NEURONAL CO-TRANSMISSION

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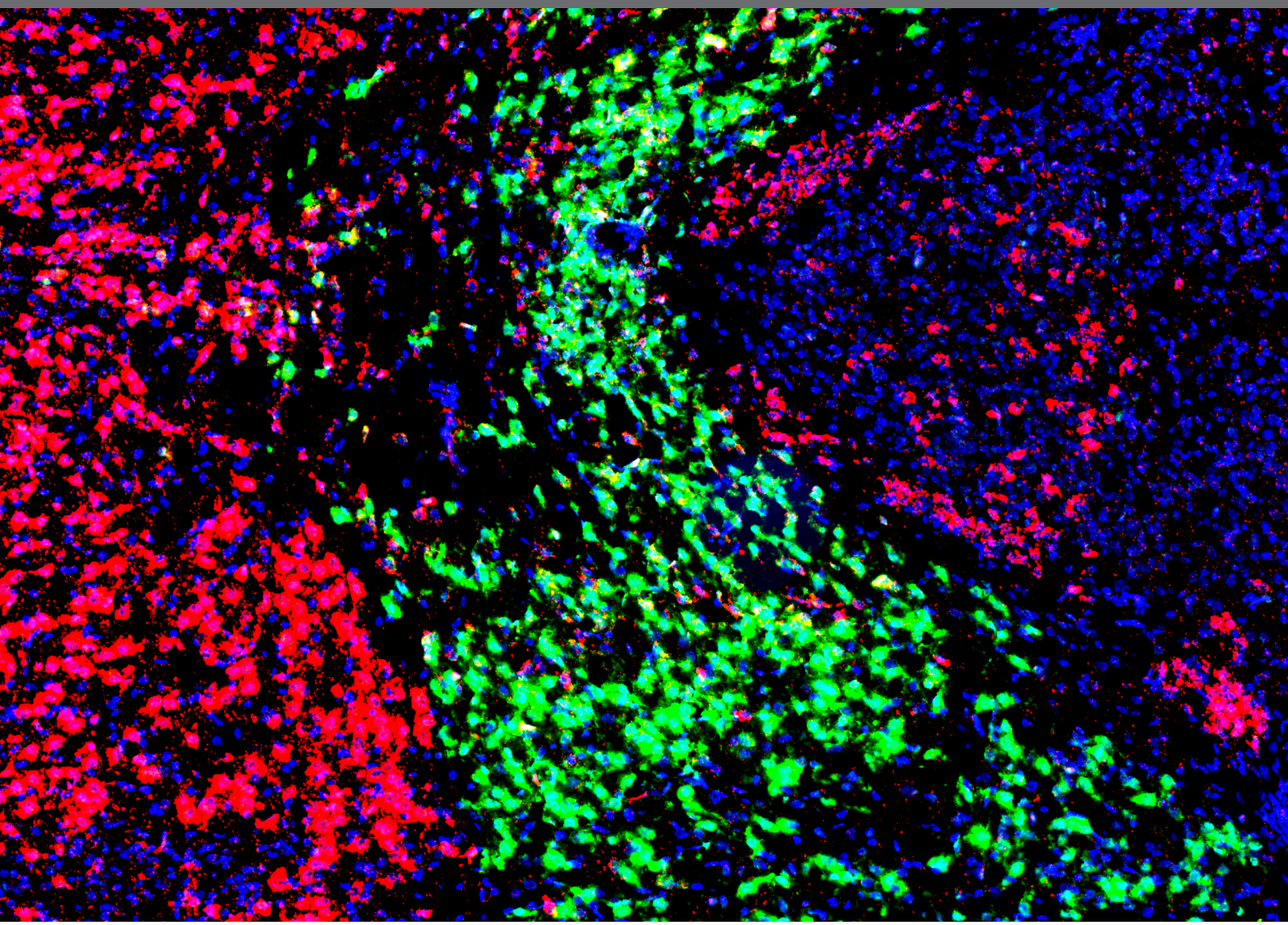
PUBLISHED IN: Frontiers in Neural Circuits and Frontiers in Cellular Neuroscience



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ISSN 1664-8714
ISBN 978-2-88945-945-2
DOI 10.3389/978-2-88945-945-2

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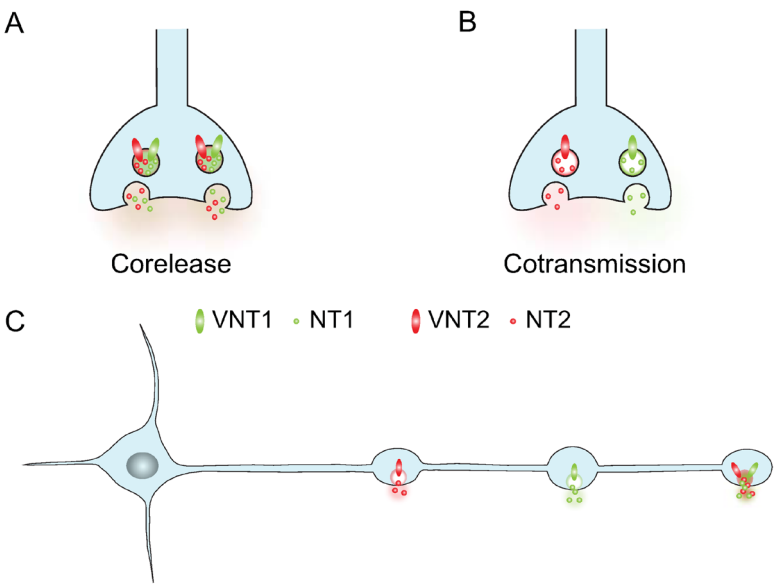
NEURONAL CO-TRANSMISSION



The co-expression of two vesicular neurotransmitter transporters by a single neuron generates multiple signaling possibilities. (A, B) Two different vesicular neurotransmitter transporters (VNT1 or VNT2) can either be addressed to the same synaptic vesicles (SVs) or segregated in different SVs. In the first case the two transmitters (NT1 and NT2) will be coreleased simultaneously, in the second one they can be differentially released. (C) Within a neuron, two different VNTs can be targeted the same or to different varicosities. With these two options, a neuron can release two different NTs from various varicosities or corelease two NTs from a single varicosity.

Image: Dr. Louis-Eric Trudeau and Dr. Salah El Mestikawy.
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Citation: Apergis-Schoute, J., Burnstock, G., Nusbaum, M. P., Parker, D., Morales, M. A., Trudeau, L.-E., Svensson, E., eds. (2019). Neuronal Co-transmission. Lausanne: Frontiers Media. doi: 10.3389/978-2-88945-945-2

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frontiers in Neural Circuits

EDITORIAL
published: 26 March 2019
doi: 10.3389/fncir.2019.00019

Editorial: Neuronal Co-transmission

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Keywords: neuropeptides, co-transmission, co-release, neurotransmitter segregation, neurotransmitter complexity

Editorial on the Research Topic

Neuronal Co-transmission

Neuronal co-transmission is now well-established as an aspect of nervous system function. However, this was not always the case, and acceptance of this important principle has required extensive work in a range of invertebrate and vertebrate systems (see Svensson et al. for a historical perspective). This work is reviewed in articles in this research topic, which we highlight in this Editorial.

A common principle highlighted by several articles in this research topic is that neuropeptides stored in dense core vesicles (DCVs) are co-localized with amines, and also with "classical" small molecule neurotransmitters stored in small synaptic vesicles in single terminals (SSVs; see Cropper et al.; Hökfelt et al.; Merighi; Nässel; Trudeau and El Mestikawy; Svensson et al.). SSVs can co-store and co-release protons and ATP, which can also elicit postsynaptic responses (Soto et al.; Svensson et al.). Soto et al. show that changes in extracellular pH activate acid sensing ion channels, and they discuss whether this is a synaptically-restricted signal or a volume modulator of neuron excitability.

Co-transmission in invertebrate neuronal circuits is highlighted by Nässel, Cropper et al., and Svensson et al.. Nässel summarizes the co-localization of neuropeptides and small molecule transmitters in *Drosophila* neuroendocrine cells, interneurons, and sensory and motor neurons, in circuits that influence learning and memory, circadian rhythms, and sensory, reproductive, developmental, and homeostatic functions. The circuit-related action of neuropeptides has been characterized in feeding circuits in the decapod crustacean stomatogastric ganglion (see the detailed discussion in Svensson et al.) and the *Aplysia californica* buccal ganglia (Cropper et al.). In *Aplysia*, analysis of the postsynaptic consequences of parallel neuropeptide actions has generated novel insights into the combinatorial effects of neuromodulators (Cropper et al.). Cropper et al. also address the role of different presynaptic activity patterns in the release of neuropeptides, an important aspect required for understanding the endogenous release of co-localized transmitters. Neuronal co-transmission has also been studied in vertebrate spinal circuits, both in dorsal horn sensory pathways and in spinal circuits involved in locomotion (see Svensson et al.).

The mammalian peripheral nervous system has been an important preparation for studying co-transmission in relation to autonomic responses, and it provided some of the key early physiological evidence for co-localization (Burnstock, 1976; see Svensson et al.). For example, in sympathetic neurons in the rat superior cervical ganglion acetylcholine and GABA are found in the same neurons, albeit segregated into different axonal varicosities (this has been defined as co-existence

Edited and reviewed by:
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Received: *13 February 2019*
Accepted: *05 March 2019*
Published: *26 March 2019*

Citation:
Apergis-Schoute J, Burnstock G, Nusbaum MP, Parker D, Morales MA, Trudeau L-E and Svensson E (2019) Editorial: Neuronal Co-transmission. Front. Neural Circuits 13:19. doi: 10.3389/fncir.2019.00019

rather than co-localization; the latter reflects two or more transmitters contained in a single terminal; Merighi, 2002). Other sympathetic neurons co-localize noradrenaline and ATP (see Svensson et al. and references therein). Merino-Jiménez et al. show that sympathetic neurons of the rat superior cervical ganglion segregate their neurotransmitters and co-transmitters to separate varicosities of single axons. They discuss whether sympathetic dysfunction in stress and hypertension correlate with changes in segregation, addressing the important question of the plasticity of transmitter systems during different functional states (Merino-Jimenez et al.).

Co-localization and co-existence occurs in multiple areas in the mammalian central nervous system, many of which are of clinical interest. For example, dopaminergic neurons in the ventral tegmental area contain glutamate and neuropeptides and project to both the nucleus accumbens and the neocortex (Pérez-López et al.; Trudeau and El Mestikawy.). In the nucleus accumbens, dopamine and glutamate, which are segregated to different varicosities, may play a role in drug addiction (Papathanou et al.). Dopamine and GABA released from ventral tegmental neurons can also influence the excitability of the prefrontal cortex (Pérez-López et al.). Dopaminergic projections from the substantia nigra and ventral tegmental area co-localize dopamine and GABA and make synapses on medial spiny neurons in the striatum. These dopamine neurons degenerate in Parkinson's disease, and understanding co-transmission from these neurons could provide novel insight into the treatment of Parkinson's disease symptoms (see Shen et al., 2018; Steinkellner et al., 2018). Trudeau and El Mestikawy specifically address the extent and functions of glutamate co-transmission in different classes of central neurons. They highlight the heterogeneous nature of glutamate co-transmission in different brain regions, and its implications for various functional processes like spatial learning, drug abuse and mood regulation. Hökfelt et al. discuss the co-localization of the neuropeptide galanin in noradrenergic neurons in the locus coeruleus. Galanin transmission is enhanced by stress and they suggest this could contribute to the induction of major depressive disorders. Co-localization of the neuropeptide orexin/hypocretin with glutamate helps regulate sleep/awake cycles. During waking, they are co-released: glutamate evokes fast excitation of histaminergic neurons and orexin/hypocretin boosts and prolongs this response by evoking a slow excitation. Dysfunction of these neurons causes narcolepsy: it is thus important to understand the functional role of this co-transmission in maintaining wakefulness (Svensson et al.).

Neuronal co-transmission has thus been extensively studied. However, there are many open questions, several of which are highlighted in this research topic. For example, we lack detailed knowledge about the subcellular organization of co-localized neurotransmitters and the presynaptic signals that differentiate their release. Merighi reviews the molecular composition and mechanisms of filling and release of large DCVs. He highlights future directions of research in the synthesis and storage of multiple transmitters in DCVs, including the possibility of selective transmitter sorting to different processes (see for example Fortin et al., 2019). An additional complexity is the combinatorial effects resulting from parallel neurotransmitter actions that can give rise to non-additive effects. This presents a challenge for any pharmacological intervention in the nervous system, as drug effects will depend on the background state of the system. Determining the general principles of these complex actions is thus a pressing need. New methodological developments that combine electrophysiology with optogenetic activation/inhibition of specific neuronal populations has started to provide insight into the functional roles and co-release of co-localized neurotransmitters (Papathanou et al.; Pérez-López et al.; Svensson et al.). The use of genetically modified animals (see Nässel; Svensson et al.) is also likely to provide important insights into the functional significance of co-transmission.

AUTHOR CONTRIBUTIONS

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

FUNDING

This work was supported by Byggmästare Olle Engkvist foundation and Major Gösta Linds foundation (ES), and from National Institute of Neurological Disease and Stroke Grant NS029436 (MN), Canadian Institutes of Health Research and the Brain Canada (Multi-Investigator Research Initiative) and Krembil Foundations (L-ET), Consejo Nacional de Ciencia y Tecnología (CONACYT) Mexico (Grant #128332) (MM).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neural Circuits

REVIEW
published: 21 January 2019
doi: 10.3389/fncir.2018.00117

General Principles of Neuronal Co-transmission: Insights From Multiple Model Systems

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It is now accepted that neurons contain and release multiple transmitter substances. However, we still have only limited insight into the regulation and functional effects of this co-transmission. Given that there are 200 or more neurotransmitters, the chemical complexity of the nervous system is daunting. This is made more-so by the fact that their interacting effects can generate diverse non-linear and novel consequences. The relatively poor history of pharmacological approaches likely reflects the fact that manipulating a transmitter system will not necessarily mimic its roles within the normal chemical environment of the nervous system (e.g., when it acts in parallel with co-transmitters). In this article, co-transmission is discussed in a range of systems [from invertebrate and lower vertebrate models, up to the mammalian peripheral and central nervous system (CNS)] to highlight approaches used, degree of understanding, and open questions and future directions. Finally, we offer some outlines of what we consider to be the general principles of co-transmission, as well as what we think are the most pressing general aspects that need to be addressed to move forward in our understanding of co-transmission.

Keywords: corelease, neurotransmitter complexity, neuromodulation, neuropeptides, colocalization

OPEN ACCESS

Edited by:
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Received: *13 September 2018*
Accepted: *14 December 2018*
Published: *21 January 2019*

Citation:
Svensson E, Apergis-Schoute J, Burnstock G, Nusbaum MP, Parker D and Schiöth HB (2019) General Principles of Neuronal Co-transmission: Insights From Multiple Model Systems. Front. Neural Circuits 12:117. doi: 10.3389/fncir.2018.00117

INTRODUCTION

Co-localization reflects the presence of two or more substances within single synaptic terminals. This suggests that two or more transmitters can be released (co-release) to act as messengers (co-transmission). However, co-localization does not necessarily mean co-release or co-transmission: one or more co-localized substances may not be released, and if released they may lack functional effects, at least on the assayed neuron/circuit/behavior. Criteria to establish a co-transmitter match those for single transmitters, including evidence for their release, the existence of receptors, and inactivating and removal mechanisms under physiological conditions.

Co-localized substances have been defined in different ways, for example (neuro)transmitter or (neuro)modulator, slow/fast, ionotropic/metabotropic, or conventional/modulatory. The terminology was widely discussed in the past (Kupfermann, 1991), but there are exceptions to the various classification schemes. We know that single substances can serve different roles from transmitter, modulator, trophic factor, etc., depending on where and when they are released and the receptors to which they bind. Thus, amino acid transmitters can generate fast or slow, classical

or modulatory, ionotropic or metabotropic, and signaling or trophic effects (Balazs, 2006). While amines and neuropeptides were considered to only generate slow, G protein receptor-mediated metabotropic responses, exceptions exist: the peptide FMRFamide activates ionotropic receptors (Cottrell, 1997), and 5-HT_3 receptors are an ionotropic monoaminergic receptor (Barnes et al., 2009).

One general co-localization principle is that amino acid transmitters [glutamate, glycine, γ-aminobutyric acid (GABA), but also acetylcholine (ACh)] are contained in small synaptic vesicles (SSVs) located at active zones, monoamines are contained in small light or dense-core vesicles (SDCVs), and neuropeptides in large DCVs (LDCVs) located away from active zones (Hökfelt et al., 2003). In comparison to SSVs, DCV release is relatively poorly understood (Bulgari et al., 2018). While SNARE complexes and synaptic proteins are used for Ca^{2+}-dependent DCV release, DCVs lack synapsins and do not form clusters at specialized release sites. This, together with their location away from the plasma membrane, tends to slow DCV release compared to SSV (Xia et al., 2009).

Co-transmission can be regulated by Ca^{2+}-dependent signals generated by the frequency and duration of spiking, which can differentially release co-localized components (Peng and Horn, 1991; Verhage et al., 1991; Vilim et al., 1996a; de Wit et al., 2009). The typical effect is SSV release with low rates of presynaptic spiking, with recruitment of SDCV release as the duration and/or frequency of spiking increases. LDCV release tends to occur with higher frequency or burst spiking. High frequency spiking could in turn reduce SSV-mediated transmission as releasable vesicles become depleted, their release is actively depressed, or their postsynaptic effects undergo desensitization.

Transmitters can also co-localize in single vesicles (Jonas et al., 1998; Vilim et al., 2000; Merighi et al., 2011). While this suggests obligatory co-release, differential release can still result from kiss-and-run-like mechanisms (Xia et al., 2009). Even with full fusion, differential movement of molecules through fusion pores could temporally dissociate effects, and different diffusion rates to target receptors or enzymatic degradation that generates fragments with modified biological activity could also generate temporally and spatially specific signals (de Wit et al., 2009).

A second general principle is that the co-localization and release of multiple transmitters provide flexibility to anatomically hard-wired circuits (see **Figure 1** for examples of some of these effects). This is the reason usually given for the preponderance of transmitter substances (Marder, 2012). In addition, transmitter-receptor mismatches at classical synapses suggest longer-distance "volume transmission" as a mechanism working in parallel with conventional local or "wired" synaptic transmission to generate more diffuse effects (Fuxe et al., 2010). Various factors influence volume signaling. The extracellular space is a 3-dimensional matrix containing proteoglycans that determine tortuosity and diffusion distances (e.g., μm for monoamines to mm for neuropeptides). Diffusion direction and distance can also be influenced by receptor affinities, concentration gradients, uptake

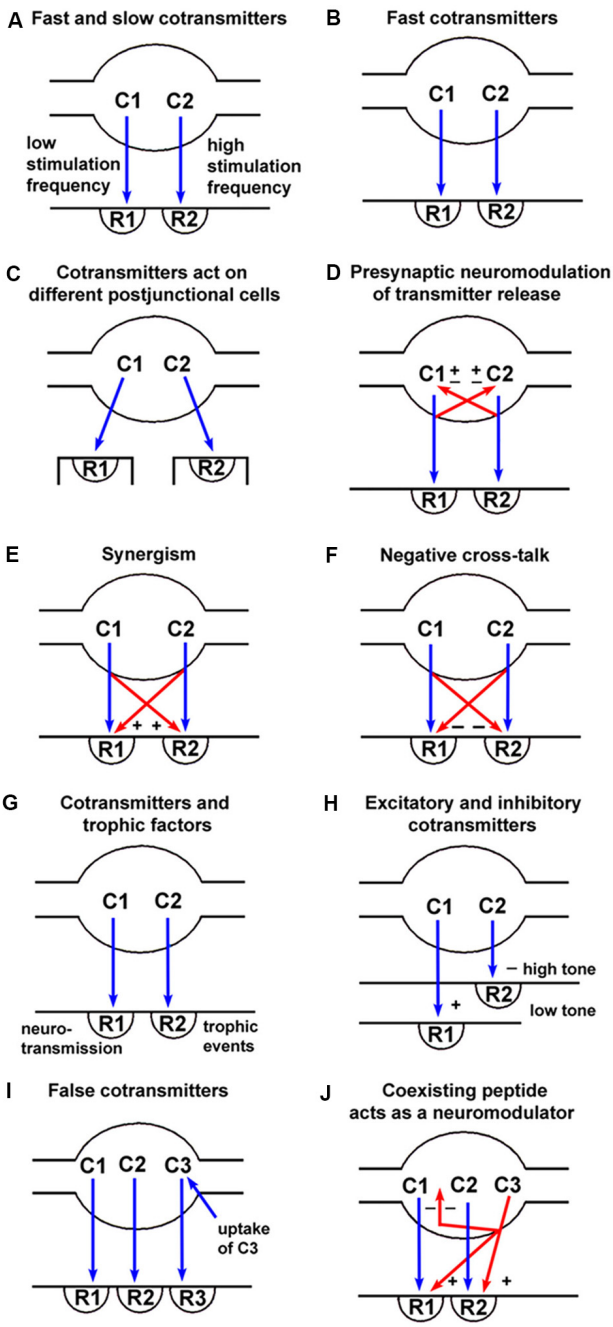

FIGURE 1 | Spectrum of signaling variations offered by co-transmission (blue arrows = neurotransmission; red arrows = pre- or post-junctional neuromodulation). **(A)** Fast transmission is usually produced by small molecules (C1) released at low frequency nerve stimulation acting on ionotropic receptors (R1), whereas slow transmission is usually produced by release of peptides (C2) or other molecules at high frequency stimulation acting on G-protein-coupled receptors (R2). **(B)** Co-transmitters C1 and C2 can both be fast messengers acting *via* ionotropic receptors (R1 and R2). **(C)** Co-transmitters C1 and C2 act on receptors (R1 and R2) localized on different postjunctional cells. **(D)** Co-transmitters C1 and C2 not only act postjunctionally *via* R1 and R2 receptors but can also act as prejunctional modulators to either inhibit (–) or enhance (+) the release of C1 and/or C2. **(E)** Co-transmitters C1 and C2 act synergistically to enhance the combined

(*Continued*)

FIGURE 1 | Continued
responses produced *via* R1 and R2 receptors. **(F)** Co-transmitters C1 and C2 act to inhibit the responses evoked *via* R1 and/or R2 receptors. **(G)** Co-transmitter C1 evokes neurotransmission *via* R1 receptors, while C2 evokes long-term (trophic) responses of postjunctional cells *via* R2 receptors. **(H)** Co-transmitter C1 produces excitation *via* R1 receptors when the postjunctional smooth muscle target has low tone, with C2 having little influence; however, when the smooth muscle tone is high, the dominant response might be relaxation produced by C2 *via* R2 receptors. **(I)** Substance C3 is taken up by nerve terminals, rather than being synthesized and stored as is true for the co-transmitters C1 and C2. C3 can then be released on nerve stimulation to act on postjunctional R3 receptors. In these circumstances, C3 would be known as a "false transmitter." **(J)** A coexisting substance C3 (often a peptide) can be synthesized and stored in a nerve, but not act directly *via* a postjunctional receptor to produce changes in postjunctional cell activity. It could, however, act as a prejunctional inhibitor (−) of the release of the co-transmitters C1 and C2, or as a postjunctional enhancer (+) of the responses mediated by R1 and R2. (Reproduced from Burnstock (2004), with permission from Elsevier).

or breakdown mechanisms, charges on extracellular matrix molecules, or "tidal" effects caused, for example, by pressure differences resulting from cerebral blood flow (Krimer et al., 1998).

A third general principle is that a single transmitter can diverge to affect multiple receptors on multiple targets, while multiple transmitters can converge onto single effectors (Swensen and Marder, 2000; Brezina, 2010; Harris-Warrick and Johnson, 2010). These effects can change depending on the functional state of the targets. Co- or simultaneous release of transmitters will create a chemical "soup" around neurons that can alter individual transmitter effects (Brezina, 2010; Harris-Warrick and Johnson, 2010). Prior modulator release could also leave a background "modulatory tone" determined by the duration of 2nd messenger pathways and the phosphorylation state of targets that will influence subsequent effects. Rather than asking if modulatory systems interact, it seems more a question of how could they not. Analyzing one modulator at a time is of obvious utility in characterizing effects, but as with any experimental approach we need to ensure that we are not constraining system variables too tightly and as a result miss aspects essential to understanding normal function. While two or more ionotropic transmitters could interact through voltage and conductance changes, two (or more) modulators have multiple potential sites of interaction, including receptor binding, G protein activation, 2nd messenger cascades, and target effectors. When scaled up to the multiple transmitters and multiple targets in networks, the potential complexity is obvious. These interactions may be designed to constrain individual co-transmitter effects to prevent "over-modulation" (Harris-Warrick and Johnson, 2010; Marder et al., 2014), to decouple the divergent effects of a single modulator to produce net changes not possible with any single modulator (Brezina, 2010), or to modulate a shift from synaptic to a cellular driven activity (McClelland and Parker, 2017). However, we also have to consider that co-released transmitters do not necessarily interact (Yang et al., 1996; Blitz and Nusbaum, 1999).

HISTORICAL PERSPECTIVES ON CO-TRANSMISSION

It was not until the early 1950's that chemical transmission was fully accepted. John Eccles, who had been the most prominent critic of chemical transmission, used newly developed micropipettes and amplification equipment to examine his electrical hypothesis of transmission. Brock et al. (1952) recorded inhibitory postsynaptic potentials (IPSPs) in cat spinal cord motor neurons, an observation that negated his electrical hypothesis (Karl Popper had encouraged him to formulate his electrical hypothesis in a form that could be negated), and as a result he accepted chemical transmission (Parker, 2018). From almost 50 years of debate on the nature of central nervous system (CNS) transmission, the chemical transmission paradigm rapidly developed, principally through the work of Bernard Katz (1966) on the statistical nature of transmission and the role of Ca^{2+}.

Much of the debate over chemical and electrical transmission was between Eccles and Henry Dale, one of the main proponents of chemical transmission during the first half of the 20th century. Eccles et al. (1954) coined the term "Dale's Principle" when suggesting that motor neurons use the same transmitter at spinal cord collaterals to Renshaw cells as they do at the neuromuscular junction (Eccles et al., 1954):

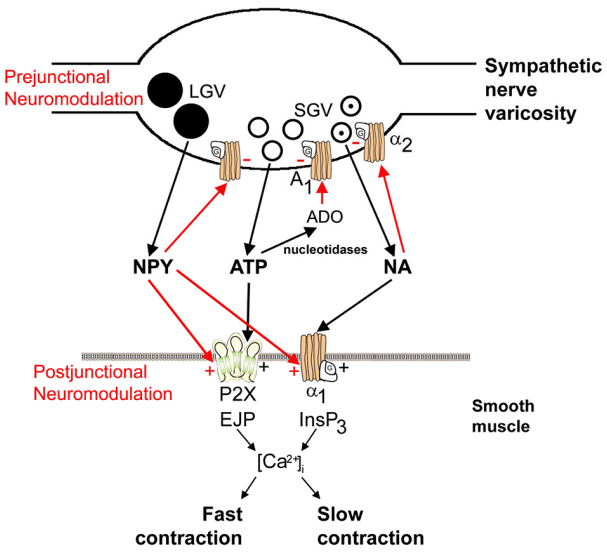

FIGURE 2 | Schematic of sympathetic co-transmission. Adenosine 5'-triphosphate (ATP) and noradrenaline (NA) released from small granular vesicles (SGVs) act on P2X and α_1 receptors on smooth muscle, respectively. ATP acting on inotropic P2X receptors evokes excitatory junction potentials (EJPs), an increase in intracellular calcium $[(Ca^{2+})]_i$ and fast contraction; while metabotropic α_1 adrenoceptors leads to production of inositol triphosphate ($InsP_3$), an increase in $(Ca^{2+})_i$ and slow contraction. Neuropeptide Y (NPY) stored in large granular vesicles (LGVs) acts after release both as a prejunctional inhibitory modulator of release of ATP and NA and as a postjunctional modulatory potentiator of the actions of ATP and NA. Soluble nucleotidases are released from nerve varicosities, and are also present as ectonucleotidases [reproduced from Burnstock and Verkhratsky (2010) with permission from Elsevier].

