary temporally during a bout of locomotion. The overall effect of the various monoamines will depend upon the balance and interaction between each neuromodulator acting at the different receptors (Doi and Ramirez, 2010; see also, Beliez et al., 2014). As discussed by Harris-Warrick (2011), the effects of each modulator "may oppose one another, but this may serve to stabilize the modulated state". Indications that variations in monoamine release may be related to changes in locomotor frequency, extensor/flexor burst area and duty cycle are presented in **Figure 8**. Here periods of steady state release are associated with stable locomotion.

Model of Descending Pathways for Initiation of Locomotion

Figure 9 illustrates a modified conceptual model of descending pathways for the production of MLR-evoked fictive locomotion in the decerebrate cat (after Noga et al., 2003). The schematic summarizes the relationships between the various descending pathways, the flexor and extensor components of the spinal locomotor central pattern generator (CPG) and their respective

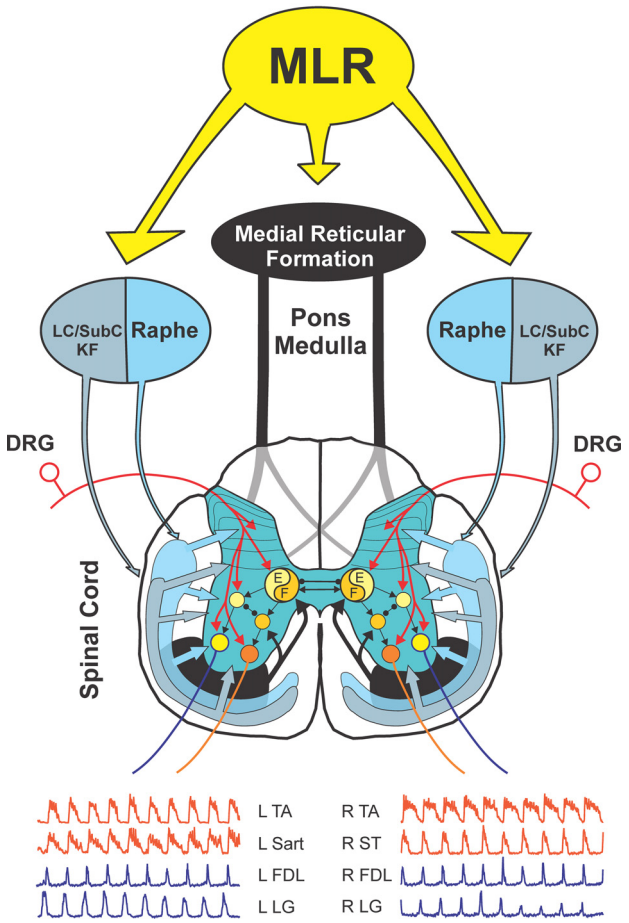

FIGURE 9 | Model of descending locomotor pathways in the cat. Stimulation of the MLR activates reticulospinal neurons which project through the VLF to activate spinal locomotor CPG neurons, in part, by the release of excitatory amino acids. This pathway is considered to comprise the primary "command pathway" for the initiation of locomotion. MLR stimulation also activates in parallel, multiple monoaminergic descending pathways during centrally-generated fictive locomotion. This is evidenced by the rapid, widespread release of NE and 5-HT in the IZ/VH, in areas with centrally-activated locomotor neurons, and in the DH, where additional neurons are activated from afferent feedback from the moving limb during real locomotion. Extracellular monoamine levels are sufficient to activate monoaminergic receptors located on spinal locomotor-activated neurons indicating that extrasynaptic or volume transmission likely plays a significant role in the modulation/generation of locomotor activity. The flexor (F) and extensor (E) components of the locomotor CPG are activated/modulated by descending bilateral reticulospinal and monoaminergic projections as well as by crossed excitatory (▶) and inhibitory (●) segmental projections from the CPG opposite to it. Sensory afferents from skin and muscles innervate spinal neurons in DH, IZ and VH to fine-tune the locomotor step cycle. Details of the flexor and extensor components of the CPG are omitted in order to emphasize general interconnections between them and their target neurons. LC, locus ceruleus; SubC, subceruleus; KF, Kölliker-Fuse; DRG, dorsal root ganglia; L, left; R, right; TA, tibialis anterior, Sart, sartorius, FDL, flexor digitorum longus; LG, lateral gastrocnemius; ST, semitendinosus.

motoneurons for bilateral hindlimb locomotion following stimulation of the MLR. In addition to the reticulospinal "command" pathway, the model incorporates the parallel, bilateral activation of ponto-medullary catecholaminergic and medullary serotonergic pathways and the subsequent spinal release of monoamines. Direct projections from the MLR or its anatomical equivalent to the output neurons of brainstem monoaminergic nuclei have been described (Edwards, 1975; Steeves and Jordan, 1984; Sotnichenko, 1985; Behbehani and Zemlan, 1986). The primary source of spinal monoaminergic innervation originates from cells in these locations (Basbaum and Fields, 1979; Wiklund et al., 1981; Stevens et al., 1982, 1985; Westlund et al., 1982; Nakazato, 1987; Clark and Proudfit, 1991a,b; Jones and Light, 1992). Raphespinal and ceruleospinal neuronal activity increases during spontaneous walking (Rasmussen et al., 1986; Jacobs and Fornal, 1995, 1999; Veasey et al., 1995) further corroborating this idea.

During bilateral hindlimb locomotion induced by unilateral stimulation of the MLR, spinal 5-HT and NE release is likely comparable on both sides. Since the majority of MLR fibers terminate ipsilaterally within the medial reticular formation (Garcia-Rill et al., 1983b; Steeves and Jordan, 1984), and since the majority of catecholaminergic fibers within the LC and dorsolateral pons innervate the ipsilateral spinal cord (Stevens et al., 1985), it is likely that a major portion of catecholamine release *on each side* of the spinal cord come from the terminals of ipsilaterally-projecting pathways. Any potential imbalance on the side opposite to stimulation must be compensated for by bilaterally-projecting catecholamine neurons (especially from the Kölliker-Fuse nucleus) and decussating at each segmental level throughout the cord (Stevens et al., 1982, 1985; Bruinstroop et al., 2012) and/or by MLR projections to the contralateral MLR and Kölliker-Fuse region (Steeves and Jordan, 1984). A similar organization has been described for activation of descending reticulospinal pathways by unilateral stimulation of the MLR where crossed segmental projections likely make up for any potential asymmetry in descending inputs (Noga et al., 2003). MLR projections to the midline raphe magnus and bilateral projections to the post-pyramidal region (Edwards, 1975; Steeves and Jordan, 1984; Sotnichenko, 1985) containing serotonergic neurons projecting to the spinal cord (Jones and Light, 1992), will likely account for bilateral release of 5-HT during locomotion. It is assumed that during spontaneous or voluntary bilateral hindlimb locomotion, MLR activity would be balanced on both sides, as would the descending reticulospinal and monoaminergic input to spinal locomotor centers on either side of the cord.

In the present study, NE release was significantly greater in the presence of organized locomotor activity vs. non-organized or tonic activity (bursting), suggesting that NE may be involved in organizing spinal circuits for coordinated motor output. Both NE and 5-HT release also co-varied in some locations with amplitude modulation of peripheral nerves. A causal relationship between spinal monoamine release and locomotor generation would imply that any left-right asymmetry seen in trials of one-sided locomotion would be due to insufficient and/or disparate activation of descending reticulospinal and monoaminergic neuronal pathways on the affected side. This implies a coupling between locomotor pattern *generation* and monoamine *levels*

in cord regions containing locomotor-activated neurons, rather than just a coupling to the stimulation. In our model, the pattern of locomotion will depend upon the amount as well as the spatial and temporal pattern (timing) of release, in addition to the spatial distribution of different functional neuronal populations relative to the site(s) of release, the receptor profiles of the neurons involved and their levels of activity. An insufficiency leading to loss or failure to generate locomotion may result from a substantially reduced signal transmission across *any* potential relay site within the pathway, or possibly be related to some mechanism responsible for central fatigue (Fornal et al., 2006).

With stimulation of the MLR leading up to the formation of locomotor activity, multiple descending pathways are activated resulting in the release of NE and 5-HT within the spinal cord. In many locations within the gray matter, this release occurs within seconds of stimulation, often preceding locomotion and peaking after locomotion ceases. Release is observed in the IZ/VH which is densely innervated by reticulospinal neurons (Peterson et al., 1975; Holstege and Kuypers, 1982; Kausz, 1991) and which contain centrally activated-neurons involved in the generation of hindlimb locomotion (see above), as well as in the DH, where neurons that are activated due to afferent feedback of the moving limb are located (Dai et al., 2005). As predicted from recordings from multiple sites in this study, monoamine release occurs across multiple laminae of the cord during locomotion. Raphe-spinal and ceruleospinal axons typically show extensive collateral branching in the spinal cord which span laminae as well as region (e.g., cervical, thoracic and lumbar; Huisman et al., 1981; Stevens et al., 1982, 1985; Westlund et al., 1982; Nakazato, 1987; Fuxe et al., 1990; Allen and Cechetto, 1994; Fields et al., 1995). Furthermore, recordings from electrophysiologically identified medullary serotonergic neurons show virtually all neurons are activated during treadmill-induced locomotion in cats (Jacobs et al., 2002). This is consistent with the idea that the descending monoaminergic system generally functions as a unit during behavioral activation to provide diffuse descending neuromodulation (see Heckman et al., 2008). Against this background of diffuse neuromodulation, specific spinal inhibitory pathways may function to focus motoneuronal excitability, as needed for specific movements (Heckman et al., 2008). In the present study, single electrode measurements revealed complex, highly unique release patterns from areas of the gray matter in some trials of evoked locomotion. It is possible that these represent unique patterns of release that are not mirrored in other areas during MLR stimulation. Such features could represent variations in the pattern of activation or recruitment (see Chandler et al., 2014; Li et al., 2016) and firing of individual monoaminergic neurons (Jacobs et al., 2002) which change over time (Fornal et al., 2006), and could be influenced by variations in the diffusion distances and/or the geometry of release sites relative to uptake and recording sites (Brumley et al., 2007). Further experiments using simultaneous voltammetric measurements across multiple recording sites would help understand the extent of diffuse neuromodulation during evoked locomotion.

AUTHOR CONTRIBUTIONS

BRN and IDH: conceptualization. BRN, RPT, SX, AT and IDH: methodology. BRN, RPT, AP and IDH: investigation. BRN, RPT, SX and AT: formal analysis. BRN and SX: visualization. BRN: writing—original draft. BRN and IH: writing—review and editing, supervision and funding acquisition.

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REFERENCES

Agnati, L. F., Zoli, M., Strömberg, I., and Fuxe, K. (1995). Intercellular communication in the brain: wiring versus volume transmission. *Neuroscience* 69, 711–726. doi: 10.1016/0306-4522(95)00308-6

Alexander, S. P. H., Davenport, A. P., Kelly, E., Marrion, N., Peters, J. A., Benson, H. E., et al. (2015). The concise guide to PHARMACOLOGY 2015/16: G protein-coupled receptors. *Br. J. Pharmacol.* 172, 5744–5869. doi: 10.1111/bph.13348

Allen, G. V., and Cechetto, D. F. (1994). Serotonergic and nonserotonergic neurons in the medullary raphe system have axon collateral projections to autonomic and somatic cell groups in the medulla and spinal cord. *J. Comp. Neurol.* 350, 357–366. doi: 10.1002/cne.903500303

Allgaier, C., Agneter, E., Feurstein, T. J., and Singer, E. A. (1992). Estimation of the biophase concentration of noradrenaline at presynaptic α_2-adrenoceptors in brain slices. *Naunyn Schmiedebergs Arch. Pharm.* 345, 402–409. doi: 10.1007/bf00176617

Alvarez, F. J., Pearson, J. C., Harrington, D., Dewey, D., Torbeck, L., and Fyffe, R. E. W. (1998). Distribution of 5-hydroxytryptamine-immunoreactive boutons on α-motoneurons in the lumbar spinal cord of adult cats. *J. Comp. Neurol.* 393, 69–83. doi: 10.1002/(SICI)1096-9861(19980330)393:1<69::AID-CNE7>3.0.CO;2-O

Antri, M., Mouffle, C., Orsal, D., and Barthe, J.-Y. (2003). 5-HT_{1A} receptors are involved in short- and long-term processes responsible for 5-HT-induced locomotor function recovery in chronic spinal rat. *Eur. J. Neurosci.* 18, 1963–1972. doi: 10.1046/j.1460-9568.2003.02916.x

Antri, M., Orsal, D., and Barthe, J.-Y. (2002). Locomotor recovery in the chronic spinal rat: effects of long-term treatment with a 5-HT_2 agonist. *Eur. J. Neurosci.* 16, 467–476. doi: 10.1046/j.1460-9568.2002.02088.x

Armstrong-James, M., and Millar, J. (1984). "High-speed cyclic voltammetry and unit recording with carbon fibre microelectrodes," in *Measurement of Neurotransmitter Release In Vivo*, ed. A. Marsden (Toronto: John Wiley and Sons), 209–226.

Aston-Jones, G., Segal, M., and Bloom, F. E. (1980). Brain aminergic axons exhibit marked variability in conduction velocity. *Brain Res.* 195, 215–222. doi: 10.1016/0006-8993(80)90880-x

Barbeau, H., and Rossignol, S. (1990). The effects of serotonergic drugs on the locomotor pattern and on cutaneous reflexes of the adult chronic spinal cat. *Brain Res.* 514, 55–67. doi: 10.1016/0006-8993(90)90435-e

Barbeau, H., and Rossignol, S. (1991). Initiation and modulation of the locomotor pattern in the adult chronic spinal cat by noradrenergic, serotonergic

and dopaminergic drugs. *Brain Res.* 546, 250–260. doi: 10.1016/0006-8993(91)91489-n

Basbaum, A. I., and Fields, H. L. (1979). The origin of descending pathways in the dorsolateral funiculus of the spinal cord of the cat and rat: further studies on the anatomy of pain modulation. *J. Comp. Neurol.* 187, 513–531. doi: 10.1002/cne.901870304

Basbaum, A. I., Godefroy, F., and Weil-Fugazza, J. (1987). A new microdissection technique for regional biochemical analysis of the rat spinal cord: serotonin, norephinephrine, dopamine and uric acid. *Brain Res.* 419, 229–238. doi: 10.1016/0006-8993(87)90588-9

Behbehani, M. M., and Zemlan, F. P. (1986). Response of nucleus raphe magnus neurons to electrical stimulation of nucleus cuneiformis: role of acetylcholine. *Brain Res.* 369, 110–118. doi: 10.1016/0006-8993(86)90518-4

Beliez, L., Barrière, G., Bertrand, S. S., and Cazalets, J. R. (2014). Multiple monoaminergic modulation of posture-locomotor network activity in the newborn rat spinal cord. *Front. Neural Cir.* 8:99. doi: 10.3389/fncir.2014.00099