"In conformity with Dale's principle that the same chemical transmitter is released from all the synaptic terminals of a neurone." While not directly stated, this was subsequently taken to mean that neurons release a single transmitter at all of their synapses (the statement was probably not worded carefully because chemical transmission had only very recently been accepted, and with only two known transmitters the possibility of co-localization was probably not of obvious concern). Dale of course never stated this principle. Eccles et al referred to a lecture by Dale in 1935 in which he asked if identification of a peripheral chemical transmitter would "furnish a hint as to the nature of the transmission process at a central synapse? The possibility has at least some value as a stimulus to further experiment" (Dale, 1935). The subsequent erroneous interpretation of Dale's Principle as one-neuron-one-transmitter led to claims by some that the principle had been invalidated when evidence of co-localization started to appear [this was even claimed long after co-localization was accepted (Nicoll and Malenka, 1998)]. As a result Eccles (1976) wrote "I proposed that Dale's Principle be defined as stating that at all the axonal branches of a neurone, there was liberation of the same transmitter substance or substances". Use of "substances" obviously removed any limit on how many transmitters were contained or released. This version of Dale's principle has, however, been negated. Sossin et al. (1990) showed that the transmitter content differed in separate processes of single *Aplysia* neurons; Blitz and Nusbaum (1999) showed likely differential release of GABA and the peptide proctolin from separate terminals of a projection neuron in the crustacean stomatogastric ganglion (STG); Sulzer and Rayport (2000) showed that dopaminergic neurons co-release glutamate from only some terminals and Ludwig and Leng (2006) showed differential release from dendrites and synaptic terminals of single neurons.

Evidence for co-localization was actually found in vertebrates and invertebrates quite soon after chemical transmission was established (Abrahams et al., 1957; Burn and Rand, 1959; Gerschenfeld et al., 1960; Singh and Singh, 1966). For example, De Robertis and Pellegrino De (1961) showed the presence of two different types of vesicle in pineal gland terminals, Kerkut et al. (1967) showed the uptake of Dopa and 5-hydroxytryptamine (5-HT) in snail neurons and Su et al. (1971) showed co-release of adenosine 5′-triphosphate (ATP) and noradrenaline (NA) at sympathetic terminals. Jaim-Etcheverry and Zieher (1973) used the term "coexistence" for the location of NA and 5-HT in the pineal gland, Brownstein et al. (1974) showed anatomical evidence for co-localization in *Aplysia* neurons and Cottrell (1976) suggested that both ACh and serotonin were released from a single snail neuron to generate fast and slower responses, respectively. The development of immunohistochemistry directly demonstrated co-localization, leading to a plethora of studies showing multiple substances in single synaptic terminals (Hökfelt et al., 2000; Hökfelt, 2009), and co-localization of transmitters has now become the norm. For reviews detailing the changing concepts of co-localization and transmission see Burnstock (1976, 2014); Potter et al. (1981); Cuello (1982); Osborne (1983); Kupfermann (1991); Lundberg (1996) and Hökfelt (2009).

PURINERGIC CO-TRANSMISSION IN THE AUTONOMIC AND CENTRAL NERVOUS SYSTEM

One of the first formal statements of the potential for co-transmission came from analyses of purinergic transmission (Burnstock, 1976), "Do some nerve cells release more than one transmitter?". The purine nucleotide ATP had been identified as a signaling molecule in 1972 (Burnstock, 1972), and shown to be a co-transmitter with NA in sympathetic nerves see **Figure 2** (Su et al., 1971; Nakanishi and Takeda, 1973; Burnstock, 1976, 1995; Langer and Pinto, 1976; Westfall et al., 1978). ATP has been subsequently found to co-localize with various transmitters in the peripheral and CNS (see **Table 1** and reviews by Westfall et al., 1978; Burnstock, 2007; Wier et al., 2009; Hill-Eubanks et al., 2010; Hnasko and Edwards, 2012; Kennedy, 2015). For example, purinergic co-transmission is involved in the sympathetic control of arterial pressure in rats (Emonnot et al., 2006); as a co-transmitter with ACh in carotid body arterial chemoreceptors (Zapata, 2007); in the human carotid body where ACh, ATP and cytokines are co-released during hypoxia (Kåhlin et al., 2014); ATP and glutamate are released ectopically from vesicles along

TABLE 1 | Table showing transmitters co-localized with adenosine 5′-triphosphate (ATP) in the peripheral and central nervous systems (CNS).

Peripheral nervous system		
Sympathetic nerves	ATP + NA + NPY	Westfall et al. (1978) and Burnstock (1990)
Parasympathetic nerves	ATP + ACh +VIP	Hoyle (1994)
Sensory-motor	ATP + CGRP + SP	Burnstock (1993)
NANC enteric nerves	ATP +NO + VIP	Belai and Burnstock (1994) and Burnstock (2001)
Motor nerves (in early development)	ATP + ACh	Henning (1997)
Central nervous system		
Cortex, caudate nucleus	ATP + ACh	Richardson and Brown (1987)
Hypothalamus, locus coeruleus	ATP + NA	Sperlágh et al. (1997) and Poelchen et al. (2001)
Hypothalamus, dorsal horn, retina	ATP + GABA	Jo and Role (2002)
Mesolimbic system	ATP + DA	Krügel et al. (2013)
Hippocampus, dorsal horn	ATP + glutamate	Mori et al. (2001) and Fujii (2004)

Modified from Abbracchio et al. (2009), with permission. ACh, acetylcholine; CGRP, calcitonin gene-related peptide; DA, dopamine; GABA, γ-aminobutyric acid; 5-HT, 5-hydroxytryptamine; NA, noradrenaline; NO, nitric oxide, NPY, neuropeptide Y; SP, substance P; VIP, vasoactive intestinal peptide.

axons to mediate neurovascular coupling *via* glial calcium signaling (Thyssen et al., 2010); co-localized ATP and NA are involved in the sympathetic thermoregulatory response to cooling (Kozyreva et al., 2015); and ATP is released from dopaminergic neurons of the mouse retina and midbrain (Ho et al., 2015). For reviews describing the physiological significance of purinergic co-transmission see Burnstock (2004, 2014).

Changes in ATP co-transmission have also been implicated in pathological states. ATP is a co-transmitter with ACh in parasympathetic nerves supplying the diseased human bladder (Palea et al., 1993), and in sympathetic nerves in spontaneously hypertensive rats (Bulloch and McGrath, 1992). ATP also appears to be a co-transmitter involved in sympathetic pain, causalgia and sympathetic dystrophy, and is enhanced in inflammatory and stress conditions (Burnstock, 1996). Changes in transmitter co-localization in disease states or after injury is a common feature in different systems (see "Spinal Cord Modulation and Co-transmission" section below), which provides strong support for a specific functional role for co-transmission.

THE *APLYSIA FEEDING CIRCUIT*

The impact of co-transmission on circuit activity has been analyzed in detail in invertebrate systems. These systems offer use conventional electrophysiological techniques to identify and determine the function of co-transmitters in physiologically identified neurons in defined neuronal circuits (Kupfermann, 1991; Nusbaum et al., 2001, 2017; Cropper et al., 2018).

The *Aplysia* feeding circuit has provided important insights into the functional role of co-transmission (Brezina, 2010). Brezina et al. (1996) and have provided a detailed analysis of how the peptide co-transmitters small cardioactive peptide and myomodulin modulate muscle contractions evoked by their small molecule co-transmitter ACh released from motor neurons onto muscle controlling feeding behavior. More significantly, this analysis has shown that transmitter-specific divergent and convergent interactive effects of the modulators on targets involved in excitation-contraction coupling (e.g., calcium channels, potassium channels, relaxation rate) can evoke novel effects not seen with either modulator individually.

Aplysia can generate two antagonistic feeding behaviors, namely ingestion and egestion. Repetitively stimulating the command neuron cerebral-buccal interneuron 2 (CBI-2) can activate the feeding central pattern generator (CPG) in the buccal ganglion to progressively produce ingestive motor programs "repetition priming of motor activity" (Cropper et al., 2014). CBI-2 co-localizes ACh, feeding circuit activating peptide (FCAP), and cerebral peptide 2 (CP-2). The neuropeptide actions diverge (CP-2 appears to act presynaptically while FCAP acts postsynaptically at the same cholinergic synapses) but their combined actions converge to potentiate fast cholinergic EPSPs in motor neurons B61/62 (**Figure 3A**; Koh et al., 2003; Koh and Weiss, 2005). Co-transmission thus allows distinct signals to be sent that regulate "conventional" transmission.

Egestion is activated by repetitive stimulation of the esophageal nerve, which induces a short-term potentiation of synaptic transmission between interneuron B20 and the follower

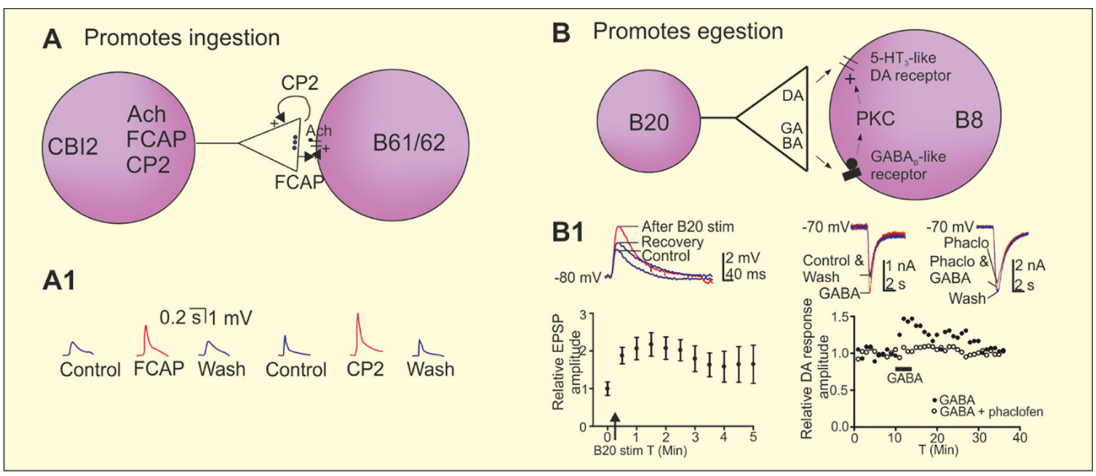

FIGURE 3 | Co-transmission in the regulation of ingestion and egestion in the *Aplysia* feeding circuit. **(A)** Repeated stimulation of the cerebral buccal interneuron 2 (CBI-2) progressively induces the ingestive motor programs in *Aplysia*. The command neuron CBI-2 uses acetylcholine (Ach) as its fast-excitatory neurotransmitter onto the motor neurons B61/62. CBI-2 also co-localize the two neuropeptides feeding circuitry activating peptide (FCAP) and cerebral peptide 2 (CP-2). Both peptides are released by high frequency stimulation of CBI-2 and contributes to post-tetanic potentiation (PTP) of the fast-cholinergic transmission at the CBI-2 to B61/62 synapses, however by different mechanisms. CP-2 is acting presynaptically and FCAP postsynaptically. **(A1)** Recording traces showing the potentiation of the cholinergic EPSP by FCAP and CP-2 in B8. **(B)** Repeated stimulation of the esophageal nerve and high frequency stimulation of the interneuron B20 that co-localizes dopamine and γ-aminobutyric acid (GABA) induces egestion and a short-term potentiation of the fast dopaminergic EPSPs between B20 and the motor neuron B8. Dopamine acts on a 5-HT_3-like receptor and GABA contributes to the potentiation of the B20 to B8 EPSP by a postsynaptic mechanism that involves activation a $GABA_B$ receptor of protein kinase C (PKC). **(B1)** High frequency stimulation of B20 potentiates the dopaminergic EPSPs in B8. GABA potentiated fast dopaminergic responses in B8 and the effect is blocked by the $GABA_B$ receptor antagonist phaclofen. Adapted from Koh et al. (2003), Koh and Weiss (2005) and Svensson et al. (2014).

motor neuron B8. B20 co-localizes GABA and dopamine (Díaz-Ríos and Miller, 2005; Svensson et al., 2014). Dopamine acts as a fast-excitatory transmitter by acting on a 5-HT_3-like receptor. GABA does not have any fast direct effect at this synapse, but can potentiate dopaminergic responses by acting on a $GABA_B$ receptor and subsequently activating protein kinase C (PKC; **Figures 3B,B1**; Svensson et al., 2014). This is an example where the "conventional" transmitter (GABA) evokes a modulatory effect and the "modulatory" transmitter (dopamine) a conventional effect. This effect of GABA is considered to be an example of intrinsic modulation by a co-transmitter as it modulates the circuit to which it belongs. Co-localization of dopamine and GABA is common. It also occurs in lamprey, and in dopaminergic neurons of the mammalian substantia nigra pars compacta and ventral tegmental area (VTA; Barreiro-Iglesias et al., 2009; Tritsch et al., 2012; Berrios et al., 2016; Ntamati and Lüscher, 2016), and in hypothalamic feeding circuits (see below).

CO-TRANSMISSION CONSEQUENCES IN THE DECAPOD CRUSTACEAN STOMATOGASTRIC NERVOUS SYSTEM

Early Contributions

Significant insight into the cellular and circuit effects of co-localized transmitters has been obtained in the isolated decapod crustacean stomatogastric nervous system (STNS: **Figure 4**; Nusbaum et al., 2001, 2017). The STNS, an extension of the decapod CNS, is composed of four ganglia plus their connecting and peripheral nerves (**Figure 4**; Nusbaum et al., 2001; Marder and Bucher, 2007). The ganglia include the paired commissural ganglia (CoGs: each containing $\geq$500 neurons) and the unpaired oesophageal (OG: 15–20 neurons) and stomatogastric (STG: 25–30 neurons) ganglia; the number of neurons per ganglion is species-specific. These ganglia contain several CPG circuits which regulate the ingestion and processing of food by the striated muscles of the foregut. As is common for CPGs, these circuits continue to operate in the isolated STNS, maintained in physiological saline, in a manner similar to their activity *in vivo* (Heinzel et al., 1993; Diehl et al., 2013; Yarger and Stein, 2015). The gastric mill (chewing) and pyloric (pumping and filtering of chewed food) circuits, both located in the STG, are extensively characterized (**Figure 4**; Marder and Bucher, 2007; Stein et al., 2007; Marder, 2012; Nusbaum et al., 2017). Despite each of these circuits being composed of a small number of neurons, they are remarkably flexible in their response to different modulatory influences and can generate many different versions of the gastric mill and pyloric rhythms.

Co-transmission studies in the STNS, primarily in the crab *Cancer borealis*, have involved manipulating the activity of identified modulatory projection neurons and sensory neurons which influence the gastric mill- and pyloric circuits (see **Table 2**: Katz and Harris-Warrick, 1990; Blitz and Nusbaum, 1999; Blitz et al., 1999; Wood et al., 2000; Wood and Nusbaum, 2002; Christie et al., 2004; Stein et al., 2007; DeLong et al., 2009a,b). As summarized below, these studies, and related ones in the lobster STNS (Meyrand et al., 2000; Thirumalai and Marder, 2002; Kwiatkowski et al., 2013), revealed that co-transmitting neurons provide many degrees of freedom to circuit outputs.

Applied vs. Endogenously Released Neuropeptides

As discussed in several sections of this review, neuropeptides, the largest and most diverse class of neurotransmitters, are commonly present as co-transmitters (Merighi et al., 2011; Taghert and Nitabach, 2012; van den Pol, 2012; Nusbaum et al., 2017; Nässel, 2018). Due to the challenges associated with studying peptidergic (co)transmission, neuropeptide actions have often been studied *via* their exogenous application to the nervous system. This approach has been considered a reasonable proxy for peptidergic transmission because neuropeptide release is envisioned to act *via* volume transmission (see above). There are, however, several reasons why exogenously applied and neurally-released peptides would not necessarily have equivalent actions (Nusbaum et al., 2017), one of which is the interaction of their effects with those of co-released transmitters. The relative influence of exogenously applied and endogenously released peptides was directly determined in *C. borealis* by comparing the pyloric rhythm response to bath-applied proctolin (RYLPT) with that evoked by separate stimulation of the three proctolinergic projection neurons [modulatory proctolin neuron (MPN), modulatory commissural neuron 1 (MCN1), MCN7] that innervate the STG (**Figure 5**). Proctolin application and stimulating each proctolin neuron all excite/modulate the pyloric rhythm, but only the pyloric rhythm configured by MPN activity was comparable to proctolin application (Nusbaum and Marder, 1989a,b; Blitz et al., 1999). MCN1 or MCN7 stimulation elicited pyloric rhythms that were different from that driven by MPN/proctolin and from each other (Blitz et al., 1999; Wood et al., 2000). Interestingly, the match between MPN stimulation and proctolin application occurred despite the fact that MPN co-releases GABA (Blitz and Nusbaum, 1999; see "Convergent and Divergent Co-transmission" section below).

Shared Co-transmitters and Circuit Targets

Neurons which use the same co-transmitters and contact the same circuit can nevertheless elicit distinct responses from that circuit. This was established in the crab STG by comparing the influence of MPN and MCN1, two projection neurons which both use proctolin and GABA as co-transmitters (**Figure 5**; Blitz et al., 1999). MCN1 also contains a second peptide co-transmitter, CabTRP Ia (Christie et al., 1997; Blitz et al., 1999). When CabTRP Ia actions were suppressed, MPN and MCN1 still elicited distinct pyloric motor patterns (Wood et al., 2000). Motivated by earlier studies in *Aplysia* that documented how effectively extracellular peptidase activity can regulate peptidergic actions (Sigvardt et al., 1986; Owens et al., 1992; Rothman et al., 1992), a proctolin peptidase inhibitor was applied to the STG, resulting in convergence of the MPN- and MCN1-elicited pyloric rhythms (Coleman et al., 1995; Wood and Nusbaum, 2002). These results suggested that the peptidergic action of these two neurons on the pyloric circuit was being

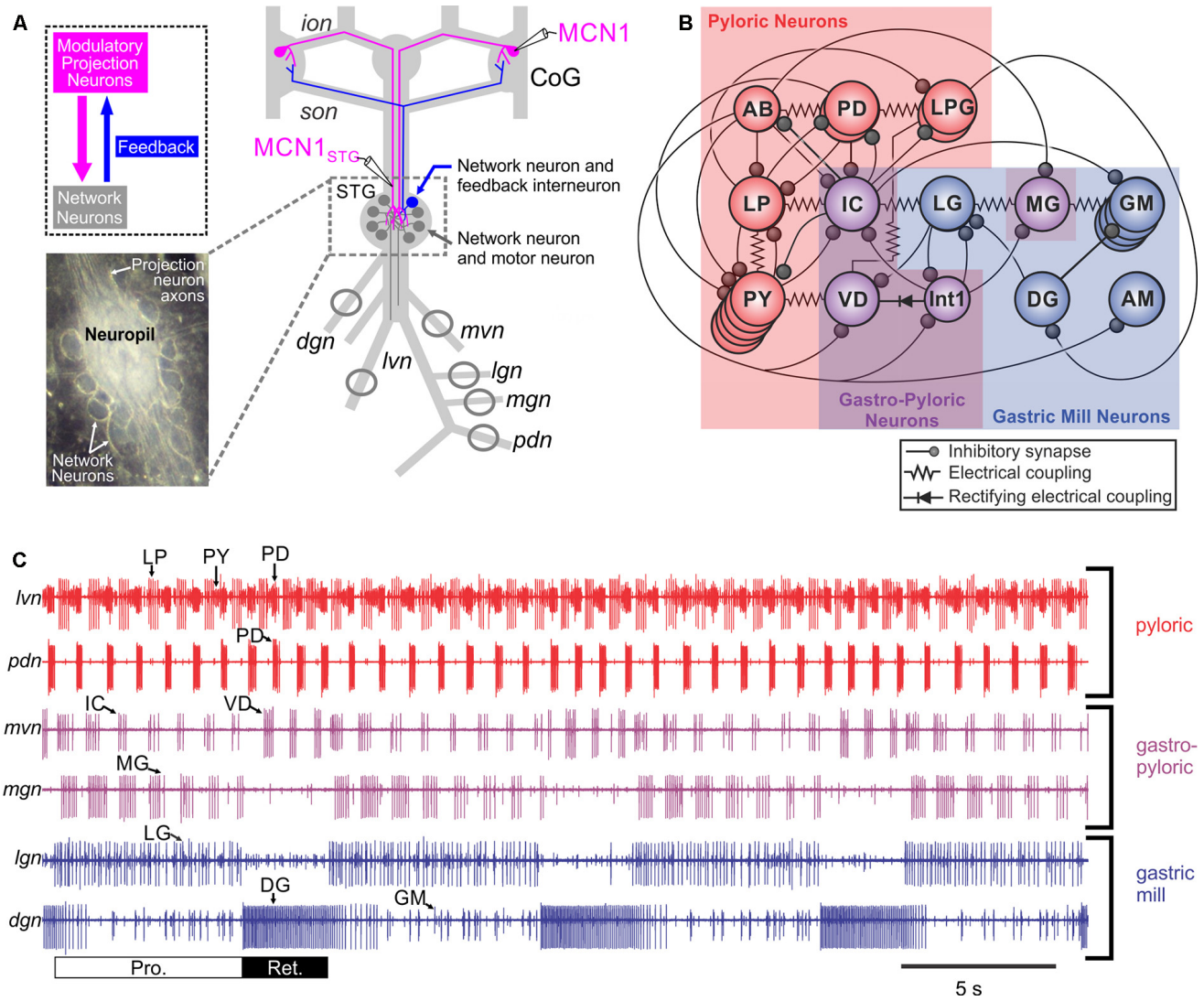

FIGURE 4 | The crab *Cancer borealis* stomatogastric nervous system (STNS). **(A)** Schematic of the isolated STNS of the crab *Cancer borealis*. The inset shows a whole-mount image of the desheathed stomatogastric ganglion (STG) under dark-field illumination (anterior, top; posterior, bottom). As it is evident in both the schematic and the inset, the 26 neuronal somata form a single layer surrounding the neuropil. Circles on nerves indicate recording sites for traces shown in part **(C)**. **(B)** Schematic of the gastric mill and pyloric circuit. The arrangement of neurons in the schematic represents the relative timing of activity for each neuron during the gastric mill and pyloric rhythms. Specifically, the neurons that exhibit pyloric rhythm-timed activity ("pyloric neurons" and "gastropyloric neurons") are displayed such that the top-row neurons are co-active, followed by the middle-row neurons and then the bottom-row neurons, after which the top-row neurons are again active. The neurons that exhibit gastric mill rhythm (GMR)-timed activity ("gastric mill neurons" and "gastropyloric neurons") are displayed such that the top-row neurons are co-active and burst in alternation with the bottom-row neurons. As shown, there are eight gastric mill circuit neuron types, one of which is present as four apparently equivalent copies (GM neurons). All eight neuron types contribute to gastric mill pattern generation, whereas only two (LG and Int1) are also rhythm generator neurons (Coleman et al., 1995; Bartos et al., 1999; Saideman et al., 2007). There are seven pyloric circuit neuron types, including the rhythm generator ("pacemaker") group AB/PD/LPG (Marder and Bucher, 2007). Three of these neuron types are present as multiple, apparently equivalent copies (PD: 2; LPG: 2; PY: 5). **(C)** Simultaneous extracellular nerve recordings of the gastric mill and pyloric rhythms during tonic stimulation of the modulatory projection neuron modulatory commissural neuron 1 (MCN1) are shown. The pyloric rhythm exhibits a rhythmically repeating triphasic pattern (for example, lateral ventricular nerve (*lvn*): PD, LP, PY) that is continuously active, *in vivo* and *in vitro*, with a cycle period of ∼1 s. The GMR (cycle period ∼10–20 s) is silent except when driven by modulatory neurons (for example, MCN1), which themselves require activation *in vivo* and *in vitro*. It is a rhythmically repeating biphasic pattern, consisting of teeth protraction (Pro.) and teeth retraction (Ret.), which drives the motor response (chewing). Note that some neurons exhibit activity patterns time-locked to both rhythms (gastropyloric neurons). CoG, commissural ganglion; *dgn*, dorsal gastric nerve; *ion*, inferior oesophageal nerve; *lgn*, lateral gastric nerve; *mgn*, medial gastric nerve; *mvn*, medial ventricular nerve; *pdn*, pyloric dilator nerve; *son*, superior oesophageal nerve; *stn*, stomatogastric nerve. Panels **(A)** and **(C)** are adapted with permission Nusbaum et al. (2017), Nature Reviews Neuroscience. Panel **(B)** is adapted with permission from Nusbaum and Beenhakker (2002), Nature. STG photo courtesy of Marie Suver, New York University, USA, and Wolfgang Stein, Illinois State University, USA.

TABLE 2 | Identified co-transmitter neurons that influence stomatogastric ganglion (STG) circuits.

Identified neuron	Co-transmitters	Target circuit(s)	Key action
[1]GPR	ACh, 5HT, AST-A	PR, GMR	PR/GMR: M/A
[1]MPN	GABA, Proct	PR, GMR	PR: M/A; GMR: I
[2]GN1/2	GABA, CCK-LI, FXRFamide-LI	PR, GMR	PR/GMR: M/A
[1]MCN1	GABA, Proct, CabTRP Ia	PR, GMR	PR: M/A; GMR: A
[1,3]IVN	HA, FXRFamide-LI	PR, GMR, OR	PR: I; GMR: A; OR: M
[4]PS	HA, Crust-MS*	PR, GMR, OR	PR/GMR/OR: M/A

*Legend: M, modulates; A, activates; I, inhibits; LI, like-immunoreactivity; *Crust-MS is a FXRF-like peptide. [1]C. borealis; [2]H. gammarus; [3]P. interruputs; [4]H. americanus. PR, pyloric rhythm; GMR, gastric mill rhythm; OR, oesophageal rhythm.*

differently sculpted, at least partly, by MPN and MCN1 releasing (a) comparable amounts of proctolin at different distances from its sites of extracellular cleavage by peptidase activity, and/or (b) different amounts of proctolin per action potential (Wood and Nusbaum, 2002).

Convergent and Divergent Co-transmission

The concepts of convergent and divergent signaling were established in the earliest co-transmission studies (Jan et al., 1979, 1980; Hökfelt et al., 1980; Lundberg et al., 1981; Jan and Jan, 1982). These seminal studies also established that neurally-released peptides can diffuse to activate receptors well beyond the boundaries of the synaptic cleft (Jan and Jan, 1982). Soon thereafter, convergent co-transmission was revealed in the arthropod (insect and crustacean) neuromuscular system (Adams and O'Shea, 1983; Bishop et al., 1987), while divergent co-transmission was documented in several systems, ranging from the *Aplysia* neuroendocrine and neuromuscular systems to the rodent thalamus (Mayeri et al., 1985; Sigvardt et al., 1986; Vilim et al., 1996a,b, 2000; Koh et al., 2003; Sun et al., 2003).