Björklund, A., and Skagerberg, G. (1979). Evidence for a major spinal cord projection from the diencephalic A11 dopamine cell group in the rat using transmitter-specific fluorescent retrograde tracing. *Brain Res.* 177, 170–175. doi: 10.1016/0006-8993(79)90927-2

Bras, H., Jankowska, E., Noga, B., and Skoog, B. (1990). Comparison of effects of various types of NA and 5-HT agonists on transmission from group II muscle afferents in the cat. *Eur. J. Neurosci.* 2, 1029–1039. doi: 10.1111/j.1460-9568.1990.tb00015.x

Brazell, M. P., Kasser, R. J., Renner, K. J., Feng, J., Moghaddam, B., and Adams, R. N. (1987). Electrocoating carbon fiber microelectrodes with Nafion improves selectivity for electroactive neurotransmitters. *J. Neurosci. Meth.* 22, 167–172. doi: 10.1016/0165-0270(87)90011-2

Bretzner, F., and Brownstone, R. M. (2013). Lhx3-Chx10 reticulospinal neurons in locomotor circuits. *J. Neurosci.* 33, 14681–14692. doi: 10.1523/JNEUROSCI.5231-12.2013

Brodin, L., Ohta, Y., Hökfelt, T., and Grillner, S. (1989). Further evidence for excitatory amino acid transmission in lamprey reticulospinal neurons: selective retrograde labeling with (^{3}H)D-aspartate. *J. Comp. Neurol.* 281, 225–233. doi: 10.1002/cne.902810206

Bruinstroop, E., Cano, G., Vanderhorst, V. G. J. M., Cavalcante, J. C., Wirth, J., Sena-Esteves, M., et al. (2012). Spinal projections of the A5, A6 (locus coeruleus) and A7 noradrenergic cell groups in rats. *J. Comp. Neurol.* 520, 1985–2001. doi: 10.1002/cne.23024

Brumley, M. R., Hentall, I. D., Pinzon, A., Kadam, B. H., Blythe, A., Sanchez, F. J., et al. (2007). Serotonin concentrations in the lumbosacral spinal cord of the adult rat following microinjection or dorsal surface application. *J Neurophysiol.* 98, 1440–1450. doi: 10.1152/jn.00309.2007

Brustein, E., and Rossignol, S. (1999). Recovery of locomotion after ventral and ventrolateral spinal lesions in the cat. II. Effects of noradrenergic and serotonergic drugs. *J. Neurophysiol.* 81, 1513–1530.

Buchanan, J. T., Brodin, L., Dale, N., and Grillner, S. (1987). Reticulospinal neurones activate excitatory amino acid receptors. *Brain Res.* 408, 321–325. doi: 10.1016/0006-8993(87)90397-0

Burke, R. E. (1999). The use of state-dependent modulation of spinal reflexes as a tool to investigate the organization of spinal interneurons. *Exp. Brain Res.* 128, 263–277. doi: 10.1007/s002210050847

Carr, P. A., Huang, A., Noga, B. R., and Jordan, L. M. (1995). Cytochemical characteristics of cat spinal neurons activated during fictive locomotion. *Brain Res. Bull.* 37, 213–218. doi: 10.1016/0361-9230(94)00271-2

Carr, P. A., Noga, B. R., Nance, D. M., and Jordan, L. M. (1994). Intracellular labeling of cat spinal neurons using a tetramethylrhodamine-dextran amine conjugate. *Brain Res. Bull.* 34, 447–451. doi: 10.1016/0361-9230(94)90017-5

Cazalets, J. R., Sqalli-Houssaini, Y., and Clarac, F. (1992). Activation of the central pattern generators for locomotion by serotonin and excitatory amino acids in neonatal rat. *J. Physiol.* 455, 187–204. doi: 10.1113/jphysiol.1992.sp019296

Chandler, D. J., Gao, W. J., and Waterhouse, B. D. (2014). Heterogeneous organization of the locus ceruleus projections to prefrontal and motorcortices. *Proc. Natl. Acad. Sci. U S A* 111, 6816–6821. doi: 10.1073/pnas.1320827111

Chau, C., Barbeau, H., and Rossignol, S. (1998). Effects of intrathecal α_1- and α_2-noradrenergic agonists and norepinephrine on locomotion in chronic spinal cats. *J. Neurophysiol.* 79, 2941–2963.

Chesler, M. (2003). Regulation and modulation of pH in the brain. *Physiol. Rev.* 83, 1183–1221. doi: 10.1152/physrev.00010.2003

Chesler, M., and Kaila, K. (1992). Modulation of pH by neuronal activity. *Trends Neurosci.* 15, 396–402. doi: 10.1016/0166-2236(92)90191-a

Christie, K. J., and Whelan, P. J. (2005). Monoaminergic establishment of rostrocaudal gradients of rhythmicity in the neonatal mouse spinal cord. *J. Neurophysiol.* 94, 1554–1564. doi: 10.1152/jn.00299.2005

Clark, F. M., and Proudfit, H. K. (1991a). The projection of locus coeruleus neurons to the spinal cord in the rat determined by anterograde tracing combined with immunocytochemistry. *Brain Res.* 538, 231–245. doi: 10.1016/0006-8993(91)90435-x

Clark, F. M., and Proudfit, H. K. (1991b). The projection of noradrenergic neurons in the A7 catecholamine cell group to the spinal cord in the rat demonstrated by anterograde tracing combined with immunocytochemistry. *Brain Res.* 547, 279–288. doi: 10.1016/0006-8993(91)90972-x

Conway, B. A., Hultborn, H., Kiehn, O., and Mintz, I. (1988). Plateau potentials in α-motoneurones induced by intravenous injection of L-DOPA and clonidine in the spinal cat. *J. Physiol.* 405, 369–384. doi: 10.1113/jphysiol.1988.sp017337

Dai, X., Noga, B. R., Douglas, J. R., and Jordan, L. M. (2005). Localization of spinal neurons activated during locomotion using the *c-fos* immunohistochemical method. *J. Neurophysiol.* 93, 3442–3452. doi: 10.1152/jn.00578.2004

Dankoski, E. C., and Wightman, R. M. (2013). Monitoring serotonin signaling on a subsecond time scale. *Front. Int. Neurosci.* 7:44. doi: 10.3389/fnint.2013.00044

Delivet-Mongrain, H., Leblond, H., and Rossignol, S. (2008). Effects of localized intraspinal injections of a noradrenergic blocker on locomotion of high decerebrate cats. *J. Neurophysiol.* 100, 907–921. doi: 10.1152/jn.90454.2008

Di Prisco, G. V., Dubuc, R., and Grillner, S. (1994). 5-HT innervation of reticulospinal neurons and other brainstem structures in lamprey. *J. Comp. Neurol.* 342, 23–34. doi: 10.1002/cne.903420104

Díaz-Ríos, M., Dombeck, D. A., Webb, W. W., and Harris-Warrick, R. M. (2007). Serotonin modulates dendritic calcium influx in commissural interneurons in the mouse spinal locomotor network. *J. Neurophysiol.* 98, 2157–2167. doi: 10.1152/jn.00430.2007

Doi, A., and Ramirez, J. M. (2010). State-dependent interactions between excitatory neuromodulators in the neuronal control of breathing. *J. Neurosci.* 30, 8251–8262. doi: 10.1523/jneurosci.5361-09.2010

Doly, S., Madeira, A., Fischer, J., Brisorgueil, M. J., Daval, G., Bernard, R., et al. (2004). The 5-HT_{2A} receptor is widely distributed in the rat spinal cord and mainly localized at the plasma membrane of postsynaptic neurons. *J. Comp. Neurol.* 472, 496–511. doi: 10.1002/cne.20082

Douglas, J. R., Noga, B. R., Dai, X., and Jordan, L. M. (1993). The effects of intrathecal administration of excitatory amino acid agonists and antagonists on the initiation of locomotion in the adult cat. *J. Neurosci.* 13, 990–1000.