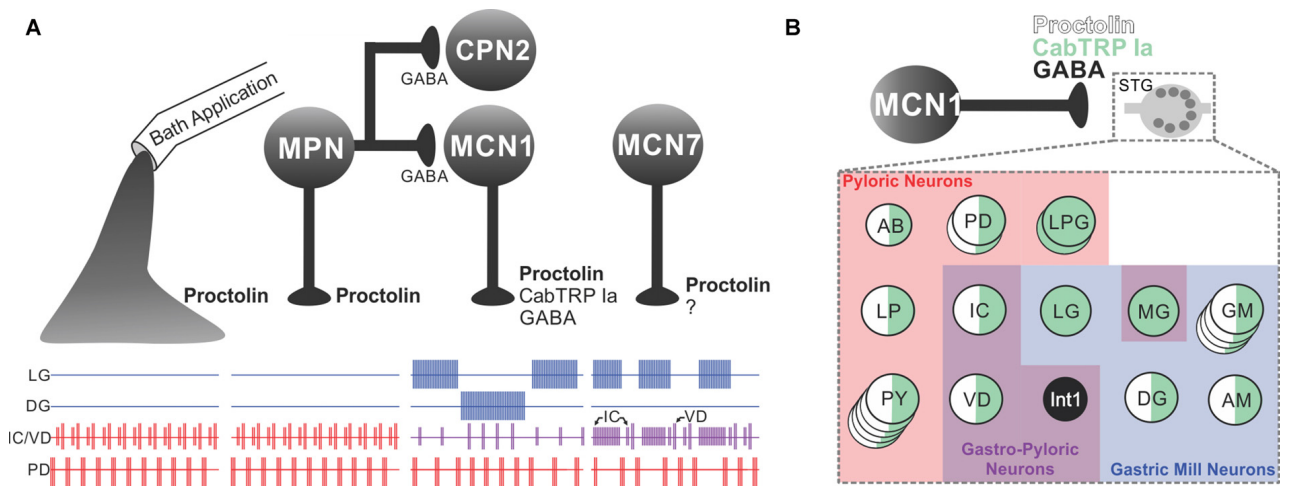

FIGURE 5 | The microcircuit response to peptidergic neuron activity is not necessarily mimicked by bath application of that neuropeptide. **(A)** Extracellular recordings of identified neurons in the crab *Cancer borealis* STG, which are active during the GMR (LG and DG neurons), pyloric rhythm (PD neuron) or both rhythms (IC and VD neurons). In the isolated crab STG, bath-applied proctolin (far left set of responses) selectively excites the pyloric rhythm (Marder et al., 1986; Nusbaum and Marder, 1989a). This action mimics the response to activation of only one [modulatory proctolin neuron (MPN)] of the three proctolinergic projection neurons that innervate the STG (MPN, MCN1 and MCN7), even though MPN also contains a small-molecule co-transmitter (GABA; Blitz et al., 1999). As indicated, MPN also inhibits two projection neurons [MCN1 and commissural projection neuron 2 (CPN2)] by releasing GABA from a separate axon projecting to a separate location (CoG; Blitz and Nusbaum, 1997, 1999). The other two proctolinergic projection neurons (MCN1 and MCN7) also influence STG microcircuit activity but elicit activity patterns from the circuit neurons that are distinct from proctolin bath application (Coleman and Nusbaum, 1994; Blitz et al., 1999). MCN1-released *C. borealis* tachykinin-related peptide Ia (CabTRP Ia) and GABA are pivotal for MCN1 activation of the GMR, whereas its release of CabTRP Ia and proctolin dominates its excitation of the pyloric rhythm (see part **B**). The MCN7 actions on these rhythms result partly from proctolin and probably also from one or more yet-to-be-identified co-transmitters (indicated by "?"). In the figure, pyloric rhythm activity is shown in red; GMR activity is shown in blue; gastropyloric activity is shown in purple. **(B)** In the crab STG, MCN1 influences all pyloric, gastropyloric and gastric mill neurons. The figure shows a representation of responsiveness of each STG circuit neuron to the MCN1-released co-transmitters proctolin (white w/black border), CabTRP Ia (green) and GABA (black; Swensen and Marder, 2001; Stein et al., 2007). Examples of convergent peptide co-transmitter action (proctolin and CabTRP Ia), selective peptide co-transmitter action (CabTRP Ia) and selective GABA action are shown. In some cases, the STG neuron only responds to the indicated co-transmitter (or co-transmitters; for example, Int1). In other cases, the STG neuron does respond to an additional co-transmitter but not when it is released from MCN1 (for example, LG responds to applied GABA but not GABA released from MCN1). No information is available regarding whether these co-transmitters are colocalized to all MCN1 terminals or are localized to separate terminals for their release. Panel **(A)** is adapted with permission from Nusbaum et al. (2001), Elsevier. Panel **(B)** is adapted with permission from Nusbaum et al. (2017), Nature Reviews Neuroscience.

There are many potential consequences of convergent and divergent co-transmission for circuit operation. For example, each could have linear or non-linear actions on circuit neurons and/or the circuit output. Additionally, different neurons in the same circuit could be targets of convergence or divergence (Wood et al., 2000; Thirumalai and Marder, 2002; Stein et al., 2007; Nusbaum et al., 2017). Given the myriad potential degrees of freedom provided by co-transmission, elucidating the cellular and synaptic mechanisms underlying their impact on circuit activity is facilitated by intracellular access to all circuit neurons.

One of the earliest studies of convergent peptidergic co-transmission examined the MCN1 (proctolin, CabTRP Ia) influence on the crab pyloric rhythm (Wood et al., 2000). This study compared the pyloric rhythm in response to normal MCN1 input relative to its input after CabTRP Ia actions were suppressed, and showed that both peptides contributed to the effects of MCN1 and had comparable actions on each pyloric circuit neuron. Their relative influence, however, appeared to be unequal because when CabTRP Ia actions were suppressed the pyloric cycle frequency and activity level of pyloric neurons remained >50% larger than without MCN1 stimulation. This interpretation, however, was inconclusive because proctolin and CabTRP Ia activate the same voltage-dependent ionic current (called I_{MI}) in STG neurons, albeit by binding to different receptors (Swensen and Marder, 2000, 2001; DeLong et al., 2009b). This convergence suggested a ceiling effect due to the combined action of both peptides activating all available I_{MI} so that, when CabTRP Ia actions were suppressed, more I_{MI} might have been available for proctolin to activate. I_{MI} activation can be limiting in STG neurons (Garcia et al., 2015), but in this case there appeared to be no such limitation because with the CabTRP Ia actions present, the peptidergic excitation of several pyloric neurons by MCN1 was increased in the presence of a proctolin peptidase inhibitor relative to normal MCN1 stimulation (Wood and Nusbaum, 2002).

Divergent co-transmission can act on separate circuit neurons and separate circuits, providing several different types of functional flexibility. These include: (1) influencing separate target neurons in the same circuit to collectively affect the circuit-level response; (2) selectively influencing release of a subset of co-transmitters; (3) enabling temporally separate responses from different circuit neurons; and (4) displaying spatially separate co-transmitter actions that influence different circuits. In the American lobster (*Homarus americanus*) STNS, a projection neuron that innervates the STG contains the peptides RPCH and CabTRP Ia (Thirumalai and Marder, 2002). This is the only source of CabTRP Ia in the *H. americanus* STG neuropil, while there is one additional RPCH neuron innervating this STG. Co-applying these peptides, but not their separate application, to the isolated STG activates the complete pyloric rhythm sequence of AB/PD, LP and PY neuron bursting. Applying them separately reveals that their actions converge to excite the pyloric pacemaker group AB/PD, but RPCH only activates the LP neuron while CabTRP Ia activates only the PY neurons. Divergent actions also result from the influence of the muscle stretch-sensitive sensory neuron GPR, which contains 5-HT, ACh and AST-A peptide (A-type allatostatin; Beltz et al., 1984; Katz et al., 1989; Skiebe and Schneider, 1994; Szabo et al., 2011). GPR primarily uses divergent co-transmission to modulate the pyloric- and GMRs, with the caveat that its AST actions remain to be determined. GPR has convergent 5-HT and ACh actions on only one pyloric circuit neuron (IC neuron), while it evokes only cholinergic EPSPs in the VD neuron and only serotonergic modulatory responses in the remaining circuit neurons (AB, PD, LPG, LP, PY). Collectively, these actions modify the pyloric cycle frequency and the pattern of the ongoing rhythm (Katz and Harris-Warrick, 1989, 1990). The relative influence of the cholinergic EPSPs and 5-HT modulation remains to be determined, but the latter effect clearly outlasts the former. As a further wrinkle, GPR that is rhythmically active *in situ* with a cycle period (∼10–20 s), that is much slower than the pyloric rhythm (∼1 s), due to the GMR-timed stretch of the muscles that GPR innervates. Thus, its pyloric circuit modulation waxes and wanes along with the gastric mill cycle motor pattern.

Selective Regulation of Co-transmitter Release

GPR also influences an ongoing MCN1-driven GMR, prolonging the retractor phase without altering the protractor phase duration (Beenhakker et al., 2005). This GPR action again results from divergent co-transmission, as it is suppressed by a 5-HT receptor antagonist (DeLong et al., 2009a). GPR has divergent co-transmitter actions on all three GMR generator neurons [LG, Int1, $MCN1_{STG}$ (axon terminals in STG)] but only its 5-HT-mediated inhibition of $MCN1_{STG}$ is effective during this GMR (Beenhakker et al., 2005; DeLong et al., 2009a). Interestingly, this 5-HT inhibition of $MCN1_{STG}$ selectively suppresses the MCN1 peptidergic (CabTRP Ia) excitation of LG without altering its GABAergic action onto Int1. The ability to separately change the amount of each co-transmitter released from a neuron can increase the functional flexibility of a co-transmitting neuron in a state-dependent manner.

Temporally distinct effects underlie the MCN1 projection neuron activation of the GMR, a result of its divergent co-transmitter excitation of the GMR generator neurons LG and Int1 (Coleman et al., 1995; Stein et al., 2007). During the GMR, MCN1 releases its co-transmitters during the retraction phase, but while Int1 is active during retraction, LG is active during protraction. This sequential activation is accomplished in part by MCN1 eliciting a fast, ionotropic (GABA) excitation of Int1 and a slow, metabotropic (CabTRP Ia) excitation of LG (see also Schöne et al., 2014).

Spatially separate co-transmitter actions on different circuits have also been demonstrated. In addition to its proctolinergic excitation of the pyloric rhythm in the crab STG (Nusbaum and Marder, 1989a,b), MPN projects an axon to each CoG where it produces a GABAergic inhibition of the projection neurons MCN1 and CPN2 (Blitz and Nusbaum, 1997, 1999). Although MCN1/CPN2 are excited by

applied proctolin, they do not respond to MPN stimulation when the GABAergic inhibition is pharmacologically suppressed, suggesting that MPN does not release proctolin in the CoG (Blitz and Nusbaum, 1999; Marder, 1999). The MPN actions in the CoG ensure a proctolin-specific modulation of the pyloric rhythm in the STG, and prevent MCN1/CPN2 activation of the GMR. Similarly, the IVN (*C. borealis*, *Panulirus interruptus*)/PS (*H. americanus*) neuron has divergent co-transmitter actions in the STG and CoG which directly and indirectly influence the STG circuits, as well as influencing the oesophageal circuit in the CoG (Russell and Hartline, 1981; Sigvardt and Mulloney, 1982; Claiborne and Selverston, 1984; Marder and Eisen, 1984; Christie et al., 2004; Kwiatkowski et al., 2013).

Species-Specific Co-transmission

While there is considerable conservation of structure and function in the STNS across species of lobster, crab, crayfish and shrimp (Böhm et al., 2001; Marder and Bucher, 2007; Dickinson et al., 2008; Hui et al., 2011; Tuszynski et al., 2015), species-dependent differences in the gastric mill and pyloric rhythms are readily recognizable. In a few cases, the apparently species-equivalent co-transmitting projection neurons have been studied in different crabs and lobsters. This includes comparison of MPN (*C. borealis*) and GN1/2 (GABA neuron 1/2: *Homarus gammarus*), as well as IVN (*C. borealis*, *P. interruptus*) and PS (*H. gammarus*, Russell and Hartline, 1981; Sigvardt and Mulloney, 1982; Claiborne and Selverston, 1984; Marder and Eisen, 1984; Meyrand et al., 2000; Christie et al., 2004; Kwiatkowski et al., 2013). The results of these studies highlight species-dependent similarities and differences in the co-transmitter content and function of the apparently same projection neuron. For example, the small molecule transmitter is unchanged (MPN, GN1/2: GABA; IVN, PS: histamine) but the peptide co-transmitter(s) differ(s) (**Table 2**). However, even when a co-transmitter was conserved across species, it did not always perform the same function, and in some cases the changed peptide co-transmitter did perform a comparable function.

Co-transmission Consequences: Future Directions

With the continual development of stimulation and imaging techniques of ever-increasing resolution, the impact of co-transmission on circuit activity has blossomed to include many more model systems (Barker et al., 2016; Qiu et al., 2016; Granger et al., 2017; Nusbaum et al., 2017; Nässel, 2018). These recent studies have revealed conservation of mechanisms across species and circuits (e.g., convergent and divergent co-transmission; different temporal dynamics of ionotropic and metabotropic co-transmission; focal regulation of co-transmitter release), as well as diverse new ways in which activity is modified by co-transmission. It is already clear that the flexibility imparted to circuit output by co-transmission firmly places the parallel goal of determining the connectome for particular behaviors as a necessary but not sufficient foundation for understanding the neuronal basis of behavior (Bargmann, 2012; Bargmann and Marder, 2013; Meinertzhagen, 2018).

Despite the already evident diversity of mechanisms by which co-transmission alters circuit output, the future promises more surprises. For example, to date most co-transmission studies, in the STNS and elsewhere, have focused on the circuit response to single co-transmitter inputs/populations. However, it is likely that circuit operation *in vivo* receives parallel input from different neurons. This raises the issue of whether the consequences of parallel co-transmission will be evident from studying the impact of the individual components. Moreover, given that the impact of even individual co-transmitting inputs on circuit output is state-dependent (e.g., dependent on the physiological state of the target circuit, as well as the firing pattern and relative amounts of co-transmitters released by the inputs), each such study will need to be performed under rigorously defined conditions. Ultimately, such studies may require a blend of *in vitro* and *in vivo* recordings and manipulations to best establish both mechanism and behaviorally appropriate "state."

There are also likely to be new functions revealed for co-transmitters. For example, in the *C. borealis* STG the co-release of the peptide proctolin from the projection neuron MCN1 does not directly influence any of the GMR generator neurons (Stein et al., 2007). However, a recent study suggests that MCN1-released proctolin may well indirectly influence GMR generation by slowing the enzymatic degradation of co-released CabTRP Ia. Specifically, [des-Arg^1] proctolin was recently identified as a cleavage product of scorpion venom which effectively inhibits the endopeptidase neprilysin in arthropods (Duzzi et al., 2016). Neprilysin is likely the extracellular peptidase in the STG neuropil that cleaves and inactivates CabTRP Ia (Wood et al., 2000), and [des-Arg^1] proctolin is the first cleavage product of proctolin in the STG (Coleman et al., 1994; Wood et al., 2000).

As already established in some systems, the release of different co-transmitters can be separately regulated and this regulation can occur focally, such as at particular axon terminals (DeLong et al., 2009a; Nusbaum et al., 2017). Under such conditions, the same neuron(s) can release different relative amounts of its co-transmitter complement from different release sites. Such compartmentalization further challenges investigators aiming to elucidate the cellular and synaptic mechanisms by which co-transmission affects neural signaling.

Understanding the impact of co-transmission, even on circuits composed of a small number of neurons, has benefitted from the use of computational models (DeLong et al., 2009b). As co-transmission studies scale up, both in terms of circuit size and the number of degrees of freedom made possible by co-transmission, hopes of attaining deep insight into the functional consequences of such events will be buoyed by the ever-increasing collaboration between experimentalists, theorists and modelers.

SPINAL CORD MODULATION AND CO-TRANSMISSION

The spinal cord contains numerous transmitters in descending, sensory, and intraspinal systems (Hökfelt, 2009). These generally lack organizing principles, with the exception of 5-HT which Jacobs and Fornal (1993) proposed biases motor over sensory activity (Jacobs and Fornal, 1993). To illustrate the transmitter complexity of the spinal cord, consider the dorsal horn (this differs between regions and species, and thus this summary is not definitive; see Todd, 2010). Transmitter co-localization is common, as are ligand–receptor mismatches indicative of volume transmission.

Most nociceptive Aδ and C fibers terminate in laminae I–II, and mechanoreceptive Aβ-fibers in laminae III–VI. Laminae I-III contains densely packed neurons: most are local inhibitory interneurons, the remainder local excitatory or projection neurons (Todd, 2010). Glutamate is released from primary afferents and from local and descending neurons. Metabotropic glutamate receptors are concentrated on local interneurons in lamina II, while ionotropic glutamate receptors are found in all dorsal horn laminae and on primary afferent terminals. GABAergic neurons are found in laminae I-III. These neurons activate $GABA_A$ and $GABA_B$ receptors to regulate nociceptive and mechanosensory inputs. GABA receptor levels are reduced by peripheral nerve lesions, suggesting they are located on afferent terminals and GABA exerts its effects presynaptically. Glycine receptors are found in lamina 1 and II, but at greater levels in deeper laminae. Glycine receptor levels are not significantly affected by peripheral lesions, suggesting localization on dorsal horn neurons [glycine receptors may be present on some low threshold mechanosensory afferent terminals (Todd, 2010)]. Glycine and GABA can co-localize, and glycine and $GABA_A$ receptors are found at many postsynaptic specializations in laminae I-III. ACh receptors are also present in lamina III-V, and a cholinergic plexus in lamina II-III receives inputs from unmyelinated and myelinated axons. Conventional transmitters can thus regulate specific afferent inputs presynaptically and/or postsynaptically.

The monoamines 5-HT and NA are found at all levels of the dorsal horn where they can act on dorsal horn neurons or afferent terminals. Both monoamines are predominantly released from descending brainstem neurons (these neurons provided key evidence of transmitter co-localization, see Hökfelt, 2009). For example, 5-HT is released from different raphe nuclei to affect sensory, motor and autonomic functions (Ghosh and Pearse, 2014). Rostral raphe neurons project to the dorsal horn and contain 5-HT and possibly GABA, while caudal raphe neurons project ventrally and co-localize 5-HT, glutamate, substance P, and TRH (Hökfelt et al., 2000). 5-HT is released synaptically in the ventral spinal cord, but paracrinally in the dorsal horn (Perrier and Cotel, 2015).

Neuropeptides are concentrated in laminae I and II [e.g., TRH, enkephalins, bombesin, substance P, vasoactive intestinal peptide (VIP), somatostatin, neurotensin, Cholecystokinin (CCK), neuropeptide Y (NPY), galanin], but also at deeper levels [e.g., substance P, enkephalins, somatostatin (Todd, 2010)]. Neuropeptides are found in descending or afferent neurons [(e.g., Galanin, CGRP SP, somatostatin, VIP, and CCK; peptides seem to be absent in Aβ fibers), and dorsal horn neurons (e.g., neurotensin and NPY)]. There are no absolute divisions in terms of where individual peptides are found, for example, substance P is found in Aδ and C afferents, in dorsal horn neurons, and in descending neurons (Jessell et al., 1979). Receptor localization is also diffuse: opiate receptor levels are reduced but not abolished following dorsal rhizotomy, suggesting they are located presynaptically on afferent terminals and postsynaptically on dorsal horn neurons. Peptides co-localize with amino acid transmitters: neurotensin, somatostatin and neurokinin B in glutamatergic neurons, galanin and NPY in GABAergic neurons, and others (e.g., enkephalins) in both glutamatergic and GABAergic neurons (Zhang et al., 1993; Xu et al., 2008; Sardella et al., 2011). Peptides also co-localize: met-enkephalin neurons contain tachykinins and somatostatin. As tachykinins and somatostatin do not co-localize there are probably distinct sub-populations of enkephalin-containing cells. CGRP co-localizes with substance P, somatostatin, or galanin. Galanin and substance P, and substance P and somatostatin co-localize, but galanin and somatostatin do not (Ju et al., 1987), again suggesting different sub-populations of peptidergic neurons.

This multiplicity of transmitters is a conserved feature, but is it necessary? Mammalian neurotransmitters serve diverse functions in bacteria and plants, and were presumably co-opted for neuronal signaling (Roshchina, 2010). The specific patterns of location and co-localization suggest functional relevance. This is supported by changes in transmitter systems with injury or disease. For example, galanin levels are up-regulated after dorsal root transection (Xu et al., 2008). Peptide receptor levels also change: inflammation increases delta opiod receptors in dorsal root ganglia and dorsal horn neurons to enhance endogenous analgesia, whereas substance P receptors are internalized (Merighi et al., 2011).

In principle one transmitter acting on multiple receptors could evoke excitation, inhibition, and modulation (Eccles, 1982). However, selecting these effects would be difficult if receptors were located at the same postsynaptic sites. Presynaptic regulation would also be difficult as autoreceptor activation would result whenever the transmitter was released. Spatially separating synapses/receptors could segregate effects, but this places demands on spinal cord size and organization. Differential effects could occur if receptors serving different functions had different thresholds, but this would be limited. Consider presynaptic regulation of transmitter release from Neuron1 by Neuron2. A high threshold receptor on Neuron1 terminals would prevent its activation when Neuron1 released transmitter, but this would demand greater release from Neuron2 to activate the receptor to evoke presynaptic regulation. Any autoreceptors on Neuron2 would then need an even higher threshold to prevent their activation, leading to ever increasing thresholds and release levels as system complexity increased. Threshold level regulation would also limit signaling distances, reducing the opportunity for volume transmission (Fuxe et al., 2010). As a specific example

consider the activity-dependent potentiation of nociceptive transmission ("wind-up") in the dorsal horn (Dubner and Ruda, 1992). Substance P released from nociceptive afferents potentiates NMDA responses to postsynaptically increase nociceptive responses: this can be reduced by opioid mediated pre- or postsynaptic inhibition. For wind-up to occur using only glutamate, increased glutamate release following nociceptor activation could activate higher threshold NMDA-receptors to trigger Ca^{2+}-dependent 2nd messenger pathways that potentiate nociceptive signaling. Descending glutamatergic inputs could act on mGluRs to pre- or postsynaptically inhibit responses, but to prevent mGluR activation by afferent glutamate release would require the higher activation threshold or spatial separation outlined above. Multiple transmitters are clearly advantageous.

Studying Spinal Cord Modulation

Analyses of spinal cord motor outputs and their modulation have typically used fictive locomotion (pharmacologically or electrically-evoked activity recorded from ventral roots in isolated spinal cords). However, assumptions that fictive activity matches normal locomotion has been questioned on experimental and conceptual grounds see Ayers et al. (1983); McClellan (1990); Musienko et al. (2012) and Parker and Srivastava (2013) and references therein. In lamprey, modulation of fictive and actual locomotion differs (Kemnitz et al., 1995; Becker and Parker, 2015). We thus need to ensure that fictive effects give physiologically-relevant information.

Given the difficulty of specifically activating modulatory systems, endogenous release is typically studied by blocking uptake. However, this does not determine how or when release occurs to evoke specific effects. Exogenous application allows known concentrations of one or more transmitters to be examined at specific times, but as normal spatial and temporal signals are not mimicked, effects may not be physiologically-relevant [exogenous and endogenous 5-HT have different effects on locomotor outputs and motor neuron excitability (Perrier and Cotel, 2015)]. Given the number of transmitters and possible combinations, we cannot examine co-transmission by analyzing all exogenous interactions individually, but have to try to determine the rules underlying these interactions (Furness et al., 1989).

Understanding functional effects requires a characterized network where convergent and divergent modulation of identified circuit components can be characterized (Harris-Warrick and Johnson, 2010). Gaps in even the simplest spinal cord locomotor networks (see Parker, 2006, 2010) mean that caution is needed in claiming understanding of how modulators evoke their effects. The effects of substance P and 5-HT in the lamprey, a lower vertebrate spinal cord model, will be used to illustrate limits to our understanding of spinal cord neuromodulation and modulator interactions.

Spinal Cord Modulation and Interactions in Lamprey

5-HT slows the frequency of fictive locomotion in lamprey (Harris-Warrick and Cohen, 1985), and reduces the slow afterhyperpolarization (sAHP) following an action potential (Van Dongen et al., 1986a). These effects were causally linked (Grillner et al., 1995): sAHP summation terminates spiking; the 5-HT-mediated reduction of the sAHP thus prolongs spiking; this will delay locomotor burst termination, thus slowing the locomotor burst frequency. This scheme was supported by a computer simulation (Hellgren et al., 1992), albeit requiring *ad hoc* adjustments of the network architecture (see Parker, 2006). In addition, the claimed causal link rested on two assumptions: that 5-HT affected the sAHP in appropriate network neurons; and that the sAHP reduction was the only effect of 5-HT. The first assumption remains uncertain (Parker, 2006), while the second was unlikely given divergent modulator effects and that 5-HT could hyperpolarize spinal cord neurons (Harris-Warrick and Cohen, 1985). 5-HT modulation of inhibitory and excitatory synaptic inputs to motor neurons was subsequently shown (Parker, 2006). 5-HT's net synaptic effect is reduced excitation, which can slow simulated and fictive network activity (Brodin et al., 1985). Two simulations (Hellgren et al., 1992; Kozlov et al., 2001) thus show the same effect using different cellular assumptions. Several divergent mechanisms individually or in combination could thus underlie the network effects of 5-HT, but their relative causal influences remaining unknown.

Substance P evokes a long-term increase in the frequency and improvement in the regularity of fictive activity in the lamprey (Parker et al., 1998) and neonatal rat (Barthe and Clarac, 1997). In lamprey, the long-term burst frequency effect is NMDA-, PKC-, and protein synthesis-dependent: the burst regularity effect is protein kinase A-dependent but NMDA- and protein synthesis-independent (Parker et al., 1998). Substance P has varied cellular and synaptic effects (Parker et al., 1997; Parker, 2006), and thus like 5-HT conforms to the general principle of divergent cell and synapse-specific modulation (Harris-Warrick et al., 1998). While the induction of the long-term burst frequency effect depends on postsynaptic NMDA receptor potentiation (Parker et al., 1998), its maintenance and the mechanisms underlying the improved burst regularity are unknown (Bevan and Parker, 2004; Parker and Bevan, 2007). As with 5-HT, the complexity of even this simpler spinal cord system means that the network effects of substance P cannot be causally reduced to cellular mechanisms (Parker and Grillner, 2000).

5-HT and substance P also illustrate modulator interactions. Both are found in a ventromedial spinal cord plexus where 5-HT and dopamine co-localize: tachykinins co-localize in a subset of 5-HT/dopamine cells (Van Dongen et al., 1986b). Exogenous 5-HT application blocks the presynaptic and postsynaptic effects of substance P on glutamatergic transmission and the long-term network modulation. Dopamine does not interact with substance P, but removes the 5-HT-mediated block of the presynaptic substance P effect to allow a short-term increase in glutamatergic synaptic transmission and the network burst frequency. As dopamine does not remove the 5-HT-mediated block of the NMDA receptor potentiation by substance P, the long-term network effect remains blocked (Svensson et al., 2001).

Other neuropeptide interactions also occur in lamprey. Peptides can modulate various reflex responses (Ullström et al., 1999). CCK and peptide YY (PYY) are co-transmitters in descending glutamatergic brainstem neurons, and CGRP and NPY co-transmitters in glutamatergic sensory neurons. All reduce the amplitude of low-frequency-evoked reticulospinal inputs (again when applied exogenously), but the sensory peptide effects are blocked by the brainstem peptides (Parker, 2000).

Spinal Cord Injury

Neuromodulation of remaining sensory or motor networks offers the potential for interventions after spinal cord injury (SCI; Rossignol and Frigon, 2011). However, despite extensive effort there is still little indication of an optimal pharmacological approach. This reflects the difficulty of understanding how even single modulators evoke their effects (see above), which makes rational targeted interventions difficult. Time after injury and the extent of the lesion also need to be considered, as these can evoke state-dependent variability that alter drug or transmitter effects (Rossignol and Frigon, 2011; Parker, 2015). As systemically-applied drugs and certain transmitters will work through volume transmission, changes in the extracellular space in the acute or chronic phases after SCI could change the spatial and temporal characteristics of endogenous or exogenous interactions.

5-HT is the best studied transmitter/modulator after SCI (Antri et al., 2003; Musienko et al., 2011). 5-HT is released from different raphe nuclei to affect sensory, motor and autonomic functions (Ghosh and Pearse, 2014). Intraspinal 5-HT systems in lower vertebrates that co-localize other amines and peptides (Schotland et al., 1996) can also appear after SCI in mammals (Ghosh and Pearse, 2014). There are over 30 5-HT receptor subtypes located presynaptically, postsynaptically, or extrasynaptically in the spinal cord (Jordan et al., 2008; Cotel et al., 2013). 5-HT usually slows locomotor activity, but its effects differ in different systems (Sillar et al., 1998), and on whether fictive or actual locomotion is examined (Kemnitz et al., 1995; Becker and Parker, 2015), and 5-HT can excite or inhibit motor neurons (Perrier and Cotel, 2015). This diversity presumably reflects the net effect of activating multiple 5-HT receptors.