Doyle, C. A., and Maxwell, D. J. (1991). Ultrastructural analysis of noradrenergic nerve terminals in the cat lumbosacral spinal dorsal horn: a dopamine-β-hydroxylase immunocytochemical study. *Brain Res.* 563, 329–333. doi: 10.1016/0006-8993(91)91557-h

Edwards, S. B. (1975). Autoradiographic studies of the projections of the midbrain reticular formation: descending projections of the nucleus cuneiformis. *J. Comp. Neurol.* 161, 341–358. doi: 10.1002/cne.901610306

El Manira, A., Parker, D., Krieger, P., Wikström, M., and Grillner, S. (1998). "Presynaptic inhibition of synaptic transmission from sensory, interneuronal and supraspinal neurons to spinal target cells in lamprey," in *Presynaptic Inhibition and Neural Control*, eds P. Rudomin, R. Romo and L. M. Mendell (New York, NY: Oxford University Press), 329–348.

Feraboli-Lohnherr, D., Barthe, J. Y., and Orsal, D. (1999). Serotonin-induced activation of the network for locomotion in adult spinal rats. *J. Neurosci. Res.* 55, 87–98. doi: 10.1002/(SICI)1097-4547(19990101)55:1<87::AID-JNR10>3.0.CO;2-#

Fields, H. L., Malick, A., and Burstein, R. (1995). Dorsal horn projection targets of ON and OFF cells in the rostral ventromedial medulla. *J. Neurophysiol.* 74, 1742–1759.

Fleetwood-Walker, S. M., and Coote, J. H. (1981). Contribution of noradrenaline-, dopamine- and adrenaline-containing axons to the innervation of different regions of the spinal cord of the cat. *Brain Res.* 206, 95–106. doi: 10.1016/0006-8993(81)90103-7

Fornal, C., Auerbach, S., and Jacobs, B. L. (1985). Activity of serotonin-containing neurons in nucleus raphe magnus in freely moving cats. *Exp. Neurol.* 88, 590–608. doi: 10.1016/0014-4886(85)90074-3

Fornal, C. A., Martín-Cora, F. J., and Jacobs, B. L. (2006). "Fatigue" of medullary but not mesencephalic raphe serotonergic neurons during locomotion in cats. *Brain Res.* 1072, 55–61. doi: 10.1016/j.brainres.2005.12.007

Frazer, A., Gerhardt, G. A., and Daws, L. C. (1999). New views of biogenic amine transporter function: implications for neuropsychopharmacology. *Int. J. Neuropsychopharmacol.* 2, 305–320. doi: 10.1017/s1461145799001625

Fuxe, K., Dahlström, A., Höistad, M., Marcellino, D., Jansson, A., Rivera, A., et al. (2007). From the Golgi-Cajal mapping to the transmitter-based characterization of the neuronal networks leading to two modes of brain communication: wiring and volume transmission. *Brain Res. Rev.* 55, 17–54. doi: 10.1016/j.brainresrev.2007.02.009

Fuxe, K., Tinner, B., Bjelke, B., Angati, L. F., Verhofstad, A., Steinbusch, H. G. W., et al. (1990). Monoaminergic and peptidergic innervation of the intermediolateral horn of the spinal cord. I. Distribution patterns of nerve terminal networks. *Eur. J. Neurosci.* 2, 430–450. doi: 10.1111/j.1460-9568.1990.tb00435.x

Garcia-Rill, E. (1986). The basal ganglia and the locomotor regions. *Brain Res. Rev.* 11, 47–63. doi: 10.1016/0165-0173(86)90009-3

Garcia-Rill, E., and Skinner, R. D. (1987). The mesencephalic locomotor region. II. Projections to reticulospinal neurons. *Brain Res.* 411, 13–20. doi: 10.1016/0006-8993(87)90676-7

Garcia-Rill, E., Skinner, R. D., and Fitzgerald, J. A. (1983a). Activity in the mesencephalic locomotor region during locomotion. *Exp. Neurol.* 82, 609–622. doi: 10.1016/0014-4886(83)90084-5

Garcia-Rill, E., Skinner, R. D., Gilmore, S. A., and Owings, R. (1983b). Connections of the mesencephalic locomotor region (MLR) II. Afferents and efferents. *Brain Res. Bull.* 10, 63–71. doi: 10.1016/0361-9230(83)90076-x

Gerin, C., Becquet, D., and Privat, A. (1995). Direct evidence for the link between monoaminergic descending pathways and motor activity. I. A study with microdialysis probes implanted in the ventral funiculus of the spinal cord. *Brain Res.* 704, 191–201. doi: 10.1016/0006-8993(95)01111-0

Gerin, C., Legrand, A., and Privat, A. (1994). Study of 5-HT release with a chronically implanted microdialysis probe in the ventral horn of the spinal cord of unrestrained rats during exercise on a treadmill. *J. Neurosci. Methods* 52, 129–141. doi: 10.1016/0165-0270(94)90121-x

Gerin, C., and Privat, A. (1998). Direct evidence for the link between monoaminergic descending pathways and motor activity. II. A study with microdialysis probes implanted in the ventral horn of the spinal cord. *Brain Res.* 794, 169–173. doi: 10.1016/S0006-8993(98)00278-9

Gerin, C. G., Smith, K., Hill, S., Hill, A., and Madueke, I. C. (2011). Motor activity affects dopaminergic and noradrenergic systems of the dorsal horn of the rat lumbar spinal cord. *Synapse* 65, 1282–1288. doi: 10.1002/syn.20948

Gerin, C., Teilhac, J.-R., Smith, K., and Privat, A. (2008). Motor activity induces release of serotonin in the dorsal horn of the rat lumbar spinal cord. *Neurosci. Lett.* 436, 91–95. doi: 10.1016/j.neulet.2008.01.081

Giroux, N., Reader, T. A., and Rossignol, S. (2001). Comparison of the effect of intrathecal administration of clonidine and yohimbine on the locomotion of intact and spinal cats. *J. Neurophysiol.* 85, 2516–2536.

Goetz, L., Piallat, B., Bhattacharjee, M., Mathieu, H., David, O., and Charbardès, S. (2016). On the role of the pedunculopontine nucleus and mesencephalic reticular formation in locomotion in nonhuman primates. *J. Neurosci.* 36, 4917–4929. doi: 10.1523/JNEUROSCI.2514-15.2016

Green, R. W., and Carpenter, D. O. (1985). Actions of neurotransmitters on pontine medial reticular formation neurons of the cat. *J. Neurophysiol.* 54, 520–531.