Damage to serotonergic pathways has been implicated in various aspects of SCI, including paralysis, spasticity, and neuropathic pain. Descending 5-HT and noradrenergic inputs presynaptically inhibit proprioceptive and nociceptive afferents and interneuronal pathways through $G_i$protein-coupled receptors e.g., 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, and α2-adrenergic receptors (Nardone et al., 2015). Damage to these pathways thus disinhibits sensory inputs leading to spasticity (Li et al., 2004). Motor neuron hyperexcitability also contributes to spasticity in rats and humans (Li et al., 2004; Norton et al., 2008). 5-HT and NA normally facilitate motor neuron function through 5-HT_2 and α1-mediated persistent sodium and calcium currents (Perrier et al., 2003). After SCI motor neurons are initially unexcitable (Li et al., 2004; Heckmann et al., 2005), leading to arreflexia and spinal shock, but large persistent calcium and sodium currents subsequently develop that increase motor neuron excitability. This can occur through constitutive activation of 5-HT_2 and α1-receptors in motor neurons (Harvey et al., 2006; Murray et al., 2010) or denervation supersensitivity of 5-HT receptors and downregulation of 5-HT uptake (Husch et al., 2012). Interestingly, contusion injuries that spare some serotonergic projections lack these effects despite 5-HT receptors being upregulated (Hayashi et al., 2010).

Various aminergic receptors are upregulated after SCI (Rossignol et al., 2001). While the simplest interpretation is that these receptors evoke the same effects in lesioned and unlesioned spinal cords, this may not be the case. Somatostatin, GABA and 5-HT modulation differ after SCI in lamprey (Svensson et al., 2013; Becker and Parker, 2015), and aminergic and glutamatergic effects differ after SCI in mammals (Giroux et al., 1998, 2003). The potential differences in transmitter effects after SCI need to be understood if targeted pharmacological interventions are to be effective.

We lack sufficient insight into modulator effects and their interactions after SCI. Endogenously released neuromodulators can evoke diverse sensory and motor effects through wired and volume transmission. We need to know if and how these effects differ after SCI. Concentration-dependent effects and production of metabolites with different effects along volume transmission pathways could produce varied temporal and spatial-dependent signals from a single transmitter, all of which could be altered by injury-induced changes in the extracellular space. Interestingly, removal of proteoglycans, intended to promote axonal regeneration across lesions (Muir et al., 2017), will also alter the extracellular space, and it should be considered if any functional effects seen with proteoglycan removal reflects changes in volume transmission rather than regeneration. Finally, changes in functional properties, which are ubiquitous below lesion sites could evoke state-dependent changes. It seems unlikely that modulation after SCI could be reduced to a single variable. Rational interventions require greater insight into spinal cord modulation and co-transmission before and after SCI.

HYPOTHALAMIC CO-RELEASE

While model systems allow detailed analyses of neuronal and circuit-level consequences of co-transmission, we ultimately have to understand these effects in more complex systems. A recent approach to investigating the synaptic consequences of co-transmission is optogenetic circuit mapping strategies, where light-sensitive opsins are virally-expressed in neurochemically-distinct neurons that are subsequently activated *ex vivo* in the presence and absence of pharmacological blocking agents. This significant development allows endogenous transmitter release and post-synaptic activity to be examined, instead of relying on exogenous application (Qiu et al., 2016). This has shown that whether the co-release of fast and relatively slow-acting transmitters act co-operatively, in an additive fashion, or independently of each other to influence the

output of post-synaptic targets seems to differ amongst various systems.

Antagonistic Inputs Onto Hypothalamic Arousal Circuits Regulate Wakefulness and Sleep

Using an optogenetic approach in acute hypothalamic slices, Schöne et al. (2014) demonstrated that hypocretin/orexin (HO) and glutamate released from HO neurons act independently of each other to influence postsynaptic histamine neuronal targets on different timescales (Schöne et al., 2014). This circuit plays an important role in arousal and maintaining wakefulness, highlighting the importance of this projection in sleep-related disorders (Huang et al., 2001). HO-expressing neurons exclusively reside in the hypothalamus and project ubiquitously throughout the brain (Tsunematsu and Yamanaka, 2012). Shortly after their discovery in the late 1990s, genetic knockout studies showed that HO plays an important role in arousal as it relates to wakefulness and for generating appropriate reward-seeking behavior specifically in regards to maintaining energy balance (de Lecea et al., 2006; Sakurai, 2007). With the development of novel circuit mapping strategies, experiments began to reveal not only the HO pathways involved in mediating these processes, but also the underlying synaptic mechanisms. *In vivo* optogenetic circuit analyses demonstrated that HO inputs to the tuberomammillary nucleus (TMN), where histamine neurons reside, are critical for maintaining wakefulness (Huang et al., 2001). Moreover, *ex vivo* interrogation of this circuit has shed light on the synaptic mechanisms through which HO acts on the histamine arousal circuit. Schöne et al. (2014; **Figure 6**) demonstrated that HO neurons excite histamine neurons through both glutamate and HO release that together cooperate in facilitating histamine neuron firing. But instead of having an additive effect, light-evoked glutamate and HO released from HO-expressing neurons act independently of each other, as pharmacological blockade of each receptor type selectively affected that receptor without altering responses to the co-transmitter. These findings indicate that co-transmission of glutamate and HO on spike responses could co-exist in the same postsynaptic cell and non-redundantly activate histamine arousal circuits for maintaining wakefulness. At low firing frequencies HO neurons generate a glutamate-mediated tonic excitatory tone in histamine neurons, while at higher firing frequencies HO peptides are released and can sustain the excitatory tone of histamine neurons long after the HO-expressing neurons fall silent. These results are consistent with, and expand on previous studies demonstrating that HO activity promotes awakening in a frequency-dependent manner (Adamantidis et al., 2007), while loss of HO neurons results in narcolepsy (Thannickal et al., 2000; Hara et al., 2001). Lack of HO peptides or HO type-2 G protein coupled receptors (HOR2), the subtype expressed by histamine neurons, results in a similar phenotype, highlighting the clinical significance of HO neurons (Chemelli et al., 1999; Lin et al., 1999) and their projection onto histamine neurons.

The hypothalamic HO wakefulness system is complemented by the sleep-promoting melanin-concentrating hormone (MCH) system. Hypothalamic MCH neurons send widespread central projections to support a diverse set of neural processes (Girardi et al., 2018). Locally in the hypothalamus, however, they play an important facilitating role in NREM and REM sleep (Ferreira et al., 2017). Similar to HO neurons, their axons synapse onto TMN histamine where they reduce histamine neuron activity through GABAergic synaptic transmission (Jego et al., 2013). Moreover, high frequency photo-stimulation results in an increase in the frequency of inhibitory IPSPs while the overall amplitude remained the same. Repeating the stimulation protocol in MCH receptor 1 knockout mice did not result in an increase in IPSP frequency leading the authors to conclude that the evoked IPSP potentiation was likely due to synaptically-released MCH that acts presynaptically on MCH terminals to increase GABA release. The net effect is a greater suppression of histamine neuronal activity with increases in MCH activity. A similar presynaptic mechanism of action has been documented in HO neurons where elevated HO activity increased GABAergic tone in MCH neurons (Apergis-Schoute et al., 2015). In contrast to HO's ability to enhance histamine neuronal output, thereby promoting wakefulness, the combined inhibitory impact of GABA and MCH co-transmission onto histamine neurons is thought to reduce histamine release and as a result contribute to the sleep-promoting feature of MCH neuronal transmission.

Hypothalamic Cell Populations Important for Maintaining Energy Homeostasis Are Functionally Connected

Distinct hypothalamic regions are involved in food-seeking behavior related to maintaining energy balance. The hypothalamic melanocortin system in particular has received much attention, as the melanocortin receptor 4 (MC4R) in the paraventricular nucleus of the hypothalamus (PVH) can differentially impact food intake, see Krashes et al. (2016). Importantly, this depends on projections from the arcuate nucleus, another hypothalamic brain region known to be important in regulating food intake relating to energy homeostasis. Arcuate agouti-related protein expressing neurons (AgRP) and pro-opiomelanocortin (POMC)-containing neurons have opposing actions on PVH neurons that can increase or decrease food intake (Sternson, 2013), implicating these cell populations in hunger and satiety states, respectively. Zhang and van den Pol (2016) examined a third arcuate cell population, one that contains the dopamine precursor enzyme tyrosine hydroxylase (TH), and showed that short-term optogenetic activation of arcuate-TH neurons transiently increased food intake in transgenic TH-cre mice, while long-term disruption in arcuate-TH activity reduced their body weight measured over the course of months (Zhang and van den Pol, 2016). The increased food intake can be partially attributed to synaptic influences of arcuate-TH neurons on PVH neurons, as photo-stimulating their axons *ex vivo* led to inhibition of satiety-signaling PVH neurons. Interestingly, at high firing frequencies arcuate-TH

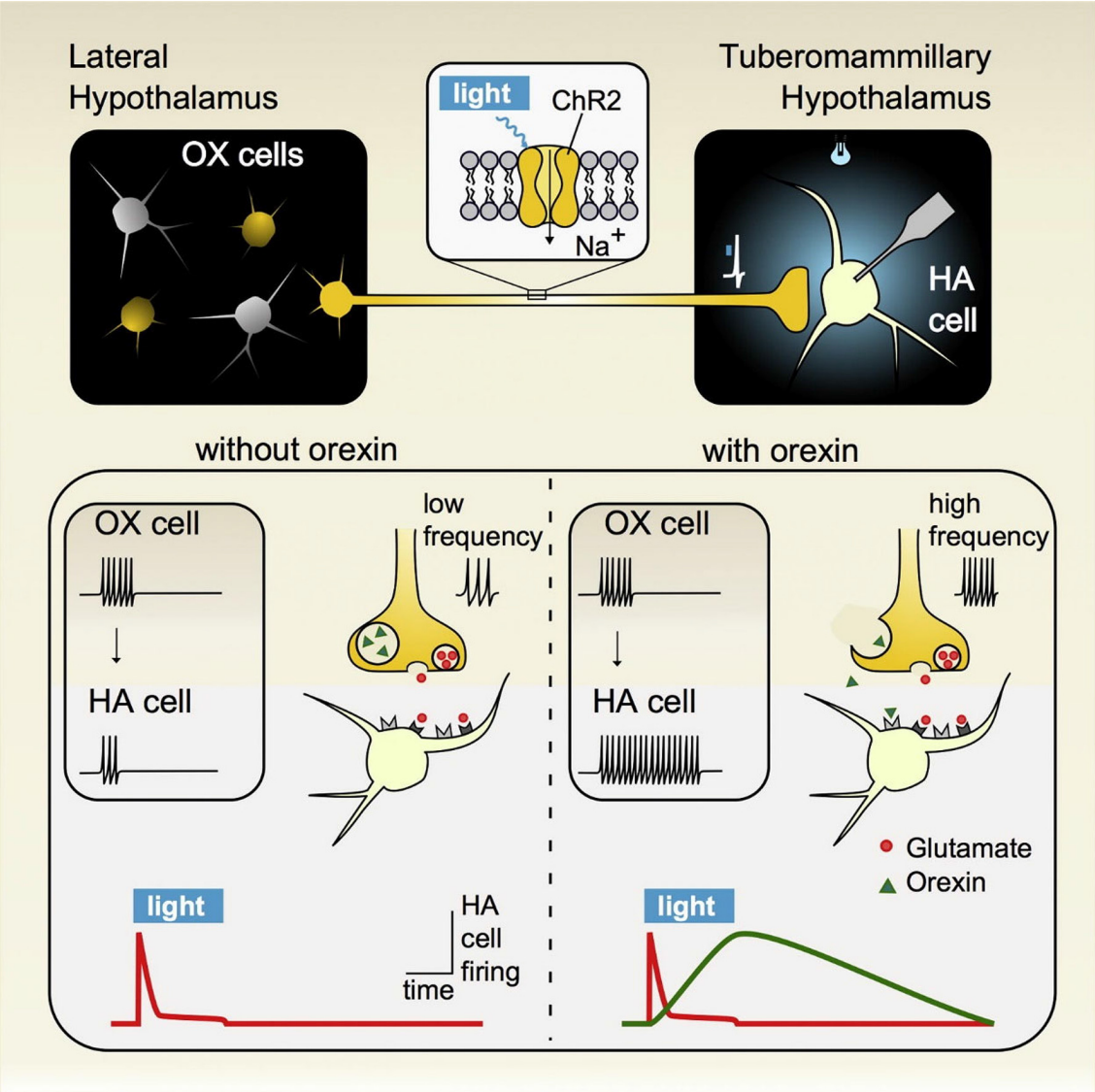

FIGURE 6 | Activation of hypothalamic orexin neurons that impact wakefulness-promoting histamine neurons generates distinct signature excitatory responses resulting from the co-release of glutamate and the neuropeptide hypocretin/orexin (HO). (Top panel) A cre-recombinase approach was used to express the light-activating excitatory protein channelrhodopsin (ChR2) in orexin neurons in orexin-cre transgenic mice. Brief pulses of light were sufficient to evoke transmitter release from orexin terminals that then impacted postsynaptic histamine neurons, which were identified by intrinsic signature currents and *post hoc* immunoprocessing for histamine decarboxylase reactivity. (Bottom panel) Low frequency stimulation led to the synaptic release of glutamate only while under high firing regimes both glutamate and orexin were co-released. In response to this co-transmission, the evoked responses measured on histamine neurons were sequential, where glutamate release and its corresponding postsynaptic excitation was fast and transient, lasting only while orexin neurons were active while orexin independently excited histamine neurons in a delayed fashion and whose response outlasted the stimulation duration. These results indicate that glutamate/orexin cotransmission may translate distinct features of orexin activity into parallel, nonredundant signals for regulating distinct circuits important for generating appropriate levels of arousal for maintaining wakefulness. Figure adapted with permission from Schöne et al. (2014) and the publisher.

neurons co-released GABA and dopamine resulting in an inhibition of PVH neurons. In contrast to co-transmission of HO and glutamate from HO neurons, GABA and dopamine release from arcuate-TH neurons had additive post-synaptic inhibitory effects. Investigation of whether synaptic inputs from arcuate-TH to AgRP or POMC can facilitate food intake by respectively exciting or inhibiting these arcuate populations found that photo-activating arcuate-TH terminals resulted in a GABA-mediated inhibition of POMC neurons while no synaptic transmission was measured on AgRP neurons. In subsequent *ex vivo* experiments, bath-applied dopamine inhibited POMC neurons and excited AgRP neurons but whether or not these opposing effects were due to synaptic dopamine release from arcuate-TH neurons was not directly tested. When neuronal excitability was monitored using cFos as a molecular marker of activity, *ex vivo* whole-cell recording techniques showed

that a state of hunger markedly increased arcuate-TH activity. Together, these results are consistent with the premise that by acting on hypothalamic circuits that regulate appetite, arcuate-TH neurons can drive hunger-related food intake. In the arcuate to PVH pathway, these neurons can synaptically influence post-synaptic targets that regulate appetite through GABA and dopamine co-release. Although activation and inhibition of arcuate-TH neurons has revealed an important role for these cells in generating feeding behavior, the exact contribution of GABA and dopamine co-transmission from arcuate-TH neurons for normal food intake is yet unresolved.

Functionally Connected Hypothalamic Circuits Are Important for Balancing Homeostatic Processes Critical for Survival

Through direct and indirect synaptic influences on one another, hypothalamic circuits with complementary appetite, sleep/wake, and other important homeostatic functions are thought to be under the influence of various sensory and physiological cues. When the system is imbalanced these signals, through synaptic processes within hypothalamic circuits, can add weight to complementary circuits for shifting the system towards homeostatic responses. This constant interplay between opposing systems ultimately changes the animal's state and defines which behavior is appropriate for survival. It is of particular interest that a number of neural disorders that involve disturbances in elementary drives critical for survival involve disruptions in hypothalamic circuits, in particular ones containing specialized neuropeptides (Krude et al., 1998; Yeo et al., 1998; Thannickal et al., 2000). Disruptions in their synthesis or transmission have often been linked to specific disorders, but what is not clear is whether or not these neuropeptide systems co-release other transmitters and if so how they interact in generating appropriate behavior. In light of this, a better understanding on the relationship between transmitter release by neurochemically-distinct hypothalamic cell populations and their post-synaptic impact will shed light on the synaptic mechanisms that regulate homeostatic processes important for survival.

RODENT NEOCORTEX AND BASAL GANGLIA

The Neocortical Neuronal Circuit

In mammals, the neocortical circuitry is relatively well-described (Markram et al., 2015) and suitable for investigations into co-transmission and neurotransmitter interactions. The neocortex is richly innervated by peptidergic interneurons containing tachykinins, enkephalins, somatostatin, NPY, VIP and CCK. The anatomical basis for these peptides is to some extent known and many of them have been localized to GABAergic interneurons (e.g., basket and Martinotti cells; **Figure 7A**). However, relatively little is known about their modulatory effects, interactions, or co-release (Markram et al., 2004; Rudy et al., 2011). Neuromodulation of short-term synaptic plasticity (metaplasticity) is an important factor for the regulation and operation of oscillatory hard-wired neuronal networks, which could contribute to fine-tuning the neocortical activity (Parker and Grillner, 1999; Abraham, 2008). Since the modulatory effects of amines and neuropeptides are long lasting, they can interact, even if their release is both temporally and spatially separate, a phenomenon known as metamodulation (Katz and Edwards, 1999; Svensson et al., 2001).

The neocortex is also innervated by aminergic and cholinergic projections originating from the midbrain that co-localize other transmitters such as glutamate and GABA, as well as neuropeptides (Kabanova et al., 2015; Root et al., 2016; Schultz, 2016a; Morales and Margolis, 2017), conforming to the general principle of amino acid, aminergic, and peptidergic co-localization outlined above. Dopaminergic inputs from VTA to the cortex co-localize glutamate, as well as the neuropeptides neurotensin and/or CCK (Figure 6A of Kabanova et al., 2015; Morales and Margolis, 2017). The co-localization of dopamine and glutamate is common and found in systems from lamprey to the human brain (Root et al., 2016). The midbrain dopaminergic system generates a reward signal critical for motor skill learning (Hosp et al., 2011; Kunori et al., 2014; Rioult-Pedotti et al., 2015; Schultz, 2016a), and dopaminergic modulation has also been implicated in working memory in the prefrontal cortex (Goldman-Rakic, 1995). By combining optogenetics with electrophysiological patch-clamp techniques, VTA projections have been found to make excitatory synapses onto prefrontal glutamatergic and GABAergic neurons (Kabanova et al., 2015; Pérez-López et al., 2018). Dopamine could promote memory formation by generating a reward signal that makes the cortical network adaptable and plastic, changing neuronal circuit activity to generate new motor skills (Goldman-Rakic, 1995; Hosp et al., 2011; Kawai et al., 2015; Schultz, 2016a). Dopaminergic neurons could reduce errors by generating instructive feedback signals (Hosp et al., 2011; Kawai et al., 2015).

Cholinergic neurons from the basal forebrain co-localize GABA (Saunders et al., 2015a,b; Granger et al., 2016) and thus activate nicotinic and $GABA_A$ receptors in cortical neurons (**Figure 7A1**). The role of ACh/GABA co-transmission is not well understood, but may contribute to motor memory formation in the primary motor cortex (Conner et al., 2010; Saunders et al., 2015a,b; Tritsch et al., 2016). Interestingly, this cholinergic/GABAergic pathway contributes to rehabilitation of motor functions after cortical injuries, and degenerates in Alzheimer's disease, making the functional role of this co-transmission of clinical interest (Tuszynski et al., 2015; Wang et al., 2016). However, the behavioral consequences of the modulatory interactions resulting from their potential co-release are poorly understood (El Mestikawy et al., 2011; Saunders et al., 2015a,b).

The Basal Ganglia and Substantia Nigra Pars Compacta

GABAergic medium spiny neurons (MSN) in the basal ganglia can be divided into two functional populations, the direct and

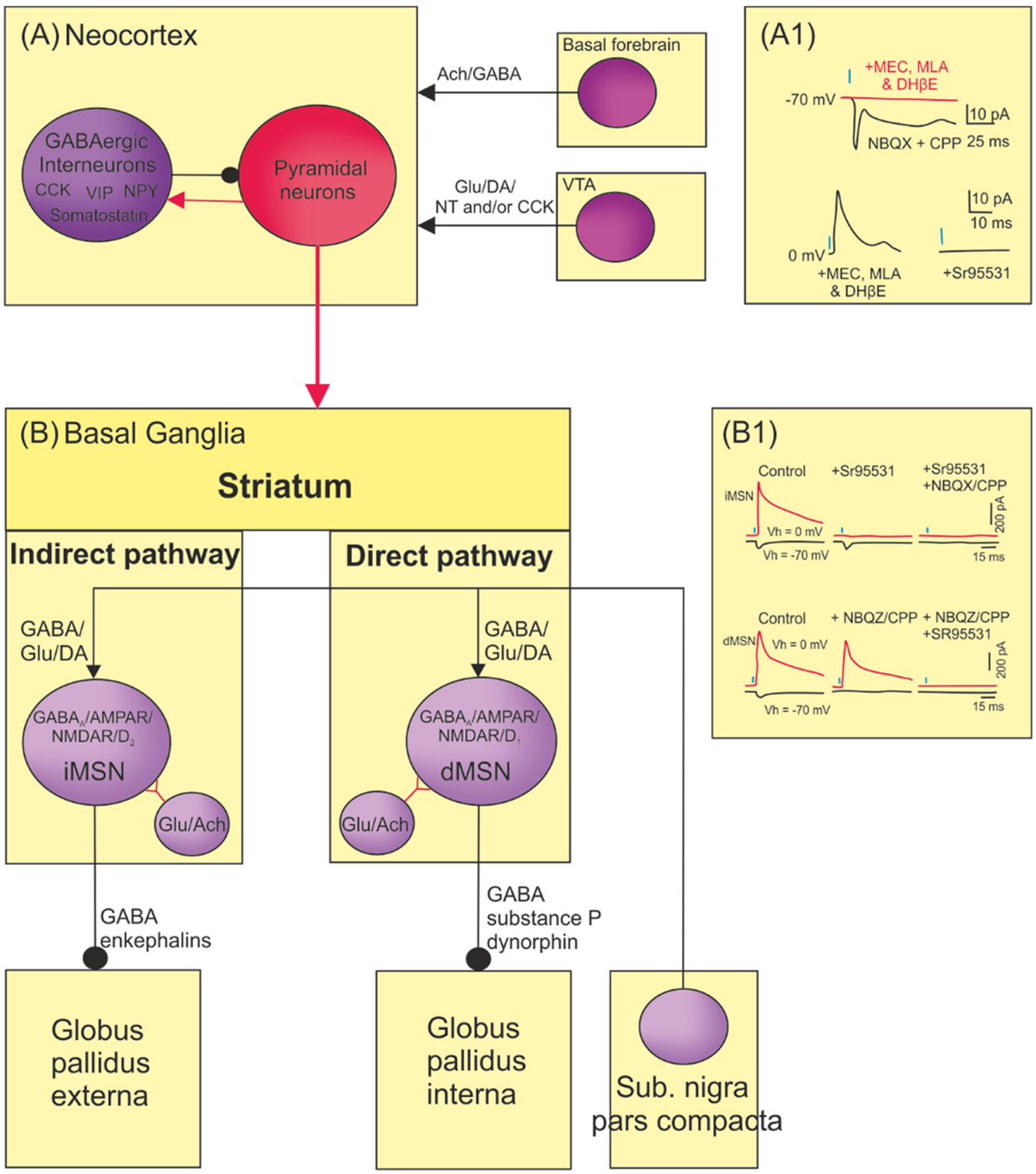

FIGURE 7 | Co-transmission in higher brain circuits (neocortex and basal ganglia). **(A)** In the cortical column GABAergic interneurons co-localize a range of different neuropeptides that can contribute to the tuning of activity of the cortical column. The neocortex also receives innervation from subcortical nuclei that can influence the tuning of cortical network activity. The projection from ventral tegmental area (VTA) co-localize glutamate, dopamine and/or the neuropeptides neurotensin and CCK. The cholinergic projections from forebrain neurons co-release GABA and thus activates nAchR and $GABA_A$ receptors in cortical neurons. **(A1)**. Traces showing nicotinergic receptor and $GABA_A$ receptor antagonism of optogenetically generated synaptic potentials. **(B)** In the basal ganglia, GABAergic medium spiny neurons (MSN) in the direct pathway co-localize substance P and dynorphins, while MSN in the indirect pathway co-localized enkephalins. Cholinergic interneurons in striatum co-localize glutamate and have excitatory action onto MSN. Dopaminergic neurons from substantia nigra pars compacta also co-localize and co-release GABA and glutamate. This input to striatum will thus activate $GABA_A$, AMPA, NMDA, and D_1 (dMSN) or D_2 (iMSN) receptors on MSN in the dorsal striatum. **(B1)** Traces showing that optogenetic activation of dopaminergic neurons activates $GABA_A$ receptors and glutamatergic AMPA and NMDA receptors. Adapted from Lobo (2009), El Mestikawy et al. (2011), Higley et al. (2011), Tritsch et al. (2012, 2016), Kabanova et al. (2015), Saunders et al. (2015a,b), Morales and Margolis (2017).

indirect pathways, used for the initiation and termination or inhibition of movements, respectively (Nelson and Kreitzer, 2014). GABAergic neurons of the indirect pathway co-localize the neuropeptide enkephalin and those of the direct pathway co-localize the neuropeptides substance P and dynorphin (**Figure 7B**). Excitatory interneurons in the striatum also co-localize ACh and glutamate to excite the MSN, as well as fast spiking interneurons that mediate a disynaptic GABAergic inhibition onto MSNs (Higley et al., 2011; Nelson et al., 2014; **Figure 7B**).

Dopaminergic inputs to striatum from the substantia nigra pars compacta are important for reward and the normal function of striatal neuronal circuitry (Schultz, 2016b). They also co-localize GABA and glutamate (**Figures 7B,B1**; Tritsch et al., 2012, 2016; Stensrud et al., 2014; Berrios et al., 2016; Chuhma et al., 2017). Dopamine activates D_1 receptors in the direct pathway but D_2 receptors in the indirect pathway. Glutamate activates AMPA and NMDA receptors and GABA acts on $GABA_A$ receptors (**Figure 7B1**; Tritsch et al., 2012). GABA is released from the same vesicles as dopamine and is transported into the vesicle by vesicular monoamine transporter (VMAT2). This complex synaptic arrangement combined with multiple receptors could give rise to new and non-linear modulatory actions and thus flexible regulation of MSN activity. Since dopaminergic projections degenerate in Parkinson's disease and MSNs in the indirect pathway degenerate in Huntington's chorea, it will be important to understand the functional role of co-transmission in these circuits to develop effective treatments, by either improving the efficacy of existing treatments (e.g., L-Dopa), suggesting new potential pharmacological strategies, or facilitating the effects of other approaches (e.g., as an adjunct to deep brain stimulation).

Furthermore, dopaminergic projections from the VTA to nucleus accumbens co-localize dopamine and glutamate and generate a reward signal. This input is implicated in drug addiction conditions (Morales and Margolis, 2017). However, the two different transmitters are segregated and are localized in both different vesicles and synaptic specializations (Zhang et al., 2015).

CONCLUSIONS

Co-transmission is clearly a ubiquitous feature across systems of differing complexity and serving different functions. This makes determining its general principles important to our basic understanding of nervous system signaling mechanisms. As co-transmission is found in central circuits involved in disorders like Parkinson's disease and Huntington's chorea, understanding these principles could potentially improve existing pharmacological treatments or identify new ones. However, our understanding of the functional effects of co-localized neurotransmitters in higher functions is still in its infancy.

It seems unlikely that any normal or pathological function can be reduced to a single transmitter system. Current pharmacological approaches for neurological disorders, including SCI, have limited efficacy at best. This should be reflected in more caution in the claims for treatments and for interventions in normal function (e.g., cognitive enhancement; Sahakian et al., 2015). There could be many reasons for this limited efficacy, but the most obvious is that pharmacological approaches do not mimic the normal endogenous release of (co)transmitters or neurotransmitter interactions, and thus the normal chemical environment of the relevant circuits. As shown in the more accessible simpler systems discussed here (*Aplysia*, crustacean STNS, lamprey), exogenous application does not necessarily reflect normal functional effects. Endogenous release reflects specific patterns of activity that differentially release multiple transmitters that have multiple single and interactive effects on multiple receptors. While exogenous application was a useful and necessary simplification, it essentially "averages" effects across receptors and regions. We need to become more sophisticated in our approaches. The best current approach is optogenetic activation of modulatory systems, as illustrated by studies performed in hypothalamic circuits (see above). However, we need to ensure that the elegance of optogenetic approaches does not blind us to the requirement of ensuring that we are stimulating the appropriate neurons in a physiologically-relevant manner if the studies are to have optimal efficacy (e.g., Arrigoni and Saper, 2014). Given sufficient temporal precision, we may be able to optogenetically activate neurons to selectively release co-localized amines or neuropeptides to investigate their physiological and behavioral effects. Discrete modulatory systems can be localized in discrete regions (e.g., raphe nuclei and locus coeruleus), allowing optogenetic activation of these systems to be done with some precision (Miyazaki et al., 2014). In addition, optogenetically regulating intracellular processes (e.g., vesicle filling; Rost et al., 2015) could enable manipulation of subsets of co-localized transmitter vesicles to investigate the effects of intrinsic co-release during natural behaviors. This would allow the analysis of intrinsic effects to move beyond the traditional approach of blocking uptake, which provides little insight except to say that transmitters are released somehow, under some conditions, to evoke some effect, and provides an adjunct to studies that use optogenetic activation or inhibition of neurons. As useful as the latter approach is, it does not tell us about the natural release or effects of co-localized transmitters. Optogenetic manipulation of endogenous co-release could thus significantly advance out understanding of behavioral effects, arguably the major open question in studies of co-transmission.

Much of our basic insight into the mechanisms and functional relevance of co-transmission and transmitter interactions has come from invertebrate and lower vertebrate model systems or the peripheral nervous system (e.g., autonomic purinergic signaling). The information obtained in these systems provides a basis for understanding effects in more complex circuits, and highlights the utility of fundamental research in model systems. Insofar as general principles of co-localization and co-transmission are still lacking, these classical systems are likely to continue to provide important insights.

Elucidating co-transmitter function will continue to benefit from analyses in genetically-tractable model systems like *Caenorhabditis elegans* and *Drosophila melanogaster*. For example, in *C. elegans*, the modulation of various behaviors [e.g., egg laying (Chen Y. Y. et al., 2017); aggregation

(Chen C. et al., 2017); aversive behavior (Mills et al., 2012; Clark et al., 2018] in response to specific conditions [e.g., food deprivation (Bhattacharya et al., 2014) or oxygen levels (Chen C. et al., 2017)] has been examined using anatomical, genetic, pharmacological, imaging and optogenetic approaches. These studies have included the effects amine and neuropeptide release and interactions (Mills et al., 2012; Ghosh et al., 2016), for example, through the co-release of different subsets of peptide co-transmitters from a single sensory neuron (Clark et al., 2018). As in mammals, neuropeptides form a major transmitter group in *C. elegans* (Van Bael et al., 2018a,b). There are approximately 119 neuropeptide genes, the products of which undergo posttranslational modifications to generate mature neuropeptides. These peptides form three classes: FMRFamide-like peptides (flps); insulin-like peptides (ins); and the largest group, neuropeptide-like proteins (nlps) that lack sequence similarity to FMRFamide or insulin. In common with other systems, these neuropeptides are stored in DCVs that can be released synaptically or extrasynaptically (see Janssen et al., 2010). Neuropeptides modulate at least several different *C. elegans* behaviors [e.g., FMRFamide promotes solitary over social feeding (de Bono and Bargmann, 1998; Leinwand and Chalasani, 2013)], while the nlp-12 gene influences food seeking behaviors (Bhattacharya et al., 2014; Chen C. et al., 2017; Chen Y. Y. et al., 2017; Iannacone et al., 2017; Stern et al., 2017; Buntschuh et al., 2018; Oranth et al., 2018). *Drosophila* has also provided important insights. For example, feeding reflects a hierarchical sequence of behaviors, including foraging and consummation, which in turn reflects the activation of various transmitter systems (see Ignell et al., 2009; Kahsai et al., 2010; Root et al., 2011; Choi et al., 2012; Kapan et al., 2012; Wang, 2012; Barnstedt et al., 2016; Kim et al., 2017; Nässel, 2018). Starved flies show facilitated synaptic outputs from Or42b olfactory receptor neurons (ORN) mediated by neuropeptide F (NPF), a peptide structurally and functionally related to mammalian NPY, but a tachykinin-mediated reduction of activity from Or85a ORNs: these two ORNs mediate odor-guided attraction and repulsion, respectively, and thus their net effect is attraction towards food. Neuromodulatory cascades also co-ordinate gustatory responses. The gustatory receptor neuron (GRN) Gr5a detects sugars while the Gr66a GRN detects bitter tastes. Starvation increases synaptic outputs from Gr5a through a NPF-mediated increase in the activity of the dopaminergic neuron TH-VUM. Starvation also causes the release of adipokinetic hormone (AKH) which increases the activity of NPF-releasing neurosecretory cells: these activate GABAergic neurons to reduce the activity of octopamine and tyramine-containing neurons, a sequence that ultimately reduces Gr66a activity. The net effect is increased attraction and reduced aversion to food cues. Starved flies also increase locomotor activity through AKH-mediated activation of octopaminergic neurons in the subesophageal zone (insulin inhibits these cells to signal satiety). Other neuromodulators (e.g., allatostatin-A, corazonin, drosulfakinin, and serotonin) also regulate feeding (see Kim et al., 2017 and references therein). These provide the opportunity to examine co-ordinated neuromodulator release and interactions. While electrophysiological analyses cannot typically be performed in *C. elegans*, and are less tractable in *Drosophila* than in the more accessible invertebrate systems such as *Aplysia* and the decapod crustacean STNS, activity can be tracked *via* Ca^{2+} imaging while molecular genetic approaches allow modulatory systems and their targets to be manipulated to investigate how co-localized transmitters and their interactions influence behaviors.

General Principles

The chemical organization of synaptic terminals, where SSVs containing amino acids and DCVs containing amines and peptides are located at different sites in the terminal and are released by different Ca^{2+}-dependent signals that reflect firing rates and patterns, appears to be a general feature of co-localization and co-transmission across systems. While specific releasing-stimuli can differ between neurons, lower firing frequencies tend to rapidly release SSVs, while higher frequency or burst firing elicits the relatively slow release of DCVs.

Flexibility is the usual reason given for the presence of multiple signaling molecules, each of which can elicit a different neuronal or circuit output, allowing hard-wired connectomes to generate a considerable diversity of outputs. This is a well-established general principle across systems.

Divergent and convergent signaling of modulators is also a highly conserved general principle conserved from simpler- to mammalian systems. Divergence is facilitated by the volume transmission of amines and especially peptides that allows a spatial field of effects from a point of release.

Aspects to Be Addressed

While SSV release has been studied extensively, we still lack insight into the mode and mechanisms of DCV release (Xia et al., 2009; Bulgari et al., 2018). To understand the regulation and role of co-release we need to understand the specific release parameters of the different classes of vesicles. These parameters are unlikely to be linear, each type of vesicle probably having specific activity-dependent release parameters. This relates to a major issue with respect to co-transmission, namely how endogenous activation of modulatory systems regulates the differential release of co-localized transmitters. While exogenous application of substances or blocking uptake or breakdown mechanisms have provided useful information, it is a crude approach. We need to examine and mimic *in vivo* release to understand the functional effects of co-transmission. Optogenetics could greatly facilitate the analysis of these aspects by using specific activation patterns or optogenetic subcellular modifications to influence co-localization and co-release (see above).

We also need to understand the signals carried by the interactions of various co-transmitters. There interactions can generate additive, subtractive, non-linear or novel effects in different systems (Brezina, 2010; Harris-Warrick and Johnson, 2010; Nusbaum et al., 2017). There may be some logic to the interactions between particular transmitter systems, but we currently lack insight into this possibility. Exogenous application will not be without utility in addressing this aspect: even though we can optogentically activate neurons, teasing apart the interactive effects of co-transmitters will require precise control

over what transmitters are released and in what concentrations, aspects facilitated by exogenous application. Even though exogenous application is a crude approach, fundamental insights have often relied on simplifying assumptions that generate testable hypotheses. But we must remember that insights obtained under simplifying conditions may not reflect how the system actually works.

In addition to normal release and interactions, we need to understand the influence of the functional state of targets, as state-dependent effects can influence neuromodulation. Understanding these changes will be important to understanding co-transmitter signaling. This will be especially important for treatments where the normal functional state is disturbed (e.g., spinal injury, Parkinson's disease). This could also require understanding of how modulatory systems change after injury or in disease states (highlighted here by purinergic signaling and SCI). We cannot assume that a pathological state is the normal state with a missing component. The nervous system is adaptive, and widespread diaschisis-like or homeostatic changes clearly occur in response to perturbations.

Volume transmission is a recognized feature of neuronal signaling, especially for aminergic and peptidergic transmitters. In addition to the conditions that lead to the differential release or co-release of transmitters, volume signals can be modified as the transmitters move through the extracellular space by factors that can affect the breakdown of transmitters (potentially generating breakdown products that are biologically active). This seems to be conserved from invertebrates (e.g., Coleman et al., 1994; Wood et al., 2000; Duzzi et al., 2016) to mammals (e.g., Le Greves et al., 1985). Given that extracellular diffusion is likely to present a major factor affecting volume transmission, and given that the volume of the extracellular space can be modified by neuronal activity (Østby et al., 2009), there could be a circular interaction whereby transmitter effects change the neuronal activity that in turn affects transmitter effects. While not of obvious relevance, the extracellular space could thus be a major factor in understanding co-transmission.

A final point of obvious importance is that the effects of transmitter co-localization on behavior are still poorly understood. Classical approaches in the conventional model systems discussed here have examined cellular, synaptic and circuit level effects of transmitters and neuromodulation and related this to various aspects of behavior. However, the behavioral links often reflect assumptions and extrapolations rather than direct insight (e.g., fictive locomotion in spinal cord modulation). While links to actual locomotor behavior are often claimed, these can be tenuous, and where effects have been compared to those in intact systems they can differ (see above). The best hope of understanding behavioral effects seems to lie with optogenetic manipulations and molecular genetic approaches in mouse, *Drosophila*, and *C. elegans*. However, while we can analyze the effects of activating or manipulating modulatory systems on behavior in these systems, cellular and synaptic analyses are more difficult, making it more challenging to elucidate how the observed effects are mediated at the cellular or circuit levels. We need to develop ways to link modulation more directly to behavior in the classic model systems, and to examine physiological mechanisms in the more recently introduced genetically tractable systems. The conservation of effects between systems will help us to infer general cellular and behavioral principles across systems.

It is clear that co-transmission is a core signaling mechanism by which neurons in all nervous systems operate. Work in several model systems, including those reviewed here, has revealed some general principles, and supports a remarkable diversity of mechanisms resulting from co-transmission. However, challenges remain in establishing the roles of this basic design in the normal and dysfunctional operation of neurons, circuits and behavior, particularly in the mammalian CNS. We thus anticipate that the future will provide new, and often unexpected, insights in the roles of co-transmission in nervous system function.

AUTHOR CONTRIBUTIONS

ES wrote the "The *Aplysia* Feeding Circuit" and "Rodent Neocortex and Basal Ganglia" sections. JA-S wrote the "Hypothalamic Co-release" sections and commented on the manuscript. GB wrote the "Purinergic Co-transmission in the Autonomic and Central Nervous System" section, contributed to the "Historical Perspectives on Co-transmission" section, and commented on the manuscript. MN wrote the "Co-transmission Consequences in the Decapod Crustacean Stomatogastric Nervous System" section and commented on the manuscript. DP wrote the "Introduction" and "Conclusions", the "Historical Perspectives on Co-transmission" section, and the "Spinal Cord Modulation and Co-transmission" section and commented on the manuscript. HS contributed to the "Rodent Neocortex and Basal Ganglia" section. Correspondence should be directed to the author of the relevant sections.

FUNDING

This work was supported by grants from The Swedish Medical Research Council (Vetenskapsrådet), Byggmästare Olle Engkvist Stiftelse, Major Gösta Linds Stiftelse (ES, HS) and from National Institute of Neurological Disease and Stroke Grant NS029436 (MN).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neural Circuits

MINI REVIEW
published: 02 October 2018
doi: 10.3389/fncir.2018.00078

Peptide Cotransmitters as Dynamic, Intrinsic Modulators of Network Activity

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Neurons can contain both neuropeptides and "classic" small molecule transmitters. Much progress has been made in studies designed to determine the functional significance of this arrangement in experiments conducted in invertebrates and in the vertebrate autonomic nervous system. In this review article, we describe some of this research. In particular, we review early studies that related peptide release to physiological firing patterns of neurons. Additionally, we discuss more recent experiments informed by this early work that have sought to determine the functional significance of peptide cotransmission in the situation where peptides are released from neurons that are part of (i.e., are intrinsic to) a behavior generating circuit in the CNS. In this situation, peptide release will presumably be tightly coupled to the manner in which a network is activated. For example, data obtained in early studies suggest that peptide release will be potentiated when behavior is executed rapidly and intervals between periods of neural activity are relatively short. Further, early studies demonstrated that when neural activity is maintained, there are progressive changes (e.g., increases) in the amount of peptide that is released (even in the absence of a change in neural activity). This suggests that intrinsic peptidergic modulators in the CNS are likely to exert effects that are manifested dynamically in an activity-dependent manner. This type of modulation is likely to differ markedly from the modulation that occurs when a peptide hormone is present at a relatively fixed concentration in the blood.

Keywords: neuropeptide, cotransmitter, neuromodulation, invertebrate, autonomic nervous system

OPEN ACCESS

Edited by:
Erik Svensson, Uppsala University, Sweden

Reviewed by:
Rhanor Gillette, University of Illinois at Urbana-Champaign, United States
Dick R. Nässel, Stockholm University, Sweden

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Received: *01 August 2018*
Accepted: *10 September 2018*
Published: *02 October 2018*

Citation:
Cropper EC, Jing J, Vilim FS and Weiss KR (2018) Peptide Cotransmitters as Dynamic, Intrinsic Modulators of Network Activity. Front. Neural Circuits 12:78. doi: 10.3389/fncir.2018.00078

INTRODUCTION

For more than 40 years, it has been apparent that neurons can contain both neuropeptides and "classic" small molecule transmitters. Much progress has been made in studies designed to determine the functional significance of this arrangement in experiments conducted in invertebrates and in the vertebrate autonomic nervous system. Below we describe some of these key results. We begin by discussing early experiments that studied cotransmission in preparations in which it was possible to directly monitor peptide release. These data provided (still valid) insights into the dynamics and pattern dependance of peptide release that could not be obtained in less experimentally advantageous systems. Later sections of this review article then

describe how these insights have informed more recent research that has sought to determine the physiological role of peptide cotransmitters that are intrinsic to a behavior-generating network.

PEPTIDE RELEASE

Does Peptide Release Occur During Normal Behavior?

It has long been apparent that neuropeptides can coexist with small molecule neurotransmitters. For example, Hökfelt and coworkers reported somatostatin-like immunoreactivity in noradrenergic neurons in principle ganglion cells of sympathetic ganglia in 1977 (Hökfelt et al., 1977). The demonstration of coexistence then led to the obvious question, do "co-existing" peptides function as neurotransmitters? (an alternative possibility would be that they simply act as trophic factors).

In the early 1980s the cotransmission question was addressed by Jan and Jan (1982) in experiments in the sympathetic nervous system of the bullfrog. There is general agreement that certain criteria have to be met for a substance to be classified as a neurotransmitter. Although there is some disagreement as to how many of these criteria there are, Jan and Jan (1982) were able to convincingly satisfy those that are most commonly considered crucial. For example, they demonstrated that the peptide they studied (LH-RH) is present in presynaptic terminals, and is released in a calcium dependent manner, Further, a late, slow EPSP was mimicked by application of exogenous LH-RH, and blocked by LH-RH antagonists (for a detailed discussion of this work see Nusbaum, 2017).

A further question that was subsequently raised was, under what circumstances does peptide release occur? Early experiments in the sympathetic nervous system of the pig used a radioimmunoassay (RIA) to quantify NPY-like immunoreactivity in the perfusate following low frequency (i.e., 2 Hz) nerve stimulation as compared to release induced by intermittent stimulation at a high frequency (20 Hz; Lundberg et al., 1986). Release was greater at the higher frequency. Data such as these led to the hypothesis that peptide release only occurs if neurons fire at high/excessive frequencies that are observed under pathological conditions (Hökfelt, 1991). This line of thinking led to the prediction that peptides would only be important for mediating responses to injury or stress (Hökfelt, 1991).

Subsequent invertebrate research clearly established that this is not the case. Many of these early studies were conducted in neuromuscular systems (O'Shea and Schaffer, 1985). An initial goal of this work was to verify peptide release by making direct biochemical measurements. Peptide release was induced either by raising the potassium concentration in the saline, or by stimulating motor neurons at relatively high frequencies. For example, Adams and O'Shea (1983) demonstrated proctolin release from a slow skeletal motoneuron (Ds) in the cockroach with stimulation at 50 Hz. Other lower frequencies were not tested when release was directly monitored, presumably because the method used to detect released peptides was not very sensitive. However, in other experiments in this study, peptide release was monitored indirectly, i.e., by monitoring a physiological response clearly not mediated by the release of the primary neurotransmitter (glutamate). Thus, Adams and O'Shea (1983) also demonstrated that when a burst of action potentials is triggered in Ds, a delayed slow increase in muscle tension is observed that is not associated with excitatory junctional potentials (EJPs). This delayed response was observed when Ds was stimulated at a frequency that was not specified but was clearly way below 50 Hz.

In another early invertebrate study, proctolin release was monitored in a neuromuscular preparation of the crayfish using a sensitive and quantifiable bioassay, i.e., samples were applied to a subset of muscle fibers from the main extensor muscle of the locust leg and changes in contraction frequency were noted (O'Shea and Bishop, 1982). In this situation, it was possible to detect release when motor neurons were stimulated at a fairly low frequency (e.g., 10 Hz; Bishop et al., 1987).

Other experiments were conducted in a molluscan (*Aplysia*) preparation that consists of a muscle utilized in feeding, the accessory radula closer (ARC) and its two cholinergic motor neurons (B15 and B16; Cohen et al., 1978). Initially, peptide release in this system was monitored indirectly. For example, investigators measured cAMP levels in the ARC muscle (Whim and Lloyd, 1989; Cropper et al., 1990b). In later studies, however, a sensitive RIA was developed that permitted direct detection of released material (Vilim et al., 1996a).

Research in the ARC neuromuscular system was unusual in its emphasis on mimicking naturally occurring patterns of motor neuron activity. Thus, extra junctional currents (EJCs) induced by B15 and B16 were recorded from the ARC muscle during normal feeding behavior in intact animals (Cropper et al., 1990a). Physiologically relevant patterns of neural activity were then simulated in subsequent release experiments that confirmed that the amount of peptide released depends on firing frequency in the ARC neuromuscular system (Vilim et al., 1996a, 2000) as it does in bullfrog sympathetic ganglia (e.g., Lundberg et al., 1986, 1989; Peng and Horn, 1991; **Figure 1A**). Importantly, however, release did occur at the low end of the physiological range (which is 7.5 Hz for B15 and 10 Hz for B16 (Cropper et al., 1990a; Vilim et al., 1996a,b, 2000). These data provide direct evidence for the release of peptides at physiologically relevant levels of activity, and obviously contradict the idea that peptide cotransmitters solely mediate responses to stress.

Plasticity in Peptide Cotransmitter Release

It has long been recognized that neuropeptides are generally packaged in dense core vesicles whereas small clear vesicles generally contain low molecular weight neurotransmitters. In a number of neurons, data suggest that exocytosis from the two types of vesicles occurs in different regions in the presynaptic terminal. In some cases, release from the small clear vesicles occurs in the active zone and release from peptidergic large dense core vesicles appears to occur elsewhere (e.g., Zhu et al., 1986; Vilim et al., 1996b; Lysakowski et al., 1999; Karhunen et al., 2001). Further, release from the two types of vesicles

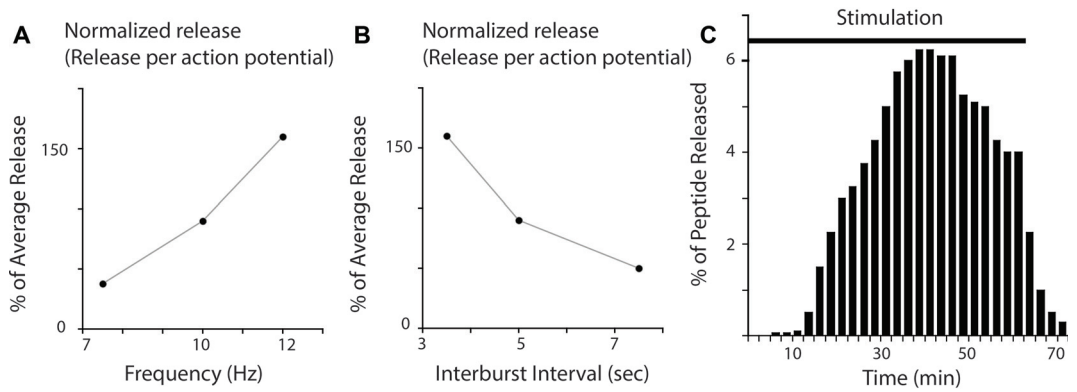

FIGURE 1 | Peptide release in *Aplysia* neuromuscular preparations. **(A)** Effect of firing frequency on peptide release. Release was measured at three different firing frequencies in experiments in which the burst duration and interburst interval were kept constant. Plotted are results corrected to give the release per action potential. Note that there is more release when firing frequency increases (results are replots of data from Vilim et al. (1996a); error bars were omitted for clarity). **(B)** Effect of interburst interval on peptide release. Release was measured at three different interburst intervals in experiments in which the burst duration and firing frequency were kept constant. Plotted are results corrected to give the release per action potential. Note that increases in interburst interval decrease peptide release (results are replots of data from Vilim et al. (1996a); error bars were omitted for clarity). **(C)** Peptide release in response to intracellular stimulation of an accessory radula closer (ARC) motor neuron (i.e., stimulation at 12 Hz for 3.5 s every 7 s). The bar indicates the period of neural stimulation. Samples of muscle perfusate were collected every 2.5 min and peptide content was determined using a radioimmunoassay (RIA). Peptide release is expressed as percentage of total release in each experiment. Note that peptide release facilitated greatly and then declined until stimulation ceased (results are replots of data from Karhunen et al. (2001); error bars were omitted for clarity).

is often differentially sensitive to increases in the intracellular calcium concentration. Release from peptidergic large dense core vesicles occurs at lower $[Ca^{2+}]_i$ (Verhage et al., 1991; Peng and Zucker, 1993; Ohnuma et al., 2001). These data suggest that the patterning of neural activity could impact peptide cotransmitter release in a manner that differs from its impact on the release of a low molecular weight transmitter.

As described above, a number of investigators have demonstrated that more peptide is released when neurons fire at higher frequencies (Lundberg et al., 1986, 1989; Peng and Horn, 1991; Vilim et al., 1996a, 2000). Obviously as firing frequency increases, there is an increase in the number of action potentials triggered in a given period of time. One method that has been used to correct for this is to calculate the amount of peptide released per action potential. Even with this correction more release at higher firing frequencies has been demonstrated in the ARC neuromuscular system (if neurons are stimulated within a physiologically relevant range; **Figure 1A**; Vilim et al., 1996a, 2000).

An additional question that has been addressed is, are periods of rest necessary to maintain peptide cotransmitter release? That this could be the case had been suggested by experiments that studied peptide hormone release from the hypothalamus (Cazalis et al., 1985). Investigators working in the bullfrog sympathetic ganglia demonstrated that rest periods are not essential, i.e., LHRH-induced slow currents were recorded from postsynaptic neurons when presynaptic neurons were stimulated continuously (Peng and Horn, 1991).

Subsequently, research conducted in the ARC neuromuscular system elaborated on these findings (Vilim et al., 1996a, 2000). With excessive stimulation, depletion of peptide cotransmitters obviously occurs. Experiments were, however designed so that all parameters chosen were behaviorally relevant. For example, motor neurons were not fired at frequencies higher than those observed during normal behavior and burst durations and interburst intervals were all within physiological limits. Under these conditions, periods of rest were actually detrimental, i.e., there was a decrease in the amount of peptide released per action potential as the interburst interval was increased (**Figure 1B**). This result suggests that effects of modulatory neuropeptides will be manifested in a manner that is at least to some extent determined by how a behavior is executed. Namely, if it is executed rapidly, effects of peptides will be more pronounced.

Additionally, ARC investigators characterized the dynamics of peptide release when neural activity was maintained for a relatively long period of time (e.g., ∼ an hour) with no change in either the motor neuron firing frequency or bursting pattern (**Figure 1C**; Vilim et al., 1996b, 2000; Brezina et al., 2000; Karhunen et al., 2001). In some of these experiments, motor neurons were stimulated at the high end of the physiological range, and pauses between bursts of activity were on the short side. Nevertheless, initially relatively little peptide was released. Over time however release facilitated and reached a peak (**Figure 1C**). Thereafter, it declined. These data suggest that even when behavior is constant, modulatory effects of peptide cotransmitters will be dynamically manifested. When a behavior is initiated, it may not be greatly impacted by peptide release. However, as it is repeated, peptidergic effects may become more pronounced (up to a point).

Taken together, these results indicate that neuropeptides are released during normal behavior. The amount of peptide released per action potential can vary greatly and be altered by the firing pattern of the neuron. Consequently, peptide

release is likely to be determined by how behavior is executed (e.g., quickly or slowly). Additionally, even when patterns of neural activity do not change, peptide release may occur dynamically, e.g., effects of modulatory peptides may become more pronounced as a behavior progresses. Below we discuss potential functional consequences of these forms of plasticity in a specific situation—in the situation where peptide cotransmitters are intrinsic to a behavior generating circuit.

PEPTIDE COTRANSMITTERS CAN BE INTRINSIC TO A BEHAVIOR GENERATING CIRCUIT

Modulatory neuropeptides are not always released as cotransmitters. In some well-characterized situations, they are released as hormones into the blood. For example, this is the case for some of the peptides that configure activity in the well-studied crustacean stomatogastric ganglion (STG; e.g., Christie et al., 1995; Marder and Bucher, 2007). This ganglion contains neurons utilized during feeding (e.g., chewing) and is located in an artery that is exposed to any substance present in the hemolymph. To give another well-characterized example, it is also the case for peptides such as eclosion hormone (EH) and ecdysis trigger hormone (ETH) that control ecdysis in insects (for review see, Taghert and Nitabach, 2012). It has therefore been suggested that neuropeptides typically act from outside motor networks to modulate output (Taghert and Nitabach, 2012).

Whether or not this is true depends on what is meant by "motor network." For example, not all modulatory input to the STG is blood borne. Peptides are also present in projection neurons that innervate this ganglion and drive activity. For example, the GABA containing modulatory commissural neuron 1 (MCN1) also contains proctolin and *C. borealis* tachykinin-related peptide 1a (CabTRP1a; Blitz et al., 1999). Experiments in intact animals have demonstrated that MCN1 is involved in the processing of exteroceptive sensory input and influences motor activity under behaviorally relevant conditions (Hedrich et al., 2011). Chemosensory stimulation of the antennae of the crab increases the MCN1 firing frequency. It also triggers a gastric mill rhythm under normal conditions, but not if the MCN1 is lesioned. Thus, MCN1 may not be part of the "motor" gastric mill network. It is however clearly part of the behavior generating circuitry as a whole.