Grillner, S., and Shik, M. L. (1973). On the descending control of the lumbosacral spinal cord from the "mesencephalic locomotor region". *Acta Physiol. Scand.* 87, 320–333. doi: 10.1111/j.1748-1716.1973.tb05396.x

Grzanna, R., and Fritschy, J.-M. (1991). Efferent projections of different subpopulations of central noradrenaline neurons. *Prog. Brain Res.* 88, 89–101. doi: 10.1016/s0079-6123(08)63801-7

Hägglund, M., Borgius, L., Dougherty, K. J., and Kiehn, O. (2010). Activation of groups of excitatory neurons in the mammalian spinal cord or hindbrain evokes locomotion. *J. Comp. Neurol.* 13, 246–252. doi: 10.1038/nn.2482

Hammar, I., Bannatyne, B. A., Maxwell, D. J., Edgley, S. A., and Jankowska, E. (2004). The actions of monoamines and distribution of noradrenergic and serotonergic contacts on different subpopulations of commissural interneurons in the cat spinal cord. *Eur. J. Neurosci.* 19, 1305–1316. doi: 10.1111/j.1460-9568.2004.03239.x

Hammar, I., Stecina, K., and Jankowska, E. (2007). Differential modulation by monoamine membrane receptor agonists of reticulospinal input to lamina VIII feline spinal commissural interneurons. *Eur. J. Neurosci.* 26, 1205–1212. doi: 10.1111/j.1460-9568.2007.05764.x

Harris-Warrick, R. M. (1988). "Chemical modulation of central pattern generators," in *Neural Control of Rhythmic Movements in Vertebrates*, eds A. H. Cohen, S. Rossignol and S. Grillner (New York, NY: John Wiley & Sons), 285–332.

Harris-Warrick, R. M. (2011). Neuromodulation and flexibility in central pattern generator networks. *Curr. Opin. Neurobiol.* 21, 685–692. doi: 10.1016/j.conb.2011.05.011

Heckman, C. J., Hyngstrom, A. S., and Johnson, M. D. (2008). Active properties of motoneurone dendrites: diffuse descending neuromodulation, focused local inhibition. *J. Physiol.* 586, 1225–1231. doi: 10.1113/jphysiol.2007.145078

Heckman, C. J., Mottram, C., Quinlan, K., Theiss, R., and Schuster, J. (2009). Motoneuron excitability: the importance of neuromodulatory inputs. *Clin. Neurophysiol.* 120, 2040–2054. doi: 10.1016/j.clinph.2009.08.009

Heien, M. L. A. V., Johnson, M. A., and Wightman, R. M. (2004). Resolving neurotransmitters detected by fast-scan cyclic voltammetry. *Anal. Chem.* 76, 5697–5704. doi: 10.1021/ac0491509

Hentall, I. D., Mesigil, R., Pinzon, A., and Noga, B. R. (2003). Temporal and spatial profiles of pontine-evoked monoamine release in the rat's spinal cord. *J. Neurophysiol.* 89, 2943–2951. doi: 10.1152/jn.00608.2002

Hentall, I. D., Pinzon, A., and Noga, B. R. (2006). Spatial and temporal patterns of serotonin release in the rat's lumbar spinal cord following electrical stimulation of the nucleus raphe magnus. *Neuroscience* 142, 893–903. doi: 10.1016/j.neuroscience.2006.06.038

Hickey, L., Li, Y., Fyson, S. J., Watson, T. C., Perrins, R., Hewinson, J., et al. (2014). Optoactivation of locus ceruleus neurons evokes bidirectional changes in thermal nociception in rats. *J. Neurosci.* 34, 4148–4160. doi: 10.1523/JNEUROSCI.4835-13.2014

Hochman, S., Garraway, S. M., Machacek, D. W., and Shay, B. L. (2001). "5-HT receptors and the neuromodulatory control of spinal cord function," in *Motor Neurobiology of the Spinal Cord. Methods and New Frontiers in Neuroscience Series*, ed. T. C. Cope (Boca Raton, FL: CRC Press), 47–87.

Holstege, G., and Kuypers, H. G. J. M. (1982). The anatomy of the brain stem pathways to the spinal cord in a cat. A labelled amino acid tracing study. *Prog. Brain Res.* 57, 145–175. doi: 10.1016/s0079-6123(08)64128-x

Holstege, J. C., Van Dijken, H., Buijs, R. M., Goedknegt, H., Gosens, T., and Bongers, C. M. (1996). Distribution of dopamine immunoreactivity in the rat, cat, and monkey spinal cord. *J. Comp. Neurol.* 376, 631–652. doi: 10.1002/(SICI)1096-9861(19961223)376:4<631::AID-CNE10>3.0.CO;2-P

Hounsgaard, J., Hultborn, H., Jespersen, B., and Kiehn, O. (1988). Bistability of α-motoneurones in the decerebrate cat and in the acute spinal cat after intravenous 5-hydroxytryptophan. *J. Physiol.* 405, 345–367. doi: 10.1113/jphysiol.1988.sp017336

Huang, A., Noga, B. R., Carr, P. A., Fedirchuk, B., and Jordan, L. M. (2000). Spinal cholinergic neurons activated during locomotion: immunohistochemical localization and electrophysiological characterization. *J. Neurophysiol.* 83, 3537–3547.

Huisman, A. M., Kuypers, H. G., and Verburgh, C. A. (1981). Quantitative differences in collateralization of the descending spinal pathways from red nucleus and other brain stem cell groups in rat as demonstrated with the multiple fluorescent retrograde tracer technique. *Brain Res.* 209, 271–286. doi: 10.1016/0006-8993(81)90153-0

Jackson, B. P., Dietz, S. M., and Wightman, R. M. (1995). Fast-scan cyclic voltammetry of 5-hydroxytryptamine. *Anal. Chem.* 67, 1115–1120. doi: 10.1021/ac00102a015

Jacobs, B. L., and Fornal, C. A. (1995). Activation of 5-HT neuronal activity during motor behavior. *Semin. Neurosci.* 7, 401–408. doi: 10.1016/1044-5765(95)90004-7

Jacobs, B. L., and Fornal, C. A. (1999). Activation of serotonergic neurons in behaving animals. *Neuropsychopharmacology* 21, 9S–15S. doi: 10.1038/sj.npp.1395336

Jacobs, B. L., Martín-Cora, F. J., and Fornal, C. A. (2002). Activity of medullary serotonergic neurons in freely moving animals. *Brain Res. Rev.* 40, 45–52. doi: 10.1016/s0165-0173(02)00187-x

Jankowska, E., Jukes, M. G. M., Lund, S., and Lundberg, A. (1967). The effect of DOPA on the spinal cord. VI. Half-centre organization of interneurons transmitting effects from the flexor reflex afferents. *Acta Physiol. Scand.* 70, 389–402. doi: 10.1111/j.1748-1716.1967.tb03637.x

Jankowska, E., Maxwell, D. J., Dolk, S., and Dahlström, A. (1997). A confocal and electron microscopic study of contacts between 5-HT fibres and dorsal horn interneurons in pathways from muscle afferents. *J. Comp. Neurol.* 387, 430–438. doi: 10.1002/(SICI)1096-9861(19971027)387:3<430::AID-CNE8>3.0.CO;2-1

Jankowska, E., Maxwell, D. J., Dolk, S., Krutki, P., Belichenko, P. V., and Dahlström, A. (1995). Contacts between serotonergic fibres and dorsal horn spinocerebellar tract neurons in the cat and rat: a confocal microscopic study. *Neuroscience* 67, 477–487. doi: 10.1016/0306-4522(95)00059-r

Jankowska, E., and Noga, B. R. (1990). Contralaterally projecting lamina VIII interneurons in middle lumbar segments in the cat. *Brain Res.* 535, 327–330. doi: 10.1016/0006-8993(90)91618-q

Jones, S. L., and Light, A. R. (1992). Serotonergic medullary raphespinal projection to the lumbar spinal cord in the rat: a retrograde immunohistochemical study. *J. Comp. Neurol.* 322, 599–610. doi: 10.1002/cne.903220413

Jordan, L. M. (1991). "Brainstem and spinal cord mechanisms for the initiation of locomotion," in *Neurobiological Basis of Human Locomotion*, eds M. Shimamura, S. Grillner and V. R. Edgerton (Tokyo: Japan Scientific Societies Press), 3–20.