In a similar vein, a number of cerebral buccal interneurons (CBIs) in the mollusc *Aplysia* are peptidergic (e.g., Phares and Lloyd, 1996; Morgan et al., 2000; Vilim et al., 2001; Koh et al., 2003; Jing et al., 2010). These cells are also projection neurons and at least some of these neurons are activated by food under physiological relevant conditions and trigger motor activity (Rosen et al., 1991; Jing and Weiss, 2005; Wu et al., 2014). For example, one cholinergic neuron (CBI-2) is a command-like neuron that can drive ingestive responses (Rosen et al., 1991; Jing and Weiss, 2005).

Lastly, peptide cotransmitters have been localized to motor neurons and sensory neurons in a number of species. Peptide-containing motor and sensory neurons are not always part of the pattern generating circuit. However, motor neurons are obviously essential for the execution of behavior and sensory neurons often trigger it. In summary, although there are a number of well-characterized examples where peptides act from outside a behavior-generating network (e.g., function as hormones), there are also clear examples of situations in which they are intrinsic to the circuit that generates a particular behavior.

INTRINSIC VS. EXTRINSIC MODULATOR RELEASE

A distinction between extrinsic and intrinsic modulation was originally made in the feeding system of *Aplysia* (Cropper et al., 1990b). The comparison there was between modulatory effects

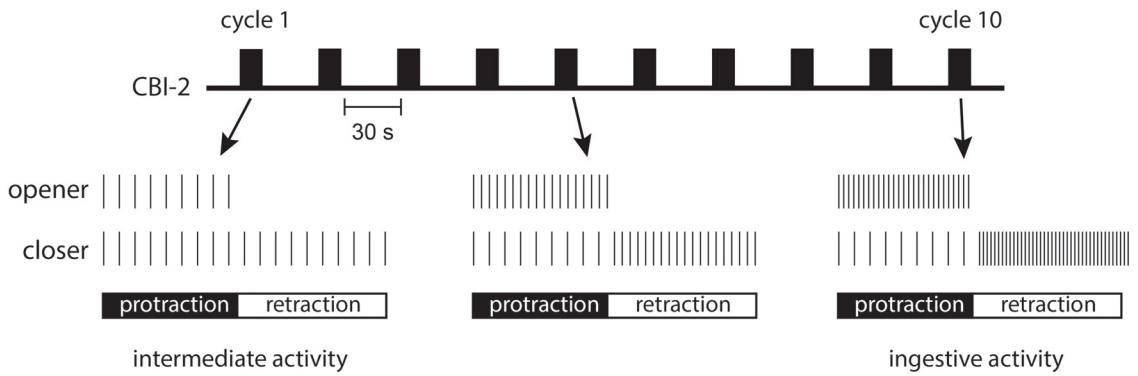

FIGURE 2 | Repetition priming is observed when cycles of activity are triggered with an inter-burst interval of 30 s as is indicated in the schematic at the top of the figure. The first cycle that is induced is referred to as having intermediate characteristics. Motor neurons fire at low frequencies and radula opener and closer motor neurons are coactive (as is schematically illustrated in the bottom two rows on the left). With repeated motor program induction, activity becomes ingestive. Radula opener motor neurons are more active during the radula protraction phase of the motor program, and radula closer motor neurons are primarily active during radula retraction (as is schematically illustrated in the bottom two rows on the right).

mediated by peptide cotransmitters in the ARC motor neurons, and modulatory input from the serotonergic metacerebral cells (MCCs). Peptide cotransmitters are obviously intrinsic to the behavior generating circuit. The MCCs were referred to as extrinsic because they are not part of the behavior generating circuitry *per se*. MCC activity does not induce a muscle contraction (Weiss et al., 1978). Further, feeding behavior is observed when the MCCs are lesioned (Rosen et al., 1983, 1989).

One difference between the two types of modulatory input arises from the fact that the release of an intrinsic modulator is likely to be tightly coupled to the manner in which the behavior is executed. This is particularly likely to be true for modulators such as peptide cotransmitters that are released in a pattern dependent manner. For example, the data reviewed above suggest that if behavior is executed rapidly, peptide release is likely to "automatically" increase. Further, if a behavior is maintained rather than terminated quickly, peptide release will progressively increase (at least for a while).

In contrast, it is not likely that such tight coupling will be observed with the release of an extrinsic modulator. In the ARC example cited above, the MCC firing frequency is at least in part determined by input that it receives from a sensory neuron that does not drive feeding motor programs (Chiel et al., 1986; Weiss et al., 1986a,b; Jing et al., 2008). Consequently, the MCCs are activated during feeding, but the MCC firing frequency is not tightly linked to variations in the activity of the behavior mediating feeding circuitry itself (Kupfermann and Weiss, 1982).

RELEASE OF "INTRINSIC" PEPTIDE COTRANSMITTERS FROM PROJECTION NEURONS

A further question is, what is the functional significance of intrinsic peptidergic neuromodulation? Obviously, the answer to this question will depend on the type of neuron that contains the peptide cotransmitter. For example, peptide cotransmitters released by motor neurons and sensory neurons are apt to exert relatively constrained effects. For example, peptides released by motor neurons are likely to modify the neuromuscular transform of one particular neuromuscular unit. In contrast, peptides released by projection neurons can exert effects that are widespread. For example, in the feeding circuit of *Aplysia* the peptides released by CBI-2 (feeding circuit activating peptide (FCAP) and cerebral peptide 2 (CP-2)) modify activity in a number of circuit elements (Morgan et al., 2000; Koh et al., 2003; Koh and Weiss, 2005, 2007; Friedman and Weiss, 2010). A more specific question is, how does peptide release from a projection neuron differ from a situation in which a modulatory peptide is released as a hormone?

In the *Aplysia* feeding circuit, FCAP and CP-2 act together to configure motor activity and make motor programs ingestive. Interestingly this occurs dynamically. Thus, when a single cycle of motor activity is triggered by CBI-2, motor neurons fire at relatively low frequencies and phase relationships are not very well defined (**Figure 2**; Proekt et al., 2004, 2007; Friedman and Weiss, 2010; Dacks et al., 2012). This type of motor activity is referred to as having intermediate characteristics. However, if CBI-2 is repeatedly stimulated with a relatively short interburst interval, program definition occurs (**Figure 2**; Proekt et al., 2004, 2007; Friedman et al., 2009; Friedman and Weiss, 2010; Dacks et al., 2012). The configuration of motor activity happens progressively with cycles of activity becoming more and more ingestive as they are repeatedly evoked. In other words, a form of repetition priming is observed, i.e., performance improves as behavior is repeated.

It is possible that postsynaptic events are partially responsible for the repetition priming that is observed in the feeding network. The CBI-2 peptides exert second messenger-mediated effects that may summate and become progressively larger when the interburst interval is short (Cropper et al., 2014). It is, however, very likely that plasticity in peptide release at least influences this process. Since effects of modulatory peptides are dose-dependent, we propose that progressive increases in the amount of peptide released are likely to impact function. This sort of progressive, activity-dependent change in the amount of peptide released is generally not observed when a peptide is released as a hormone. In conclusion, when peptides are released as cotransmitters from within a behavior mediating circuit, activity-dependent, dynamic effects may be observed that are not typical of peptide hormones. These effects may be important for the induction of phenomena such as repetition priming.

SUMMARY

Research conducted in invertebrates and in the vertebrate autonomic nervous system has played an important role in establishing that modulatory neuropeptides can function as cotransmitters and influence the generation of normal behaviors such as feeding and digestion. For example, peptides configure and reconfigure network activity and promote multitasking. Further, studies discussed in this review article have demonstrated that peptide release can be pattern and time dependent when neurons fire in physiologically relevant patterns. When peptide cotransmitters are intrinsic to a behavior generating circuit, this necessarily links peptidergic modulation to the manner in which a behavior is executed. For example, peptide release is more likely to occur when behavior occurs quickly. Further, when behavior is maintained, there can be progressive time-dependent increases in peptide cotransmitter release.

AUTHOR CONTRIBUTIONS

EC wrote the article with editorial suggestions from JJ, FV and KW.

FUNDING

This research was supported by the National Institutes of Health (Grants NS066587 and NS070583), and by the National Natural Science Foundation of China (Grants 31671097, 31371104).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular Neuroscience

REVIEW
published: 23 March 2018
doi: 10.3389/fncel.2018.00083

Substrates for Neuronal Cotransmission With Neuropeptides and Small Molecule Neurotransmitters in *Drosophila*

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It has been known for more than 40 years that individual neurons can produce more than one neurotransmitter and that neuropeptides often are colocalized with small molecule neurotransmitters (SMNs). Over the years much progress has been made in understanding the functional consequences of cotransmission in the nervous system of mammals. There are also some excellent invertebrate models that have revealed roles of coexpressed neuropeptides and SMNs in increasing complexity, flexibility, and dynamics in neuronal signaling. However, for the fly *Drosophila* there are surprisingly few functional studies on cotransmission, although there is ample evidence for colocalization of neuroactive compounds in neurons of the CNS, based both on traditional techniques and novel single cell transcriptome analysis. With the hope to trigger interest in initiating cotransmission studies, this review summarizes what is known about *Drosophila* neurons and neuronal circuits where different neuropeptides and SMNs are colocalized. Coexistence of neuroactive substances has been recorded in different neuron types such as neuroendocrine cells, interneurons, sensory cells and motor neurons. Some of the circuits highlighted here are well established in the analysis of learning and memory, circadian clock networks regulating rhythmic activity and sleep, as well as neurons and neuroendocrine cells regulating olfaction, nociception, feeding, metabolic homeostasis, diuretic functions, reproduction, and developmental processes. One emerging trait is the broad role of short neuropeptide F in cotransmission and presynaptic facilitation in a number of different neuronal circuits. This review also discusses the functional relevance of coexisting peptides in the intestine. Based on recent single cell transcriptomics data, it is likely that the neuronal systems discussed in this review are just a fraction of the total set of circuits where cotransmission occurs in *Drosophila.* Thus, a systematic search for colocalized neuroactive compounds in further neurons in anatomically defined circuits is of interest for the near future.

Keywords: fly brain, circadian clock, olfactory system, mushroom bodies, neurosecretory cells, neuromodulation, presynaptic facilitation, short neuropeptide F

OPEN ACCESS

Edited by:
Erik Svensson,
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Reviewed by:
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Received: *15 February 2018*
Accepted: *08 March 2018*
Published: *23 March 2018*

Citation:
Nässel DR (2018) Substrates for Neuronal Cotransmission With Neuropeptides and Small Molecule Neurotransmitters in Drosophila. Front. Cell. Neurosci. 12:83. doi: 10.3389/fncel.2018.00083

INTRODUCTION

Already more than 40 years ago it was proposed that individual neurons can produce more than one neurotransmitter (Burnstock, 1976), and subsequently a multitude of studies established this as a common phenomenon in central and peripheral neurons of mammals (see Hökfelt et al., 1977; Cuello, 1982; Chan-Palay and Palay, 1984; Hökfelt et al., 1987). Over the years much progress has been made in understanding the functional consequences of co-transmission in the nervous systems of vertebrates (see Svensson et al., 2001; Hnasko and Edwards, 2012; Vaaga et al., 2014). Also neurons of invertebrates, such as insects, crustaceans, and mollusks, were early on shown to co-express different neuroactive substances (O'Shea and Bishop, 1982; Adams and O'Shea, 1983; Bishop et al., 1984; Kupfermann, 1991; Weiss et al., 1992; Glantz et al., 2000; Nusbaum et al., 2001). These studies demonstrated co-expression of neuropeptides and small molecule neurotransmitters (SMNs), where the neuropeptide acts as a cotransmitter and modulates the action of the neurotransmitter (Adams and O'Shea, 1983; Blitz and Nusbaum, 1999; Glantz et al., 2000; Nusbaum et al., 2001). There were also early studies of actions of coexisting neuropeptides in neuronal circuits or at peripheral targets (Weiss et al., 1992; Nusbaum et al., 2001; Nusbaum and Blitz, 2012). From these studies, and later, it has emerged that neuropeptide cotransmission serves to increase the flexibility and the dynamic range of signaling within neuronal networks or even reconfiguring these, and thereby altering network outputs (see Marder, 2012; Nusbaum et al., 2017). It has been shown that neuropeptides can diffuse some distance within the CNS and that they can act extrasynaptically, even far from the release site [(Jan and Jan, 1982) and reviewed in (Zupanc, 1996; Nässel, 2009; Nusbaum and Blitz, 2012; van den Pol, 2012)]. This increases the flexibility of neuromodulation with action both near the synapse and away from it. Although not yet demonstrated in insects, it is known from mammals that also SMNs (e.g., monoamines and amino acids) can diffuse some distance from the synapse (synaptic spillover or parasynaptic signaling) and act on receptors away from the nearest target neuron (Agnati et al., 1995; Szapiro and Barbour, 2009). Still, it appears as if the fast SMNs are more confined to the hardwiring of the synapses, whereas the neuropeptides have freedom to act at other sites, by so-called volume transmission (Agnati et al., 1995). Another difference is the temporal scale of action with SMNs operating in a millisecond range and neuropeptides commonly over seconds, minutes or longer, and the neuropeptides may require a stronger stimulus (stronger depolarization or burst of action potentials) to be released (Merighi, 2002; Marder, 2012; van den Pol, 2012; Nusbaum et al., 2017). Some neuropeptides are only released episodically and in bulk, especially ones constituting developmental signals (see Kim et al., 2006; Park et al., 2008). It is also known that neuropeptides can act in autocrine loops to regulate release of SMNs from the same neuron by presynaptic facilitation (Merighi, 2002; Root et al., 2011) or act in retrograde feedback to input neurons (Hu et al., 2017). Furthermore, colocalized neuropeptides can engage in various forms of intrinsic and extrinsic neuromodulation, where the peptide can be released by neurons within the circuit or from ones extrinsic to it (Katz and Frost, 1996; Katz, 1998; Morgan et al., 2000; Marder and Bucher, 2007; Nusbaum and Blitz, 2012).

The repertoire of possible actions of colocalized substances in insects has been extended substantially with the discovery that also neurosecretory cells and gut endocrine cells can produce multiple peptide hormones that may act both locally in paracrine signaling, at peripheral targets and in certain neuronal circuits in the CNS to orchestrate physiology and behavior (Veenstra et al., 2008; Kahsai et al., 2010; Nässel and Winther, 2010; Veenstra and Ida, 2014; Wegener and Veenstra, 2015; Zandawala et al., 2018). More recently, studies employing single cell transcriptomics have expanded the list of colocalized neuroactive substances in *Drosophila* neurons (Abruzzi et al., 2017; Croset et al., 2017; Davie et al., 2017).

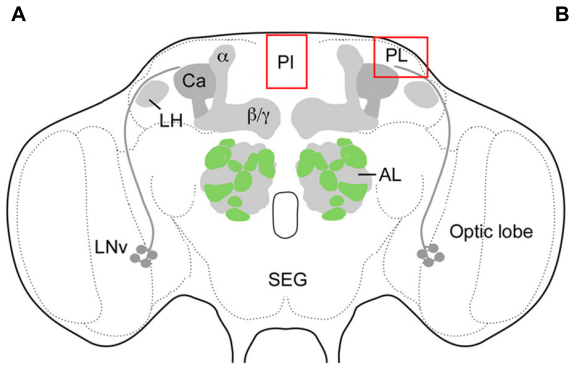
This review presents a summary of neurons and other cells in *Drosophila* that employ two or more colocalized peptides, or peptides coexisting with SMNs, including monoamines, amino acids and acetylcholine. Such coexistence occurs in neurosecretory cells, interneurons, sensory cells and motor neurons, as well as in endocrine cells of the intestine, indicating that neuropeptides act as local or more global neuromodulators, circulating hormones and hormone releasing factors or as cotransmitters of SMNs. Emphasis is on neuromodulation in clock circuits, olfactory and mechanosensory systems, mushroom bodies (MBs) and the neuromuscular junction, as well as hormone actions of peptides coreleased from neurosecretory/endocrine cells of the brain, ventral nerve cord (VNC) and intestine.

Since the terminology is somewhat diverse in different descriptions of neuroactive substances and their actions, some definitions and synonyms are provided in **Figure 1**. This figure also shows a schematic of the *Drosophila* brain with some of the structures discussed in this review. Abbreviations used in this review are listed in **Table 1**.

NEUROACTIVE COMPOUNDS THAT HAVE BEEN FOUND COLOCALIZED IN NEURONS OF *Drosophila*

Colocalization of Neuropeptides and Small Molecule Neurotransmitters

Out of all the neuropeptides and peptide hormones encoded by more than 50 genes known in *Drosophila* (see Nässel and Winther, 2010; Ida et al., 2011a,b; Jiang et al., 2013; Jung et al., 2014; Yeoh et al., 2017), very few have so far been mapped to neurons that coexpress other peptides or SMNs. This is primarily due to the fact that no systematic analysis of colocalization has been attempted. The following neuropeptides are known to be expressed in specific *Drosophila* neurons and endocrine cells that also utilize other neuroactive compounds based on



B

Neuroactive substance		Specifics
Classical neurotransmitters or Small molecule neurotransmitters (SMNs) or Fast neurotransmitters		Acetylcholine, GABA Glutamate, Glycine
(also SMNs)	Monoamines or Biogenic amines	Dopamine, Serotonin Octopamine. Histamine
Peptides:	Neuropeptide Peptide hormone Protein hormone	Encoded on precursor genes About 50 genes in *Drosophila* Cotransmitter, modulator, hormone
Neurotransmitter		Fast action; ion channels
Neuromodulator or Cotransmitter		Modulates fast transmission or Changes network properties

FIGURE 1 | Outline of *Drosophila* brain centers and some definitions. **(A)** The adult *Drosophila* brain with some of the centers/regions discussed in this review. The glomerular antennal lobes (AL) are located anteriorly. Projection neurons from the ALs supply axons to the calyx (Ca) of the mushroom bodies (MBs) and the lateral horn (LH). The MBs have three major lobes, α, β, and γ lobes. Neurosecretory cells are located in the pars intercerebralis (PI) and pars lateralis (PL), as well as in the subesophageal ganglion (SEG). Bilateral pacemaker centers of the circadian clock are located near the optic lobe and are formed by the LNv clock neurons and others not shown. **(B)** Some definitions of terms used in this review for signaling with neuroactive substances. So-called classical- or SMNs are sometimes referred to as fast neurotransmitters. The four listed ones are discussed in this review. Monoamines or biogenic amines can be subdivided into catecholamines, indolamines and phenolamines, and the four amines discussed here are listed. Neuropeptides/peptide hormones are very diverse and some larger ones are termed protein hormones. Commonly peptides/proteins of this kind act as cotransmitters, modulators or hormones over a slower and more long lasting temporal scale than the neurotransmitters. The neurotransmitters often activate ion channels leading to rapid changes in membrane potential.

immunocytochemistry, Gal4 expression or other "mapping" techniques (**Table 2**): adipokinetic hormone (AKH), allatostatin C (AstC), bursicon, Capa-pyrokinin/periviscerokinin (Capa-PK/PVK), crustacean cardioactive peptide (CCAP), corazonin (Crz), diuretic hormones 31 and 44 (DH31 and DH44), *Drosophila* insulin-like peptides 1–3, 5, and 7 (DILP1-3, 5 and 7), drosulfakinin (DSK), hugin-pyrokinin (hug-PK), IPNamide, leucokinin (LK), limostatin, myoinhibitory peptide (MIP), neuropeptide F (NPF), orcokinin B, partner of bursicon, pigment-dispersing factor (PDF), proctolin, short neuropeptide F (sNPF), and tachykinin (TK). References to these studies are provided in **Table 2**. There are also a few SMNs that have been shown to coexist with neuropeptides in *Drosophila* (**Table 2**). These are acetylcholine, GABA, glutamate and glycine and are commonly detected by indirect means. Presence of acetylcholine is in most cases based on antisera to choline acetyltransferase (Cha) and vesicular acetylcholine transporter (vAChT), or on *Cha* promoter Gal4-driven GFP expression (Yasuyama et al., 1995; Salvaterra and Kitamoto, 2001; Kolodziejczyk et al., 2008; Barnstedt et al., 2016). GABA detection relies on glutamic acid decarboxylase-1 (GAD-1) Gal4 expression and GABA or GAD-1 immunolabeling (Enell et al., 2007; Kolodziejczyk et al., 2008). Glutamate localization is indicated by immunolabeling with antisera to glutamate, vesicular glutamate transporter (vGluT) and vGluT-Gal4 expression (Mahr and Aberle, 2006; Hamasaka et al., 2007; Daniels et al., 2008; Kolodziejczyk et al., 2008).

Surprisingly, there is only one report on mapping a neuropeptide together with a biogenic amine in specific neurons of *Drosophila* (Castellanos et al., 2013), although this has been demonstrated more frequently in other insects such as moths, locusts and cockroaches and especially in mammals [summarized in (Hökfelt et al., 1987; Nässel, 2002; Nässel and Homberg, 2006)]. In the lamina of the *Drosophila* visual system there are cases of colocalized SMNs such as GABA and acetylcholine in C2 neurons, as well as acetylcholine and glutamate in L1 and L2 neurons (see Kolodziejczyk et al., 2008).

The studies discussed above are based on traditional imaging techniques and have probably only revealed the tip of the iceberg. Recently, reports on single-cell transcriptomics of dissociated *Drosophila* brain neurons discovered numerous additional patterns of colocalized neuropeptides and neuropeptides with SMNs (Abruzzi et al., 2017; Croset et al., 2017; Davie et al., 2017), shown in **Tables 3**, **4**. Although this type of analysis substantially increased the cases of likely colocalization of neuropeptides and monoamines, as well as other SMNs, it provides little information on which specific types of neurons that express the substances. Therefore it is urgent to localize the proposed SMNs and neuropeptides to specific brain neurons *in situ* using conventional mapping techniques to allow for circuit analysis. Such charting is likely to unveil a huge complexity in cotransmission in neuronal networks in the *Drosophila* brain. The examples of colocalized neuropeptides given above pertain to peptides that arise from distinct precursor genes. In the next section I will discuss coexpression of multiple neuropeptides derived from the same precursor gene.

Colocalization of Neuropeptides Derived From the Same Precursor Gene

Neuropeptide colocalization can also arise from expression of genes encoding precursors that can produce multiple

TABLE 1 | Abbreviations.

General abbreviations	Neuropeptide/neurotransmitter acronyms
ABLKs, abdominal leucokinin producing neurons	Ach, acetylcholine
CA, corpora allata	AKH, adipokinetic hormone
CC, corpora cardiaca	AstA, allatostatin A
DLPs, dorso-lateral peptidergic neurons (Crz expressing)	AstC, allatostatin C
DN, dorsal neurons (clock neurons, DN1-3)	Capa-PK, capa-pyrokinin (from *capability* gene)
DP1, dorsal paired neurons (1st abdominal ganglion)	CapaPVK, capa-periviscerokinin (from *capability* gene)
EECs, enteroendocrine cells (gut endocrines)	CCAP, crustacean cardioactive peptide
IPCs, insulin-producing cells (in brain)	Cha, choline acetyltransferase
LNCs, lateral neurosecretory cells	Crz, corazonin
LN_d, lateral neurons, dorsal	DH31 and DH44, diuretic hormones 31 and 44
LNs, local neurons (interneurons in antennal lobe)	DILP1-3, 5 and 7, *Drosophila* insulin-like peptides 1-3, 5 and 7
LN_v (l-LN_v, s-LN_v), lateral neurons, ventral (large and small)	dInR, *Drosophila* insulin receptor
MB, mushroom body	DSK, drosulfakinin
MBON, mushroom body output neuron	GAD-1, glutamic acid decarboxylase-1
MNC, median neurosecretory cell	hug-PK, hugin-pyrokinin (from *hugin* gene)
OSN, olfactory sensory neuron	LK, leucokinin
PN, projection neuron (antennal lobe)	MIP, myoinhibitory peptide (aka Allatostatin B)
SEG, subesophageal ganglion (aka subesophageal zone)	NPF, neuropeptide F
SMN, small molecule neurotransmitter	PDF, pigment-dispersing factor
VNC, ventral nerve cord	sNPF, short neuropeptide F
	TK, tachykinin (aka DTK)
	Upd1, unpaired-1(leptin-like cytokine)
	vAChT, vesicular acetylcholine transporter
	vGluT, vesicular glutamate transporter

copies of sequence-related peptide isoforms, or in a few cases precursors that generate peptides that might be functionally distinct. One example of the former is the thoracic Tv1-3 neurons that produce 8 different extended FMRFamide-like peptides derived from the same gene. Five of these peptides have variable N-terminal sequences, but a conserved FMRFamide C-terminus; the sequences of the other peptides differ more overall (Schneider and Taghert, 1988; Wegener et al., 2006). It appears that seven of these FMRFamides are functionally redundant in modulating the nerve-stimulated contraction of larval body wall muscles (Hewes et al., 1998). Several other *Drosophila* prepropeptides (peptide precursors) can give rise to multiple neuropeptides with related sequences (e.g., Ast-A, MIP, natalisin, sNPF, and TK), but so far there are no studies that suggest distinct differential functions (only slightly different potencies) of these sequence-related peptide isoforms in *Drosophila* or other insects (see Lange et al., 1995; Nässel and Winther, 2010; Jiang et al., 2013). It may be relevant to mention here that although isoform multiplication within a precursor may result in a diversification of functional neuropeptides over evolution, analysis of the genomes of 12 *Drosophila* species revealed a remarkable conservation of isoform sequences between these species (Wegener and Gorbashov, 2008). This suggests that peptides with biological activity are under stabilizing selection, but certainly some isoform diversification has occurred in some precursor genes during earlier evolution.

Some evidence for differential actions of sequence-related peptide isoforms exists in other animals. In mollusks a few sets of peptide isoforms derived from single precursors have been tested on different muscle preparations and these studies revealed that, depending on the dose, some isoforms can produce differential modulatory actions on contractions, whereas others are redundant (Brezina et al., 1995; van Golen et al., 1996). A more striking example of differential actions of sequence-related isoforms derived from a single precursor is provided by mammalian preprotachykinin A that gives rise to substance P and neurokinin A, which have different affinities for the three receptors NK1-3 that have different distributions (see Otsuka and Yoshioka, 1993).

The second variety of peptide precursors which generates colocalized peptides with distinct sequences can be exemplified by the one encoded on the *Capa* gene in *Drosophila.* This produces two distinct types of peptides Capa-PK and Capa-PVK1 and 2 (Capa1 and 2) (Kean et al., 2002). However, only the actions of the PVKs have been studied so far and the Capa-PK function remains to be determined. An excellent example of functional roles of distinct neuropeptides generated from the same precursor is from the snail *Lymnaea* where the FMRFamide gene encodes multiple peptides, several of which have sequences distinct from FMRFamide (Santama et al., 1995; Santama and Benjamin, 2000). Due to differential splicing of this gene, sets of distinct peptides (tetrapeptides and heptapeptides) are targeted to two specific neuron populations in a mutually exclusive pattern and serve in distinct aspects of heart regulation (Santama and Benjamin, 2000). In *Drosophila* the *Itp* gene is known to produce three splice forms, each giving rise to a distinct peptide (Dircksen et al., 2008). The resulting peptides, ITP, ITPL1, and ITPL2 differ somewhat in their sequences and only ITP is C-terminally amidated; these peptides are likely to display differential expression patterns and maybe distinct functions (Dircksen et al., 2008), but details are yet to be revealed.

Further discussion of functions of neuropeptides encoded by the same gene, both redundancy and differential roles can be found in (Nässel, 1996; Santama and Benjamin, 2000; Wegener and Gorbashov, 2008). As noted, the functional aspects of coexpressed neuropeptide isoforms is underexplored in *Drosophila* and certainly would merit a more systematic exploration in the future, especially for peptides derived from genes encoding Capa, NPLP1, and sNPF.

In the following sections I will discuss specific cases of colocalized substances in *Drosophila* neurons and neuronal circuits, as well as neuroendocrine cells in the CNS and elsewhere, including the intestine. When possible the functional implications of co-expressed substances will be discussed. However, I will not further deal with coexpression of neuropeptides derived from the same precursor gene.

TABLE 2 | Colocalization of neuropeptides with neuropeptides and other neuroactive substances in neurons and endocrine cells of *Drosophila* established by marker techniques[1].

Tissue[2]	Cell type[2]	Substances[3]	Reference
Brain	IPCs (NSCs; PI)	DILP1, 2, 3, 5, DSK	Brogiolo et al., 2001; Söderberg et al., 2012; Liu et al., 2016a
Brain	MNCs (NSCs; PI)	DH44, DILP2	Ohhara et al., 2018
Brain	DLP (NSCs; PL)	CRZ, sNPF, proctolin	Isaac et al., 2004; Kapan et al., 2012
Brain	ipc-1 (NSCs; PL)	ITP, sNPF, TK	Kahsai et al., 2010
Brain	l-LNv (clock neurons)	PDF, NPF, Upd1	Schlichting et al., 2016; Beshel et al., 2017
Brain	s-LNv (clock neurons)	PDF, sNPF, glycine[4]	Johard et al., 2009; Frenkel et al., 2017
Brain	5th s-LNv (clock neurons)	ITP, NPF, Ach[4]	Johard et al., 2009; Schlichting et al., 2016
Brain	LNd (clock neurons)	ITP, NPF	Johard et al., 2009
Brain	LNd (clock neurons)	sNPF, Ach[4]	Johard et al., 2009
Brain	DN1a (clock neurons)	DH31, IPNamide, Glutamate[4]	Shafer et al., 2006; Hamasaka et al., 2007; Goda et al., 2016
Brain	DN1p (clock neurons)	DH31, Glutamate[4,5]	Hamasaka et al., 2007; Kunst et al., 2014
Brain	LN (local neurons; AL)	MIP, Ach[4]	Carlsson et al., 2010
Brain	LN (local neurons; AL)	AstA, Ach[4]	Carlsson et al., 2010
Brain	LN (local neurons; AL)	TK, GABA[4]	Ignell et al., 2009
Brain	LN (local neurons; AL)	TK, MIP	Carlsson et al., 2010
Brain	LN (local neurons; AL)	TK, Ast-A	Carlsson et al., 2010
Brain	LN (local neurons; AL)	MIP, Ast-A	Carlsson et al., 2010
Brain	OSNs (sensory; AL)	sNPF, Ach[4]	Buchner et al., 1986; Nässel et al., 2008
Brain	OSNs (sensory; AL)[6]	MIP, Ach[4]	Hussain et al., 2016a
Brain	Kenyon cells (MB)	sNPF, Ach[4]	Johard et al., 2008; Barnstedt et al., 2016
Brain	NPF interneurons[7]	NPF, sNPF	Nässel et al., 2008
Brain	Small interneurons	sNPF, GABA[4]	Nässel et al., 2008
Brain	Small interneurons	sNPF, Ach[4]	Nässel et al., 2008
Brain	Small interneurons	sNPF, glutamate[4]	Nässel et al., 2008
SEG	Hugin neurons (L1)[8]	Hug-PK, Ach[4]	Schlegel et al., 2016
SEG	Large SEG neurons	Capa-PK, Hug-PK_{2-15}	Wegener et al., 2006
CC	Corpora cardiaca cells	AKH, Limostatin	Lee and Park, 2004; Alfa et al., 2015
VNC	ABLK (NSCs)	LK, DH44	Zandawala et al., 2018
VNC	DP1 (interneurons; L3)	DILP7, sNPF, Ach[4]	Nässel et al., 2008; Hu et al., 2017
VNC	CCAPa (NSCs; L3)	CCAP, Bursicon	Luan et al., 2006
VNC	CCAPp (NSCs; L3)	CCAP, Bursicon, MIP	Kim et al., 2006
VNC	Motoneurons (RP2; L3)	Proctolin, glutamate[4]	Luo et al., 2017
VNC	CRZ neurons (males)	CRZ, Ach[4]	Tayler et al., 2012
Midgut	Endocrine cells	TK, NPF	Veenstra et al., 2008
Midgut	Endocrine cells, posterior	TK, DH31	Veenstra et al., 2008
Midgut	Endocrine cells, middle	Ast-C, Orcokinin B	Veenstra and Ida, 2014
Midgut	Endocrine cells, L3	MIP, Ach[4]	LaJeunesse et al., 2010

[1]In adults, unless otherwise specified (L1 and L3, 1st and 3rd instar larvae). [2]Abbreviations (some acronyms are established names of neurons, and not explained here): SEG, subesophageal ganglion; CC, corpora cardiaca; VNC, ventral nerve cord; IPCs, insulin producing cells; NSCs, neurosecretory cells; PI, pars intercerebralis; PL, pars lateralis; AL, antennal lobe; OSNs, olfactory sensory neurons (antennae to brain); MB, mushroom body. [3]Abbreviations of peptides/transmitters as in text and in ***Table 1****. If not otherwise specified (details in text) determined by immunocytochemistry and/or Gal4 expression (details in text); in some cases antisera to biosynthetic enzymes. [4]Detected by promoter-Gal4 expression or antisera to biosynthetic enzymes [5]Note that DN1p constitute a cluster of neurons and individual ones have not been investigated; these cells appear heterogeneous in terms of transmitters/modulators (see also* ***Figure 5E****). [6]In female flies. [7]Two dorsal abdominal neurons shown in* ***Figure 3B****. [8]In hugin-PC and hugin-VNC/PH cells.*

COLOCALIZED PEPTIDES IN NEUROENDOCRINE CELLS IN THE BRAIN AND VENTRAL NERVE CORD

This section is a survey of coexpression of neuroactive compounds in neurosecretory and neuroendocrine cells of the brain and VNC, as well as in motor neurons and other efferent neurons. Neurosecretory cells release peptide hormones into the circulation to target a host of different tissues to regulate for instance metabolic homeostasis, diuresis, reproduction and developmental transitions (see Nässel and Winther, 2010). Peptide hormones are commonly released episodically and act over extended periods. Co-expressed peptide hormones may after release act on different or overlapping targets to

TABLE 3 | Neuropeptide transcripts expressed in *Drosophila* brain neurons sorted by neurotransmitter phenotype as determined by single cell transcriptomics (Davie et al., 2017).

	Peptide										
Monoamine											
Serotonin	DILP7	NPLP1	MIP	DH44	proct	NPLP2					
Dopamine				DH44			CCH2a	FMRFa			
Octopamine			MIP		Proct						DH31
Transmitter											
Cholinergic	DILP7						CCHa2	FMRFa	CCHa1	Capa	NPLP3
GABA		NPLP1						FMRFa			
Glutamate		NPLP1	MIP	DH44	Proct		CCHa2	FMRFa	CCHa1		

RNA sequence data from dissociated neurons were clustered to neuron types according to expression of specific neurotransmitter markers, such as vAChT/ChAT, vGluT, Gad1, SerT/Trh, Tdc/Vmat, and Ple/Vmat. For peptide acronyms see text and ***Table 1****. Gray lettering denotes weak expression. Data mined from Davie et al. (2017).*

TABLE 4 | Colocalized neuropeptides, monoamines, and neurotransmitters in *Drosophila* brain neurons based on single cell transcriptomics (Croset et al., 2017).

	Peptide										
Monoamine											
Dopamine	DH44	NPLP1	Gpb5	Proct							
Serotonin	**DH44**	NPLP1	Gpb5						sNPF		
Octopamine	DH44	**NPLP1**	Gpb5		SIFa	ITP	**DMS**	CAPA			
Tyramine		NPLP1	Gpb5		**SIFa**	ITP			sNPF	**MIP**	**DH31**
Transmitter											
Cholinergic	sNPF	CCHa2	TK								
GABA	DH31										
Glutamate	NPLP1	AstA									
2 or 3 of the above	DMS										

RNA sequence data from dissociated neurons were clustered to neuron types according to expression of specific neurotransmitter markers as in ***Table 3****. Bold text denotes strong expression. For peptide acronyms see text and* ***Table 1****. Gpb5, glycoprotein B5. From Croset et al. (2017).*

orchestrate organismal responses (Zandawala et al., 2018). Since neurosecretory cells have processes also within the CNS that express peptides it is not unlikely that they act within central circuits or interact with other neurosecretory cells (Kapan et al., 2012). Although not explicitly tested in insects, it might be that a neuropeptide colocalized with a typical peptide hormone acts within the CNS only, or in an autocrine regulation of hormone release at the axon termination. In the following cases of coexpressed peptide hormones are discussed, but also neuropeptides coexpressed in interneurons and efferent neurons innervating muscle fibers and reproductive organs.

Coexpression in Neurosecretory Cells in the Brain and Roles in Hormonal Signaling

In the brain of insects there are two major sets of neurosecretory cells, median neurosecretory cells (MNCs) and lateral neurosecretory cells (LNCs), both located dorsally in the protocerebrum (see Siegmund and Korge, 2001; Hartenstein, 2006) (**Figure 2A**). There are some additional neurosecretory cells in the subesophageal ganglion (SEG). In *Drosophila* the brain neurosecretory cells send axons to peripheral release sites (neurohemal areas) in the corpora cardiaca (CC) and/or corpora allata (CA) located at the junction between foregut and proventriculus anteriorly in the thorax, as well as the surface of the aorta, anterior foregut, proventriculus, and crop, thereby providing extensive release sites in contact with the open circulation (Siegmund and Korge, 2001; Hartenstein, 2006).

Peptides expressed in different sets of MNCs are *Drosophila* insulin-like peptides (DILPs) DILP1, 2, 3, and 5, sulfakinin (DSK), diuretic hormone 44 (DH44), and dromyosuppressin (DMS) (see Park et al., 2008; Nässel and Vanden Broeck, 2016) (**Figure 2A**). Probably more peptides are present in other MNCs, such as allatostatin B (AstB, also known as myoinhibitory peptide, MIP) and allatostatin C (AstC) (Williamson et al., 2001a,b; Min et al., 2016). The different LNCs express allatostatin A (AstA), corazonin (Crz), sNPF, proctolin, ion transport peptide (ITP), prothoracicotropic hormone (PTTH), and tachykinin (TK, also known as DTK) (Yoon and Stay, 1995; Mc Brayer et al., 2007; Kahsai et al., 2010; Kapan et al., 2012).

In some of the neurosecretory systems listed above colocalized peptides have been detected (**Figure 2A**). The following description pertains to adult *Drosophila* if not elsewise specified. The insulin-producing cells (IPCs) of the MNC group are known to coexpress DILP1, 2, 3, and 5, which are each encoded by a separate gene (Brogiolo et al., 2001; Rulifson et al., 2002; Liu et al., 2016a). DILP1 is only expressed transiently during pupal stages and the first few days of adult life, whereas the other DILPs are expressed throughout larval, pupal, and adult stages (see Liu et al., 2016a). Several studies have suggested that the different DILPs are regulated individually at the

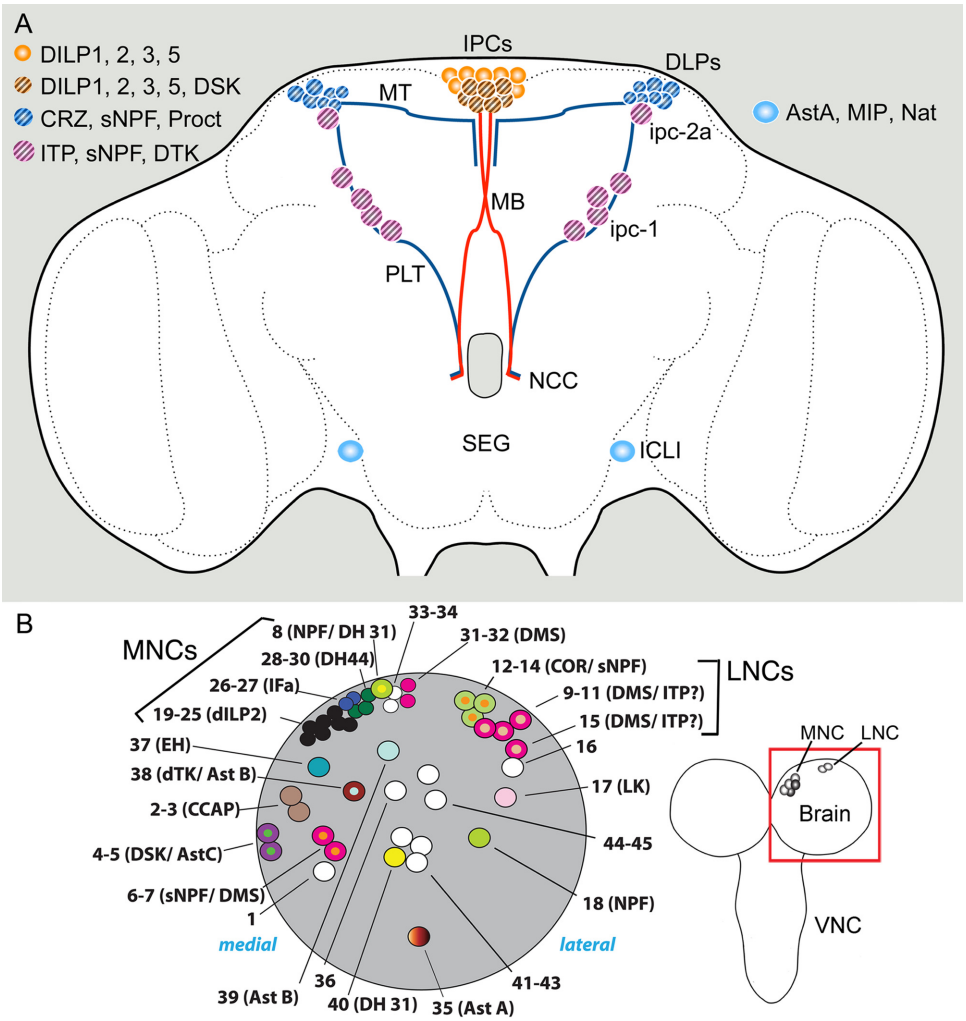


FIGURE 2 | Sets of neuroendocrine cells in the *Drosophila* brain that express colocalized neuropeptides and peptide hormones. **(A)** Neurosecretory cells and interneurons of the adult brain. The insulin producing cells (IPCs) co-express DILP1, 2, 3, and 5. Some of the IPCs (cross hatched) also produce drosulfakinins (DSK1 and 2). The IPCs are regulated by DLP neurons that produce short neuropeptide F (sNPF), corazonin (CRZ), and proctolin (Proct) (Kapan et al., 2012). These DLPs are likely to also release CRZ into the circulation via axons passing through the NCC nerves to peripheral release sites in the CC, anterior aorta and intestine (Kubrak et al., 2016). Another set of lateral neurosecretory cells (LNCs), designated ipc-1 and ipc-2a, express the peptides sNPF, tachykinin (TK) and ion transport peptide (ITP) (Kahsai et al., 2010). These neurons, like the DLPs, are parts of the LNC clusters and have axon terminations in peripheral sites overlapping those of the IPCs and the DLPs. A pair of large interneurons in the brain (ICLI) were shown to co-express AstA, MIP and natalisin Diesner et al. (2018). Further abbreviations: MT, medially projecting axon tract; PLT, posterior lateral axon tract; MB, median bundle. This figure is slightly altered and updated from Nässel et al. (2013). **(B)** Neuroendocrine cells in one hemisphere of the larval brain (dorsal view). The cells shown (numbered 1–43) are Dimmed positive cells, most of which have neuropeptides assigned to them. The *bona fide* MNCs include cells numbered 19–25, 28–30, and 31–32, the other adjacent neurons are peptidergic interneurons. Note several cases of colocalized peptides, some of which so far have not been observed in the adult brain: NPF/DH31, DMS/ITP, dTK/AstB, DSK/AstC, and sNPF/DMS (acronyms as in text). Left image from Park et al. (2008) with permission (PLOS, Open access).

transcriptional level (summarized in Ikeya et al., 2002; Grönke et al., 2010; Nässel et al., 2015; Nässel and Vanden Broeck, 2016) and also that release of the peptides from the IPCs is likely controlled separately for each DILP (Geminard et al., 2009; Kim and Neufeld, 2015). This requires that the different DILPs are located in different vesicle populations, which was proposed for DILP2 and 3 based on immunolabeling (Kim and Neufeld, 2015). There is also evidence that a multitude of different factors trigger transcriptional activation or release of the different DILPs in different combinations, further supporting that each of the colocalized peptide hormones is regulated separately [summarized in (Nässel et al., 2013; Alfa and Kim, 2016; Nässel and Vanden Broeck, 2016)]. These factors include nutrients, SMNs, neuropeptides and fat body derived factors. Taken together it is suggestive that the four DILPs colocalized in the IPCs have distinct functions during development and in the daily life of *Drosophila,* although some redundancy between the peptides has been demonstrated (Grönke et al., 2010). These DILP functions include regulation of growth, carbohydrate and lipid metabolism and storage, stress responses, fecundity and

lifespan (Brogiolo et al., 2001; Tatar et al., 2001, 2014; Rulifson et al., 2002; Grönke et al., 2010; Alfa and Kim, 2016).

In addition to the DILPs, the IPCs also produce drosulfakinins (DSK1 and 2), two cholecystokinin-like peptides (Park et al., 2008; Söderberg et al., 2012). Although DSK can be found in additional brain neurons, DSK in the IPCs seems sufficient to induce satiety in flies (Söderberg et al., 2012). Since DILPs (at least certain ones) are released after feeding, and can induce satiety, the colocalized DSK may act together with DILPs to orchestrate post-feeding physiology. In *Drosophila* DSK is also known to regulate gut function, hyperactivity and aggression (summarized in Nässel and Williams, 2014; Williams et al., 2014).

Recently it was shown that in adult flies the six DH44 producing MNCs also produce weak DILP2 expression (Ohhara et al., 2018) (see **Figures 3A**, **7A** for DH44 neurons). Thus, there are possibly novel roles of DILP2 associated with activation of the DH44 neurons.

Among the LNCs there is a set of neurons, designated DLPs, that produces Crz, sNPF and proctolin (Isaac et al., 2004; Nässel et al., 2008; Kapan et al., 2012) (**Figure 2A**). These neurons use sNPF to regulate IPCs, and Crz for systemic signaling to regulate metabolism and stress responses (Kapan et al., 2012; Kubrak et al., 2016). Both sNPF and Crz have been extensively studied in other contexts in *Drosophila*. Accumulated data suggest that in *Drosophila* Crz is involved in stress signaling, metabolism, sperm transfer, and copulation (see Veenstra, 2009; Zhao et al., 2010; Tayler et al., 2012; Kubrak et al., 2016) whereas sNPF is truly multifunctional, including roles as a pre- and postsynaptic cotransmitter [summarized in (Nässel and Wegener, 2011; Hu et al., 2017) and discussed in later sections]. Since there is no evidence for hormonal functions of sNPF it is possible that its role in DLP neurons is confined to the brain, such as being a regulator of IPCs, or even as a regulator or hormone release in the CC. The role of proctolin in DLPs has not been investigated, although in locusts proctolin (probably from similar LNCs) has been shown to trigger release of AKH from the CC and stimulate juvenile hormone biosynthesis in the CA (Clark et al., 2006).

Another set of prominent neurosecretory cells of the brain is the 10 ITP producing ipc-1 and Ipc-2a neurons with cell bodies in the LNC group and axons to the CC/CA, aorta and anterior intestine (Dircksen et al., 2008) (**Figures 2A**, **3A**). These cells were found to colocalize ITP, sNPF, and tachykinin (TK) in adult flies (Kahsai et al., 2010), and maybe in addition leucokinin (LK) at least in early larvae (Herrero et al., 2007). Knockdown of either sNPF or TK in ipc-1/Ipc-2a neurons leads to decreased survival during desiccation and starvation, as well as an increase in water loss during desiccation (Kahsai et al., 2010). The role of ITP was not studied at the time due to lethality associated with its non-conditional knockdown. It is possible that ITP acts in *Drosophila* as an antidiuretic hormone, since in the locust ITP was shown to stimulate Cl^- transport from gut lumen to the circulation and thereby reabsorb water (Audsley et al., 1992; Coast et al., 2002). Thus, the ipc-1/Ipc-2a neurons appear to utilize three different neuropeptides to control water homeostasis and responses associated with metabolic and ionic stress. While these neurons supply axon terminations also to the CA, it is possible that one or several of the peptides are involved in regulating juvenile hormone biosynthesis, together with insulins from the IPCs (Tu et al., 2005). In the flour beetle *Tribolium castaneum* knockdown of ITP induced reduced fecundity and in larvae ITP is important in molting behavior (Begum et al., 2009). It might also be of interest in the context of colocalized ITP and TK in the *Drosophila* ipc-1 neurons to note that in the silk moth *Bombyx* a receptor was identified that can be activated both by TK and a splice form of ITP (Nagai-Okatani et al., 2016).

Finally, there is a pair of large and widely arborizing *interneurons* in the lateral brain that produce at least three neuropeptides: AstA, MIP, and natalisin (Diesner et al., 2018) (**Figure 2A**). These cells are designated ICLI (Jiang et al., 2013). The peptides coexpressed in the ICLI neurons have been assigned individual functions in earlier studies, as listed next; the question is how they might act together to orchestrate a behavior or physiological function when coreleased from these neurons. The only known role of natalisin so far is in fecundity (Jiang et al., 2013), whereas the other peptides display several functions. MIP regulates mating, satiety and sleep stabilization (Oh et al., 2014; Min et al., 2016; Jang et al., 2017) and AstA is known to regulate feeding and sleep (Hergarden et al., 2012; Hentze et al., 2015; Chen et al., 2016b). These actions are probably via interneuronal pathways, some of which may converge on IPCs and insulin signaling. It would be of interest to search for further roles of natalisin since at this point it is not clear how this peptide could cooperate with AstA and MIP. Also, to grasp the compound function of the three peptides produced by the ICLI neurons it would be interesting to genetically interfere with activity of these cells. Do they regulate food ingestion and postprandial sleep, or some other behavior?

A systematic screen of peptidergic neurons in the larval brain of *Drosophila* revealed many of the same cases of coexpression seen in adults and added a few more (Park et al., 2008). The additional combinations in neurosecretory cells are dromyosuppressin (DMS) and ITP, and in other neurons of the brain DMS/sNPF, TK/MIP, NPF/DH31, and DSK/AstC (**Figure 2B**). A recent study also indicated that some of the important Hugin cells of the SEG, known to be at the interface between gustatory inputs and regulation of feeding in larvae, not only express hugin-PK, but also are likely to be cholinergic (Schlegel et al., 2016). The cell groups displaying this coexpression are the hugin-PC and hugin-VNC/PH cells.

Coexisting Peptides in Neuroendocrine Cells of the Ventral Nerve Cord

Neurosecretory cells of the VNC regulate some functions overlapping those of the brain, but also have several distinct roles (Nässel and Winther, 2010). In the VNC cells colocalized peptide hormones also appear to coordinate several targets to orchestrate physiology and behavior. Some peptidergic interneurons or efferents discussed below utilize co-expressed neuropeptides and SMNs in various ways such as feedback facilitation of input neurons or signaling to reproductive organs.

In the thoracic neuromeres there is a set of six large FMRFamide-expressing neurons (Tv1-3) with release sites in a plexus of axon terminations in the dorsal neural sheath

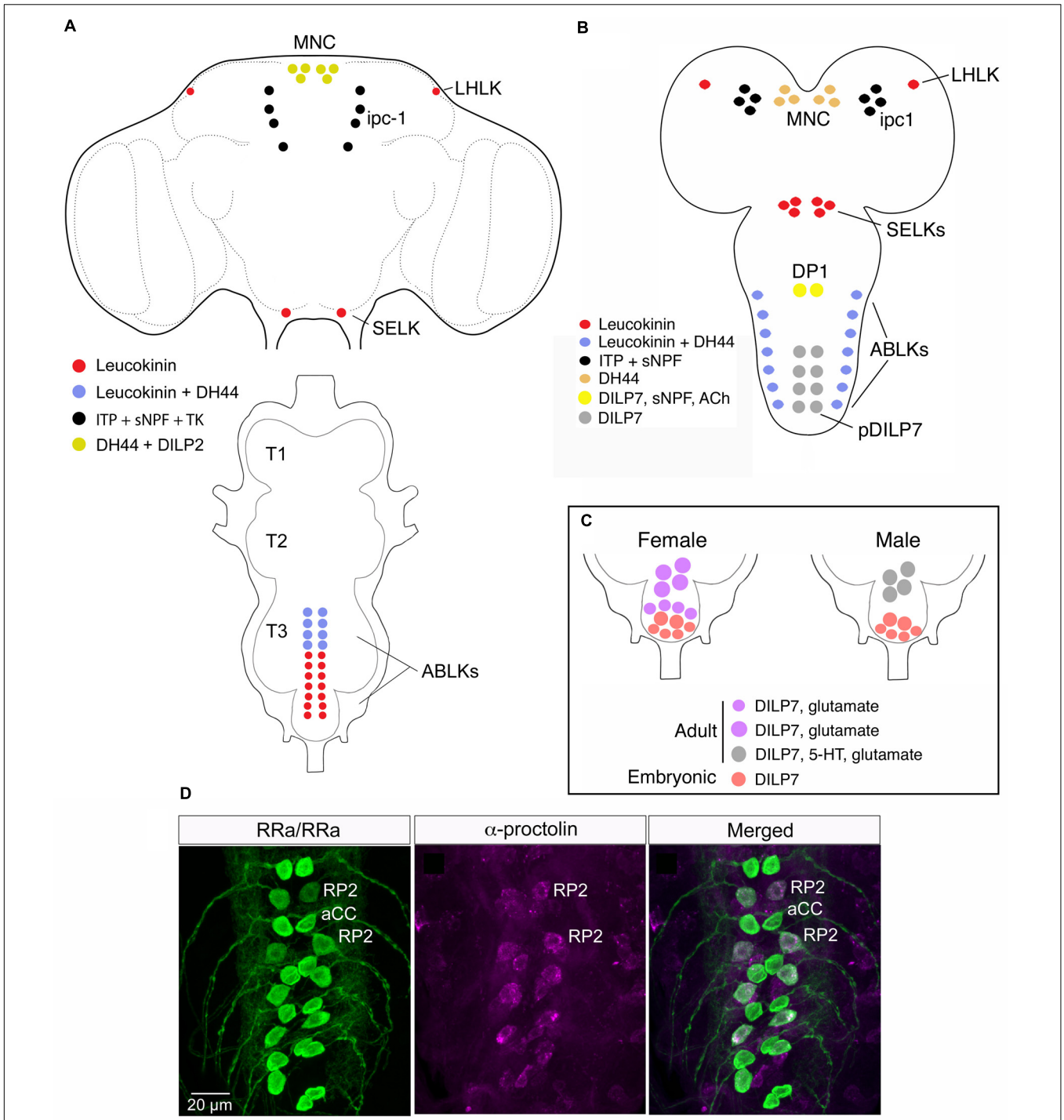


FIGURE 3 | Neuroendocrine cells colocalizing neuropeptides and neurotransmitters in the CNS of adult and larval *Drosophila*. **(A)** Neuroendocrine cells colocalizing leucokinin and diuretic hormone 44 in the adult CNS. In the brain there are 4 neurons expressing leucokinin (LK), 6 neurons producing diuretic hormone 44 (DH44) and 8 ipc-1 neurons producing ion transport peptide (ITP; shown because they express Lk-Gal4), tachykinin (TK) and sNPF. In the abdominal ganglion at least 8 of the 22 ABLKs coexpress LK and DH44. Note that in adults, but not larvae the brain MNCs coexpress DH44 and DILP2 (Ohhara et al., 2018). This figure is slightly altered from Zandawala et al. (2018). **(B)** In third instar larvae all ABLKs coexpress LK and DH44. In younger larvae the ipc-1 neurons, in addition to ITP and sNPF, express weak LK (de Haro et al., 2010; Kahsai et al., 2010). One subset of midline neurons expressing DILP7, the DP1 neurons, coexpress sNPF and acetylcholine (Ach) (Nässel et al., 2008). **(C)** In adult flies the posterior DILP7 expressing neurons in abdominal ganglia consist of neurons derived from the embryo (pDILP7 in **B**) and adult-specific ones that are generated later. There is a sex-dimorphism in that females have a larger number of adult DILP7 neurons that also produce glutamate, whereas in males the adult DILP7 neurons produce additional serotonin (5-HT) (Castellanos et al., 2013). **(D)** In each neuromere of the larval ventral nerve cord (VNC) there is a pair of midline motor neurons, aCC and RP2 that signal with glutamate. The RP2 neurons also produce proctolin from Luo et al. (2017).

of the VNC (Lundquist and Nässel, 1990; Schneider et al., 1993). In abdominal neuromeres there are segmental neurons producing different peptide hormones, such as Capa peptides, CCAP, Bursicon, LK, DH44, orcokinins, and the glycoproteins GPA2/GPB5, (Cantera and Nässel, 1992; Luan et al., 2006; Sellami et al., 2011; Chen et al., 2015; Zandawala et al., 2018) (**Figures 3A,B**). Posteriorly in abdominal ganglia there are peptidergic neurosecretory cells (or efferent neurons) that supply axon terminations to the posterior intestine. These produce PDF, ITP, AstA, proctolin, and DILP7 (Anderson et al., 1988; Nässel et al., 1993; Yoon and Stay, 1995; Kean et al., 2002; Dircksen et al., 2008; Miguel-Aliaga et al., 2008; Cognigni et al., 2011; Sellami et al., 2011).