Kausz, M. (1991). Arrangement of neurons in the medullary reticular formation and raphe nuclei projecting to thoracic, lumbar and sacral segments of the spinal cord in the cat. *Anat. Embryol.* 183, 151–163. doi: 10.1007/bf00174396

Kawagoe, K. T., Garris, P. A., and Wightman, R. M. (1993). pH-Dependent processes at Nafion®-coated carbon-fiber microelectrodes. *J. Electroanal. Chem.* 359, 193–207. doi: 10.1016/0022-0728(93)80409-b

Keithley, R. B., Heien, M. L., and Wightman, R. M. (2009). Multivariate concentration determination using principal component regression with residual analysis. *Trends Analytical Chem.* 28, 1127–1136. doi: 10.1016/j.trac.2009.07.002

Kiehn, O., Hultborn, H., and Conway, B. A. (1992). Spinal locomotor activity in acutely spinalized cats induced by intrathecal application of noradrenaline. *Neurosci. Lett.* 143, 243–246. doi: 10.1016/0304-3940(92)90274-b

Kiehn, O., and Kjærulff, O. (1996). Spatiotemporal characteristics of 5-HT and dopamine-induced rhythmic hindlimb activity in the *in vitro* neonatal rat. *J. Neurophysiol.* 75, 1472–1482.

Kuhr, R. F., and Wightman, R. M. (1986). Real-time measurement of dopamine release in rat brain. *Brain Res.* 381, 168–171. doi: 10.1016/0006-8993(86)90707-9

Li, Y., Hickey, L., Perrins, R., Werlen, E., Patel, A. A., Hirschberg, S., et al. (2016). Retrograde optogenetic characterization of the pontospinal module of the locus ceruleus with a canine adenoviral vector. *Brain Res.* 1641, 274–290. doi: 10.1016/j.brainres.2016.02.023

Liu, J., and Jordan, L. M. (2005). Stimulation of the parapyramidal region of the neonatal rat brain stem produces locomotor-like activity involving spinal 5-HT7 and 5-HT2A receptors. *J. Neurophysiol.* 94, 1392–1404. doi: 10.1152/jn.00136.2005

Lu, Y., Peters, J. L., and Michael, A. C. (1998). Direct comparisons of the response of voltammetry and microdialysis to electrically evoked release of striatal dopamine. *J. Neurochem.* 70, 584–593. doi: 10.1046/j.1471-4159.1998.70020584.x

MacDonell, C. W., Power, K. E., Chopek, J. W., Gardiner, K. R., and Gardiner, P. F. (2015). Extensor motoneurone properties are altered immediately before and during fictive locomotion in the adult decerebrate rat. *J. Physiol.* 593, 2327–2342. doi: 10.1113/JP270239

Marcoux, J., and Rossignol, S. (2000). Initiating or blocking locomotion in spinal cats by applying noradrenergic drugs to restricted lumbar spinal segments. *J. Neurosci.* 20, 8577–8585.

Marlier, L., Sandillon, F., Poulat, P., Rajaofetra, N., Geffard, M., and Privat, A. (1991). Serotonergic innervation of the dorsal horn of rat spinal cord: light and electron microscopic immunocytochemical study. *J. Neurocytol.* 20, 310–322. doi: 10.1007/bf01235548

Martin, K. F., Marsden, C. A., and Crespi, F. (1988). *In vivo* electrochemistry with carbon fibre electrodes: principles and application to neuropharmacology. *Trends Anal. Chem.* 7, 334–339. doi: 10.1016/0165-9936(88)90025-8

Matsumoto, I., Combs, M., Brannan, S., and Jones, D. J. (1990). Autoreceptor- and heteroreceptor-mediated regulation of monoamine release in spinal cord synaptosomes. *Ann. N Y Acad. Sci.* 604, 609–611. doi: 10.1111/j.1749-6632.1990.tb32040.x

Matsuyama, K., Kakajima, K., Mori, F., Aoki, M., and Mori, S. (2004). Lumbar commissural interneurons with reticulospinal inputs in the cat: morphology and discharge patterns during fictive locomotion. *J. Comp. Neurol.* 474, 546–561. doi: 10.1002/cne.20131

Maxwell, D. J., Leranth, C., and Verhofstad, A. A. J. (1983). Fine structure of serotonin-containing axons in the marginal zone of the rat spinal cord. *Brain Res.* 266, 253–259. doi: 10.1016/0006-8993(83)90656-x

Men, D.-S., Matsui, A., and Matsui, Y. (1996). Somatosensory afferent inputs release 5-HT and NA from the spinal cord. *Neurochem. Res.* 21, 1515–1519. doi: 10.1007/bf02533099

Michael, A. C., Justice, J. B., and Neill, D. B. (1985). *In vivo* chronoamperometric evaluation of DOPAC formation following electrical stimulation. *Neurosci. Lett.* 56, 365–369. doi: 10.1016/0304-3940(85)90270-8

Michael, A. C., and Wightman, R. M. (1999). Electrochemical monitoring of biogenic amine neurotransmission in real time. *J. Pharm. Biomed. Anal.* 19, 33–46. doi: 10.1016/s0731-7085(98)00145-9

Miles, G. B., and Sillar, K. T. (2011). Neuromodulation of vertebrate locomotor control networks. *Physiology (Bethesda)* 26, 393–411. doi: 10.1152/physiol.00013.2011

Miller, S., Van Der Burg, J., and Van Der Meche, F. G. A. (1975). Locomotion in the cat: basic programmes of movement. *Brain Res.* 91, 239–253. doi: 10.1016/0006-8993(75)90545-4

Mouchet, P., Manier, M., Dietl, M., Feuerstein, C., Bérod, A., Arluison, M., et al. (1986). Immunohistochemical study of catecholaminergic cell bodies in the rat spinal cord. *Brain Res. Bull.* 16, 341–353. doi: 10.1016/0361-9230(86)90055-9

Nakazato, T. (1987). Locus coeruleus neurons projecting to the forebrain and the spinal cord in the cat. *Neuroscience* 23, 529–538. doi: 10.1016/0306-4522(87)90074-1

Noga, B. R., Blythe, A. D., Brumley, M. R., Hentall, I. D., Kadam, B. H., and Sanchez, F. J. (2006). Relation between monoamine levels and parameters of fictive locomotion. *Soc. Neurosci. Abstr.* 252:23.

Noga, B. R., Blythe, A. D., Brumley, M. R., Hentall, I. D., Sanchez, F. J., and Taberner, A. M. (2007). Modulation of spinal serotonin and norepinephrine levels during locomotion and at rest: real-time measurements with multi-electrode cyclic voltammetry. *Soc. Neurosci. Abstr.* 924:13.

Noga, B. R., Bras, H., and Jankowska, E. (1992). Transmission from group II muscle afferents is depressed by stimulation of locus coeruleus/subcoeruleus, Kölliker-Fuse and raphe nuclei in the cat. *Exp. Brain Res.* 88, 502–516. doi: 10.1007/bf00228180

Noga, B. R., Fortier, P. A., Kriellaars, D. J., Dai, X., Detillieux, G. R., and Jordan, L. M. (1995a). Field potential mapping of neurons in the lumbar spinal cord activated following stimulation of the mesencephalic locomotor region. *J. Neurosci.* 15, 2203–2217.

Noga, B. R., Jankowska, E., and Skoog, B. (1995b). Depression of transmission from group II muscle afferents by electrical stimulation of the cuneiform nucleus in the cat. *Exp. Brain Res.* 105, 25–38. doi: 10.1007/bf00242179

Noga, B. R., Johnson, D. M. G., Riesgo, M. I., and Pinzon, A. (2009). Locomotor-activated neurons of the cat. I. Serotonergic innervation and co-localization of 5-HT_7, 5-HT_{2A} and 5-HT_{1A} receptors in the thoraco-lumbar spinal cord. *J. Neurophysiol.* 102, 1560–1576. doi: 10.1152/jn.91179.2008

Noga, B. R., Johnson, D. M. G., Riesgo, M. I., and Pinzon, A. (2011). Locomotor-activated neurons of the cat. II. Noradrenergic innervation and co-localization with $NA\alpha_{1a}$ and $NA\alpha_{2b}$ receptors in the thoraco-lumbar spinal cord. *J. Neurophysiol.* 105, 1835–1849. doi: 10.1152/jn.00342.2010

Noga, B. R., Kriellaars, D. J., Brownstone, R. M., and Jordan, L. M. (2003). Mechanism for activation of locomotor centers in the spinal cord by

stimulation of the mesencephalic locomotor region. *J. Neurophysiol.* 90, 1464–1478. doi: 10.1152/jn.00034.2003

Noga, B. R., Kriellaars, D. J., and Jordan, L. M. (1991). The effect of selective brainstem or spinal cord lesions on treadmill locomotion evoked by stimulation of the mesencephalic or pontomedullary locomotor regions. *Exp. Neurol.* 11, 1691–1700.

Noga, B. R., Pinzon, A., Mesigil, R. P., and Hentall, I. D. (2004). Steady-state levels of monoamines in the rat lumbar spinal cord—spatial mapping and the effect of acute spinal cord injury. *J. Neurophysiol.* 92, 567–577. doi: 10.1152/jn.01035.2003

Ohta, Y., and Grillner, S. (1989). Monosynaptic excitatory amino acid transmission from the posterior rhombencephalic reticular nucleus to spinal neurons involved in the control of locomotion in lamprey. *J. Neurophysiol.* 62, 1079–1089.

O'Neill, R. D., Lowry, J. P., and Mas, M. (1998). Monitoring brain chemistry *in vivo*: voltammetric techniques, sensors, and behavioral applications. *Crit. Rev. Neurobiol.* 12, 69–127. doi: 10.1615/critrevneurobiol.v12.i1-2.40

Orlovskii, G. N. (1970). Connexions between reticulospinal neurons and locomotor regions of the brainstem. *Biofizika* 15, 171–177.

Palij, P., and Stamford, J. A. (1994). Real-time monitoring of endogenous noradrenaline release in rat brain slices using fast cyclic voltammetry. 3. Selective detection of noradrenaline efflux in the locus coeruleus. *Brain Res.* 634, 275–282. doi: 10.1016/0006-8993(94)91930-5

Park, J., Takmakov, P., and Wightman, R. M. (2011). *In vivo* comparison of norepinephrine and dopamine release in rat brain by simultaneous measurement with fast-scan cyclic voltammetry. *J. Neurochem.* 119, 932–944. doi: 10.1111/j.1471-4159.2011.07494.x

Perreault, M.-C., Shefchyk, S. J., Jiménez, I., and McCrea, D. A. (1999). Depression of muscle and cutaneous afferent-evoked monosynaptic field potentials during fictive locomotion in the cat. *J. Physiol.* 521, 691–703. doi: 10.1111/j.1469-7793.1999.00691.x

Peters, J. L., and Michael, A. C. (1998). Modeling voltammetry and microdialysis of striatal extracellular dopamine: the impact of dopamine uptake on extraction and recovery ratios. *J. Neurochem.* 70, 594–603. doi: 10.1046/j.1471-4159.1998.70020594.x

Peterson, B. W., Maunz, R. A., Pitts, N. G., and Mackel, R. G. (1975). Patterns of projection and branching of reticulospinal neurons. *Exp. Brain Res.* 23, 333–351. doi: 10.1007/bf00238019

Phillips, P. E. M., Robinson, D. L., Stuber, G. D., Carelli, R. M., and Wightman, R. M. (2002). "Real-time measurements of phasic changes in extracellular dopamine concentration in freely moving rats by fast-scan cyclic voltammetry," in *Methods in Molecular Medicine, Vol 79: Drugs of Abuse: Neurological Reviews and Protocols*, ed. J. Q. Wang (Totawa, NJ: Humana Press Inc.), 443–464.

Poulat, P., Marlier, L., Rajaofetra, N., and Privat, A. (1992). 5-Hydroxytryptamine, substance P and thyrotropin-releasing hormone synapses in the intermediolateral cell column of the rat thoracic spinal cord. *Neurosci. Lett.* 136, 19–22. doi: 10.1016/0304-3940(92)90637-m

Rajaofetra, N., Ridet, J.-L., Poulat, P., Marlier, L., Sandillon, F., Geffard, M., et al. (1992). Immunocytochemical mapping of noradrenergic projections to the rat spinal cord with an antiserum against noradrenaline. *J. Neurocytol.* 21, 481–494. doi: 10.1007/bf01186952

Rasmussen, K., Morilak, D. A., and Jacobs, B. L. (1986). Single unit activity of locus coeruleus neurons in the freely moving cat. I. During naturalistic behaviors and in response to simple and complex stimuli. *Brain Res.* 371, 324–334. doi: 10.1016/0006-8993(86)90370-7

Rexed, B. (1954). A cytoachitectonic atlas of the spinal cord in the cat. *J. Comp. Neurol.* 100, 297–379. doi: 10.1002/cne.901000205

Ridet, J. L., Rajaofetra, N., Teilhac, J. R., Geffard, M., and Privat, A. (1993). Evidence for non-synaptic serotonergic and noradrenergic innervation of the rat dorsal horn and possible involvement of neuron-glia interactions. *Neuroscience* 52, 143–157. doi: 10.1016/0306-4522(93)90189-m

Ridet, J.-L., Sandillon, F., Rajaofetra, N., Geffard, M., and Privat, A. (1992). Spinal dopaminergic system of the rat: light and electron microscopic study using an antiserum against dopamine, with particular emphasis on synaptic incidence. *Brain Res.* 598, 233–241. doi: 10.1016/0006-8993(92)90188-f

Ridet, J.-L., Tamir, H., and Privat, A. (1994). Direct immunocytochemical localization of 5-hydroxytryptamine receptors in the adult rat spinal cord: a light and electron microscopic study using an anti-idiotypic antiserum. *J. Neurosci. Res.* 38, 109–121. doi: 10.1002/jnr.490380114

Rivot, J. P., Ory-Lavollee, L., and Chiang, C. Y. (1983). Differential pulse voltammetry in the dorsal horn of the spinal cord of the anaesthetized rat: are the voltammograms related to 5-HT and/or to 5-HIAA? *Brain Res.* 275, 311–319. doi: 10.1016/0006-8993(83)90992-7

Roberts, C., Price, G. W., Gaster, L., Jones, B. J., Middlemiss, D. N., and Routledge, C. (1997). Importance of 5-HT_{1B} receptor selectivity for 5-HT terminal autoreceptor activity: an *in vivo* microdialysis study in the freely moving guinea-pig. *Neuropharmacology* 36, 549–557. doi: 10.1016/s0028-3908(97)00026-9

Schmidt, B. J., and Jordan, L. M. (2000). The role of serotonin in reflex modulation and locomotor rhythm production in the mammalian spinal cord. *Brain Res. Bull.* 53, 689–710. doi: 10.1016/s0361-9230(00)00402-0

Shik, M. L., Severin, F. V., and Orlovskii, G. N. (1966). Control of walking and running by means of electrical stimulation of the mid-brain. *Biofizika* 11, 659–666.

Shik, M. L., Severin, F. V., and Orlovskii, G. N. (1967). Structures of the brain stem responsible for evoked locomotion. *Fiziol. Zh. SSSR Im. I M Sechenova* 53, 1125–1132.

Skagerberg, G., Björklund, A., Lindvall, O., and Schmidt, R. (1982). Origin and termination of the diencephalo-spinal dopamine system in the rat. *Brain Res. Bull.* 9, 237–244. doi: 10.1016/0361-9230(82)90136-8

Skoog, B., and Noga, B. R. (1991). Do noradrenergic descending tract fibres contribute to the depression of transmission from group II muscle afferents following brainstem stimulation in the cat? *Neurosci. Lett.* 134, 5–8. doi: 10.1016/0304-3940(91)90495-f

Sotnichenko, T. S. (1985). Differentiation of efferent projections of the medial (cuneiform nucleus) and lateral regions of the reticular formation in cat midbrain. *Neurophysiology* 17, 466–471. doi: 10.1007/bf01052183

Sqalli-Houssaini, Y., and Cazalets, J.-R. (2000). Noradrenergic control of locomotor networks in the *in vitro* spinal cord of the neonatal rat. *Brain Res.* 852, 100–109. doi: 10.1016/s0006-8993(99)02219-2

Stamford, J. A. (1986). *In vivo* voltammetry: some methodological considerations. *J. Neurosci. Methods* 17, 1–29. doi: 10.1016/0165-0270(86)90031-2

Stamford, J. A. (1989). *In vivo* voltammetry—prospects for the next decade. *Trends Neurosci.* 12, 407–412. doi: 10.1016/0166-2236(89)90081-7

Stamford, J. A., Cresp, I. F., and Marsden, C. A. (1992). "*In vivo* voltammetric methods for monitoring amine release and metabolism," in *Monitoring Neuronal Activity*, ed. J. A. Stamford (New York, NY: Oxford University Press), 113–145.

Steeves, J. D., and Jordan, L. M. (1980). Localization of a descending pathway in the spinal cord which is necessary for controlled treadmill locomotion. *Neurosci. Lett.* 20, 283–288. doi: 10.1016/0304-3940(80)90161-5

Steeves, J. D., and Jordan, L. M. (1984). Autoradiographic demonstration of the projections from the mesencephalic locomotor region. *Brain Res.* 307, 263–276. doi: 10.1016/0006-8993(84)90480-3

Steeves, J. D., Jordan, L. M., and Lake, N. (1976). The close proximity of catecholamine containing cells to the 'mesencephalic locomotor region' (MLR). *Brain Res.* 100, 663–670. doi: 10.1016/0006-8993(75)90166-3

Steeves, J. D., Schmidt, B. J., Skovgaard, B. J., and Jordan, L. M. (1980). The effect of noradrenaline and 5-hydroxytryptamine depletion on locomotion in the cat. *Brain Res.* 185, 349–362. doi: 10.1016/0006-8993(80)91073-2

Stevens, R. T., Apkarian, A. V., and Hodge, C. J. Jr. (1985). Funicular course of catecholamine fibers innervating the lumbar spinal cord of the cat. *Brain Res.* 336, 243–251. doi: 10.1016/0006-8993(85)90651-1

Stevens, R. T., Hodge, C. J. Jr., and Apkarian, A. V. (1982). Kölliker-Fuse nucleus: the principal source of pontine catecholaminergic cells projecting to the lumbar spinal cord of cat. *Brain Res.* 239, 589–594. doi: 10.1016/0006-8993(82)90533-9

Syková, E., and Svoboda, J. (1990). Extracellular alkaline-acid-alkaline transients in the rat spinal cord evoked by peripheral stimulation. *Brain Res.* 512, 181–189. doi: 10.1016/0006-8993(90)90625-l

Szokol, K., Glover, J. C., and Perreault, M. C. (2011). Organization of functional synaptic connections between medullary reticulospinal neurons and lumbar descending commissural interneurons in the neonatal mouse. *J. Neurosci.* 31, 4731–4742. doi: 10.1523/JNEUROSCI.5486-10.2011

Tyce, G. M., and Yaksh, T. L. (1981). Monoamine release from cat spinal cord by somatic stimuli: an intrinsic modulatory system. *J. Physiol.* 314, 513–529. doi: 10.1113/jphysiol.1981.sp013722

Veasey, S. C., Fornal, C. A., Metzler, C. W., and Jacobs, B. L. (1995). Response of serotonergic caudal raphe neurons in relation to specific motor activities in freely moving cats. *J. Neurosci.* 15, 5346–5359.

Viala, D., and Buser, P. (1969). The effects of DOPA and 5-HTP on rhythmic efferent discharges in hind limb nerves in the rabbit. *Brain Res.* 12, 437–443. doi: 10.1016/0006-8993(69)90011-0

Viala, D., Valin, A., and Buser, P. (1974). Relationship between the late reflex discharge and locomotor movements in acute spinal cats and rabbits treated with DOPA. *Arch. Ital. Biol.* 112, 299–306.

Wessendorf, M. W., Proudfit, H. K., and Anderson, E. G. (1981). The identification of serotonergic neurons in the nucleus raphe magnus by conduction velocity. *Brain Res.* 214, 168–173. doi: 10.1016/0006-8993(81)90449-2

Westlund, K. N., Bowker, R. M., Ziegler, M. G., and Coulter, J. D. (1982). Descending noradrenergic projections and their spinal terminations. *Prog. Brain Res.* 57, 219–238. doi: 10.1016/s0079-6123(08)64131-x

Wightman, R. M., Amatore, C., Engstrom, R. C., Hale, P. D., Kristensen, E. W., Kuhr, W. G., et al. (1988). Real-time characterization of dopamine overflow and uptake in the rat striatum. *Neuroscience* 25, 513–523. doi: 10.1016/0306-4522(88)90255-2

Wiklund, L., Léger, L., and Persson, M. (1981). Monoamine cell distribution in the cat brain stem. A fluorescence histochemical study with quantification of indolaminergic and locus coeruleus cell groups. *J. Comp. Neurol.* 203, 613–647. doi: 10.1002/cne.902030405

Zar, J. H. (1974). "Circular distribution," in *Biostatistical Analysis*, eds W. D. McElroy and C. P. Swanson (Englewood Cliffs, NJ: Prentice-Hall), 310–327.

Zhou, F. C., Tao-Cheng, J. H., Segu, L., Patel, T., and Wang, Y. (1998). Serotonin transporters are located on the axons beyond the synaptic junctions: anatomical and functional evidence. *Brain Res.* 805, 241–254. doi: 10.1016/s0006-8993(98)00691-x

Zoli, M., Torri, C., Ferrari, R., Jansson, A., Zini, I., Fuxe, K., et al. (1998). The emergence of the volume transmission concept. *Brain Res. Rev.* 26, 136–147. doi: 10.1016/S0165-0173(97)00048-9

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