Neurons of the abdominal ganglia display several cases of coexpressed peptides (**Figures 3A,B**). In *Drosophila* larvae there are seven pairs of segmental neurosecretory cells in the abdominal ganglia (A1–A7) that express LK, designated ABLKs (Cantera and Nässel, 1992; de Haro et al., 2010) (**Figure 3B**). In adults the number of ABLKs is 20–22, by addition of 4–6 larger cells anteriorly in the abdominal ganglion (Luo et al., 2013; Alvarez-Rivero et al., 2017) (**Figure 3A**). LK is one of several diuretic hormones in *Drosophila* (Terhzaz et al., 1999) and is likely to be released into the circulation from the ABLKs that have axon terminations along the abdominal body wall and heart. LK acts on stellate cells of the Malpighian tubules to increase fluid secretion across the epithelium (Terhzaz et al., 1999; Halberg et al., 2015). Recently it was found that the ABLKs also express another diuretic peptide, the corticotropin-releasing factor-like DH44 (Zandawala et al., 2018) (**Figures 3A,B**). DH44 acts on another cell type in Malpighian tubules, the principal cells, also to stimulate fluid secretion (Cabrero et al., 2002). Thus, it appears as if the ABLKs release LK and DH44, and that both regulate diuresis, but via different epithelial cell types and intracellular mechanisms [Cl^- transport and cAMP mediated transport, respectively (Cabrero et al., 2002, 2014)]. Furthermore, the effect of the two hormones on secretion is additive (Zandawala et al., 2018). Both LK and DH44 have been shown to also regulate survival during stress induced by desiccation (Liu et al., 2015; Cannell et al., 2016). By targeted knockdown of each peptide in ABLKs it was found that either DH44 or LK signaling from these cells is sufficient for the regulation of resistance to desiccation, as well as ionic and starvation stress (Zandawala et al., 2018). However, regulation of food intake was only affected by DH44 knockdown in ABLKs. It should be noted that these two peptides are produced, but not colocalized, also in other neurons of the CNS, and that in these neurons DH44 and LK may serve other functions related to the circadian clock, sleep, metabolism, and reproduction (Lee et al., 2015; Cavey et al., 2016; Murakami et al., 2016).

There is a set of segmentally distributed neuroendocrine cells along the midline of abdominal ganglia that express DILP7 both in larval and adult abdominal ganglia (Miguel-Aliaga et al., 2008; Castellanos et al., 2013) (**Figures 3B,C**). In the most anterior pair, the interneurons DP1 with axons ascending to the brain, DILP7 is colocalized with sNPF and Cha expression (Nässel et al., 2008) (**Figure 3B**). It was found that the larval DP1 neurons are part of a circuit that integrates nociceptive (mechanosensory) inputs utilized in an escape response (Hu et al., 2017). In this circuit the DP1 neurons signal with sNPF back to their input neurons, specific nociceptive sensory neurons, and DILP7 is not necessary for the integrative function of the circuit. Probably acetylcholine is also not involved in this response since blocking regular neurotransmission has no effect on the response (Hu et al., 2017).

The posterior DILP7 neurons are more heterogeneous, especially in adult flies. Some are interneurons, others are efferents with axons to the hindgut or reproductive organs; additionally some neurons are adult-specific and sexually dimorphic (Castellanos et al., 2013) as shown in **Figure 3C**. The embryo-derived cells express only DILP7 in both sexes, whereas the neurons specific to the adult in males produce DILP7, serotonin and glutamate and innervate the seminal vesicle, but are not required for fertility (Castellanos et al., 2013). The female post-embryonic cells only express DILP7 and glutamate, and appear to be motor neurons that innervate the oviduct and are required for fertility.

Another case of sex-specific expression of a neuropeptide and a coexpressed neurotransmitter was demonstrated in the abdominal ganglia of adult flies where males have a set of Crz producing interneurons that also express Cha (Tayler et al., 2012). These neurons act via efferent Crz-receptor expressing serotonergic neurons that innervate the male accessory glands where they regulate sperm transfer. The role of acetylcholine in the signal transfer to the serotonergic neurons was not investigated.

In summary of the above section, one can infer that colocalized peptide hormones may orchestrate organismal physiology by acting on relevant targets, and that neuropeptides can provide feedback signals to presynaptic neurons. However, the functional role of coexpressed neuropeptides and SMNs remains to be explored in VNC interneurons and efferents.

PROCTOLIN AS A COTRANSMITTER OF GLUTAMATE IN MOTOR NEURONS OF THE VENTRAL NERVE CORD

When the neuropeptide proctolin was first mapped to neurons in insects it was noted that in the cockroach *Periplaneta americana* certain slow skeletal motor neurons produce this peptide in addition to glutamate (Adams and O'Shea, 1983). Neuron stimulation or depolarization by potassium application triggered proctolin release onto the target muscle, a coxal depressor. A cotransmitter role was suggested since proctolin produces a delayed and sustained muscle contraction without actually depolarizing the muscle (Adams and O'Shea, 1983). This response differed from the normal response of muscle to activation of the Ds motor neurons, which is a transient depolarization and rapid contraction typical of glutamate stimulation (Adams and O'Shea, 1983).

Also in *Drosophila* certain motor neurons express proctolin (Anderson et al., 1988; Taylor et al., 2004). In *Drosophila* larvae especially the segmental mid-line motor neurons designated RP2

coexpress glutamate and proctolin (Luo et al., 2017) (**Figure 3D**). Application of proctolin onto body wall muscle in *Drosophila* larvae indicated muscle fiber specific actions and induced dose dependent slow contractions (Ormerod et al., 2016). Proctolin also potentiated nerve-evoked muscle contractions. Knockdown of proctolin receptor decreased thermal preference and larval crawling at higher temperature (Ormerod et al., 2016).

COLOCALIZED NEUROPEPTIDES IN ABDOMINAL NEURONS COORDINATE ECDYSIS MOTOR BEHAVIOR

In larval *Drosophila* there are sets of neuroendocrine cells in abdominal ganglia that regulate motor activity during molting behavior (**Figure 4**). These neurons express colocalized CCAP and bursicon and a subset produces also a third peptide, MIP (Kim et al., 2006; Luan et al., 2006). As seen in **Figure 4**, these neurons together with other abdominal and thoracic neurons that use CCAP, FMRFamide or LK express receptors for ecdysis triggering hormone (ETH) and when activated by ETH they signal in a temporal sequence (with feedback inhibition) to coordinate the ecdysis motor behavior (Kim et al., 2006, 2015). It is not yet clear how the co-localized MIP and CCAP act together in generating the ecdysis motor pattern, but bursicon released from some of the CCAP neurons is likely to act on other targets that regulate post-ecdysis phenomena (Luan et al., 2006). Also the role of DH44 coexpressed in the LK producing ABLKs (Zandawala et al., 2018) is not known. Since LK recently was found to play a hormonal role in regulating fluid transport in trachea during larval molts (Kim et al., 2018), it is possible that coreleased DH44 acts in a similar fashion.

COEXPRESSED NEUROPEPTIDES AND NEUROTRANSMITTERS IN THE CLOCK NEURONS OF THE BRAIN

Daily activity and physiology of animals is synchronized with the 24 h cycle of earth's rotation around its axis with the aid of an endogenous circadian clock. In *Drosophila* the master clock is situated in the brain and consists of about 150 neurons in 8 bilateral groups (Nitabach and Taghert, 2008; Schlichting et al., 2016) shown in **Figures 5A,B**. The first neuropeptide to be associated with clock neurons was PDF, expressed by small and large lateral ventral neurons, s-LN_vs and l-LN_vs (Helfrich-Förster, 1995; Renn et al., 1999). In addition to PDF, several other neuropeptides have been mapped to different clock neurons in *Drosophila* (Johard et al., 2009; Hermann et al., 2012; Kunst et al., 2014; Schlichting et al., 2016; Abruzzi et al., 2017). In several sets of clock neurons different combinations of these neuropeptides are colocalized (**Figures 5C–E** and **Table 2**). As will be shown below, most studies of clock neuron peptides have analyzed their functions one by one, but in some cases it is evident that colocalized peptides target different neurons of the clock network, and it has also been shown that PDF and an unidentified SMN act together on a common set of target neurons (Choi et al., 2012).

The two sets of main pacemaker neurons (morning and evening oscillators) the l-LN_vs/s-LN_vs and LN_ds display different combinations of the neuropeptides PDF, NPF, sNPF, and ITP (**Figures 5C,D**) and some subsets of neurons also produce SMNs such as acetylcholine, and glycine (**Table 2**). The l-LN_vs are known to express PDF and NPF (at least in some cells), as well as the cytokine unpaired 1 (Upd1), and the 5th s-LN_v produces ITP, NPF and acetylcholine (Johard et al., 2009; Schlichting et al., 2016; Beshel et al., 2017). Single cell transcriptomics identified further neuropeptide candidates in LNds: DH44, AstC, DMS, Hugin peptides, and trissin (Abruzzi et al., 2017) (**Figure 5E**). The dorsal neurons (DNs) are located in three clusters DN1-3 and are diverse in terms of neuroactive substances. The two DN1a (anterior) neurons produce glutamate, IPNamide (from NPLP1 precursor) and diuretic hormone 31 (DH31) (Shafer et al., 2006; Hamasaka et al., 2007; Goda et al., 2016). The other DN1 neuron cluster located more posteriorly (DN1p) as a group expresses transcripts for DH31, NPF, sNPF, CCHamide1, and CNMamide (Abruzzi et al., 2017) (**Figure 5E**). This peptide transcript expression was determined by RNA sequencing of dissociated DN1p neurons, and it is therefore not clear in which specific DN1p neurons they are located or to what extent the neuropeptides are colocalized. However, DH31 was mapped to some DN1p neurons by immunolabeling (Kunst et al., 2014).

Of all these peptides PDF is the one extensively investigated for distinct roles in the clock network and as an output of LN_vs (see Nitabach and Taghert, 2008; Taghert and Nitabach, 2012; Schlichting et al., 2016). Also sNPF and NPF play defined roles within the network, but for the other peptides the main information available is on network outputs monitored as activity, sleep or other behaviors. With the exception of colocalized sNPF and PDF, discussed below, there are no clear data on the outcome of cotransmission by neurons in the clock circuit. However, to provide an idea of what the individual neuropeptides/SMNs do, and how one could approach analysis of cotransmission, I briefly summarize the known signaling roles of these substances in the clock network.

As seen in **Figure 5D**, PDF from both small and large LN_vs act on sets of clock neurons that generate evening activity (evening oscillators) and the large LN_vs signal to small ones. The l-LN_vs receive light inputs both from the compound eyes and the extraretinal photoreceptors of the eyelet, whereas the s-LN_vs only from the latter (Schlichting et al., 2016). All groups of neurons in the network shown in **Figure 5D** (M, E1, and E2 oscillators), except l-LN_vs, express the PDF receptor (PDFR), and s-LN_vs receive light information from large cells, but also seem to utilize the PDFR as autoreceptors (see Taghert and Nitabach, 2012). More specifically, these autoreceptors inhibit s-LN_v activity and PDF release and thereby play a role in setting the phase of daily outputs, including locomotor activity (Liang et al., 2017). The PDF signaling to other clock neurons is inhibitory and causes delays in calcium activity in follower neurons, LNd and DN3 (see **Figure 6**).

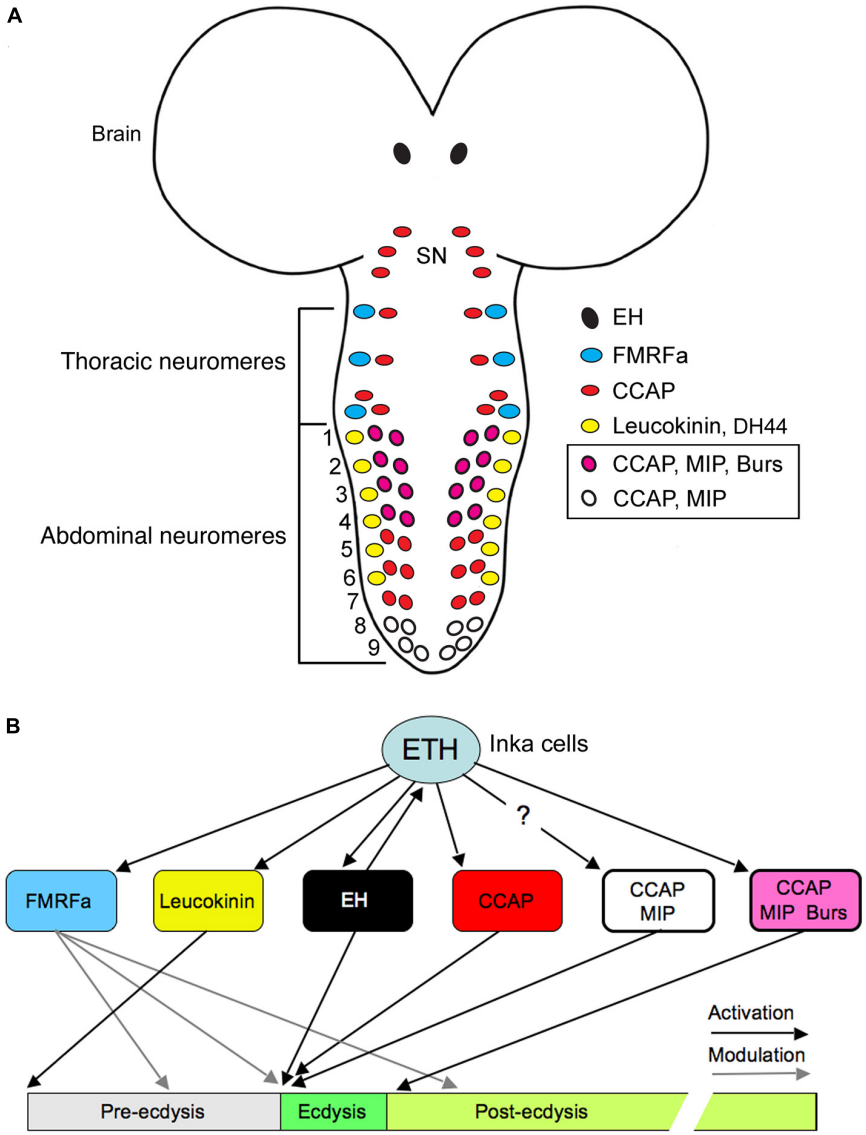
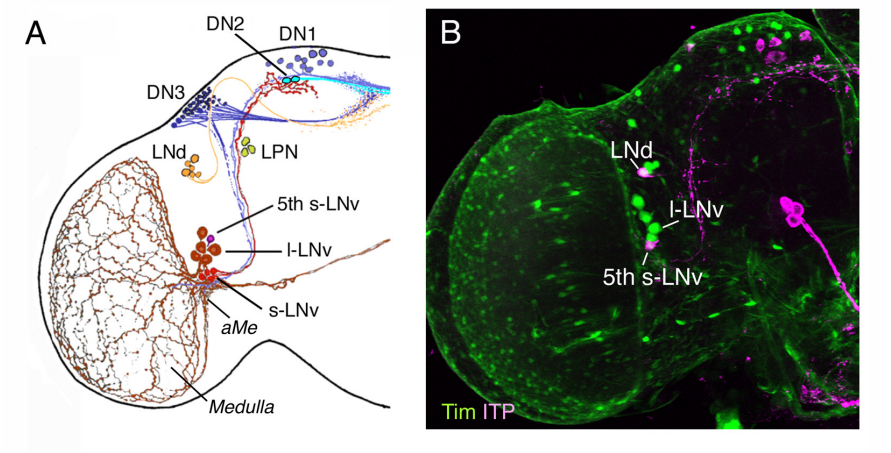


FIGURE 4 | Neurons and neuropeptides that control ecdysis motor activity in *Drosophila*. **(A)** Schematic depiction of peptidergic neurons in the larval CNS that express the ecdysis triggering hormone receptor ETHR-A. These neurons respond to ETH in a sequence and trigger ecdysis behavior as shown in **(B)**. The color-coding depicts the expression of various neuropeptides in the neurons (note that some colocalize 2–3 peptides). ETH is released from peritracheal Inka cells (not shown). SN, subesophageal neuromeres 1–3; ETH, ecdysis triggering hormone; FMRFa, FMRFamide; EH, eclosion hormone; CCAP, crustacean cardioactive peptide; MIP myoinhibitory peptide; burs, bursicon. Redrawn from Kim et al. (2006), Nässel and Winther (2010).

Some other neuropeptides, such as sNPF and NPF, found in clock neurons also act in the clock network in patterning daily rhythms. Release of sNPF from s-LN$_v$s and LNds acts to sculpt the DN1 activity period at night by suppressing DN1 activity at other times (Liang et al., 2017). The s-LN$_v$s receive negative PDF feedback in an autocrine loop, and both sNPF and PDF suppress Ca^{2+} levels in other pacemakers (**Figure 6**), thereby providing a neuropeptide-mediated chain of sequential inhibition and delay in the network that ensures phase-setting of neuronal activity (pacemaker entrainment) (Liang et al., 2017). This interplay between sNPF and PDF in a defined network is also a good example how a single neuron type (s-LN$_v$) can use sNPF and PDF to target different neurons (**Figure 6**). Another example of division of labor between sNPF and PDF released from s-LN$_v$s will be discussed below in the context of a developmental transition.

NPF was found in 1–3 of the l-LN$_v$s and in the 5th s-LN$_v$ and function was analyzed in flies with the NPF expressing clock neurons genetically ablated (Hermann et al., 2012). Such ablation eliminates the neurons with both NPF and other colocalized substances and results in flies with prolonged free-running period in constant darkness, an advanced phase of the evening activity peak and reduced amplitude of this peak. Further experiments suggested that this phenotype arose from ablation of the NPF



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Cell	Neuropeptide							
LNd	NPF	sNPF	DH44	AstC	DMS	Hugin	Trissin	ITP
LNv	NPF	sNPF	DH31	PDF				
DN1	NPF	sNPF	DH31	CCH1a	CNMa			

FIGURE 5 | Several neuropeptides are colocalized in different types of clock neurons in *Drosophila*. **(A)** The different types of clock neurons in one brain hemisphere. There are lateral ventral neurons (s-LNv and l-LNv), lateral dorsal neurons (LNd), dorsal neurons (DN1–DN3), and a set of lateral posterior neurons (LPN). aMe, accessory medulla. This panel is originally from Helfrich-Förster et al. (2007), but slightly modified in Johard et al. (2009). **(B)** Expression of Tim-Gal4-driven GFP (green) and immunolabeling for ion transport peptide (ITP; magenta). From Johard et al. (2009). **(C)** Neuropeptides in LNd and LNv neurons are colocalized in different patterns. The figure is compiled from data in Johard et al. (2009) and Schlichting et al. (2016). Note that the s-LNvs also produce the amino acid transmitter glycine (Frenkel et al., 2017) and l- LNvs the cytokine Upd1 (Beshel et al., 2017). **(D)** The clock output generates daily activity with peaks in the morning (M) and evening (E) and low activity at night and mid-day. This activity is generated by clock neurons that act as morning (M) and evening (E1–E3) oscillators. These express different combinations of neuropeptides and some interactions between LNvs and other neurons are known to be by means of pigment-dispersing factor (PDF) shown in red arrows. The roles of other peptides are less known so far (but, see **Figure 6**). This figure is compiled in part from data in (Schlichting et al., 2016). **(E)** This table shows additional neuropeptides in clock neurons indicated by singe cell transcriptomics. Note that the cell types listed were assayed as groups and there is no information on peptides in subtypes of neurons in these groups. Thus it is not known to what extent the neuropeptide transcripts are coexpressed in specific neurons; this is especially prominent for the relatively large group of DN1 neurons, which may be heterogeneous. Data mined from Abruzzi et al. (2017).

expressing LNds and the 5th s-LN$_v$ (Hermann et al., 2012). Diminishing NPF by RNAi in clock neurons only had a minor effect and slightly advanced the evening activity phase. With simultaneous knockdown of both PDF and NPF the effect was stronger and resembled that seen after ablation of the neurons. However, the specific role of NPF in the clock circuit needs to be further characterized by future experiments, and especially the combined roles of the neuropeptides, known to be colocalized with NPF, require further study.

Some neuropeptide roles in the clock neurons involve aspects other than signaling within the network to generate rhythmic activity patterns. ITP release from the 5-th s-LN$_v$s is under clock control and knockdown of ITP in these cells and LNds results in reduced evening activity of the flies and an increase in

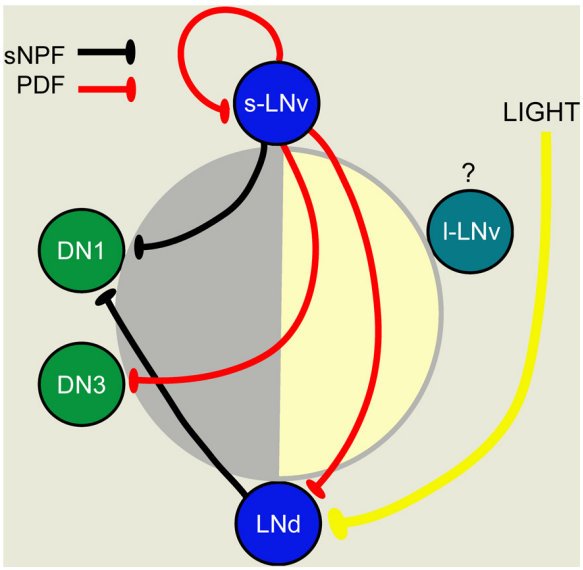

FIGURE 6 | A scheme depicting PDF-, sNPF-, and light-mediated interactions that orchestrate sequential Ca^{2+} activity phases in different pacemaker groups. Each pacemaker group is represented by one neuron. The position of cells in the day-night circle (yellow–gray) indicates the peak phase of Ca^{2+}. Both PDF and sNPF signals inhibit the target neurons and suppress these from being active when the sender neurons (s-LNv for PDF; s-LNV and LNd for sNPF) are active. Light cycles act together with PDF to delay Ca^{2+} phases in LNds. This figure was redrawn from Liang et al. (2017).

night activity (Hermann-Luibl et al., 2014). These authors also demonstrated that interference with ITP did not affect PERIOD (PER) cycling, suggesting that the peptide is part of an output pathway rather than being a signal within the clock network. Knockdown of both ITP and PDF resulted in hyperactive flies that were arrhythmic in constant darkness, and also displayed reduced sleep both during mid-day and night (Hermann-Luibl et al., 2014).

Neuropeptide F in clock neurons regulate aspects of mating behavior (Lee et al., 2006; Hamasaka et al., 2010; Kim et al., 2013), sleep-wake behavior (Chung et al., 2017), and indirectly the peptide regulates circadian gene expression in the fat body (Erion et al., 2016). Again, it is not known whether combined actions of co-expressed peptides affect these behaviors. Another peptide, sNPF, has been implicated in regulation of sleep: this peptide in s-LN_vs is promoting sleep without affecting feeding (Shang et al., 2013). DH31 in the clock system was demonstrated as a wake-promoting neuropeptide acting before dawn (Kunst et al., 2014). Finally, DH31, and to a lesser extent PDF, acting on DN2 neurons regulate night time temperature preferences in flies and PDF mainly regulates locomotor activity rhythm (Goda et al., 2016). Interestingly, these authors propose that DH31 acts via the somewhat promiscuous PDF receptor in DN2 neurons to decrease temperature preference at night onset.

Another aspect of clock outputs is regulation of developmental transitions such as shedding the old cuticle (ecdysis) in insects. Timing of molts relies on production of the steroid hormone ecdysone in the prothoracic gland and is regulated by PTTH produced in two pairs of LNCs (Mc Brayer et al., 2007). A recent study revealed that timing of the final molt, the adult emergence from the puparium (eclosion), is regulated by the s-LN_vs signaling with sNPF, but not PDF, to the PTTH neurons (Selcho et al., 2017). This s-LN_v signal thereby serves to coordinate the central clock with the local one in the prothoracic gland (Selcho et al., 2017). Of interest in this review: this is a clear example of a distinct separation of functions of two colocalized neuropeptides.

What about the SMNs in the clock neurons? DN1s were shown to promote sleep by glutamate release that inhibits pacemaker neurons (both morning and evening oscillators) and a feed-back circuit ensures generation of the mid-day siesta and night sleep, especially in males (Guo et al., 2016). The inhibitory neurotransmitter glycine in LN_vs contributes to synchronization of the circadian network (Frenkel et al., 2017). These authors showed that diminishing the glycine production in LN_vs increases the period length, without affecting the locomotor activity rhythm of the flies. Thus, fast inhibitory neurotransmission in addition to PDF plays a role in synchronizing the clock circuit, and it was proposed that PDF and glycine released from s-LN_vs might signal to the LNds to affect the period of activity (Frenkel et al., 2017). An earlier study provided evidence that activation of PDF autoreceptors on s-LN_vs modulates release of PDF and a non-identified SMN resulting in a rhythm acceleration and increased morning activity (Choi et al., 2012). The SMN mediating this light-induced phase-shift might be glycine.

Do signal molecules from clock neurons act on circuits outside the *bona fide* clock network to regulate behavior that is not directly involving locomotor activity or sleep? A recent finding was that the leptin-like cytokine Upd1 (Unpaired 1) is produced by LNv clock neurons (Beshel et al., 2017). The Upd1 receptor Domeless (Dome) is expressed in several NPF neurons in the brain, known to be orexigenic, and disruption of Upd1 signaling leads to increased food attraction and food ingestion as well as weight increase. These findings suggest that clock neuron-derived Upd1 suppresses NPF neuron activity and thereby food intake. It is not clear from the study whether NPF expressing clock neurons are among the ones expressing the Upd1 receptor Dome, or if the effect is on NPF neurons outside the principal clock circuit.

In summary of the above, it is known that in addition to locomotor activity and sleep, some of the outputs of the clock regulate specific behaviors such as feeding and reproduction. A few specific outputs of the clock are relevant to consider in the light of the coexpressed neuropeptides in the system. One pathway that was recently discovered consists of connections between clock neurons and output neurons regulating locomotor activity, without affecting feeding rhythm (King et al., 2017). This pathway, shown in **Figure 7A**, comprises connections from s-LN_v neurons to DN1s, that signal to DH44 neurons in the PI, which in turn connect to Hugin neurons in the SEG that via descending axons regulate glutamatergic premotor neurons in the VNC (Cavanaugh et al., 2014; King et al., 2017). These connections were established both by GRASP technique (GFP reconstitution across synaptic partners) and genetic manipulations. In this pathway it is postulated (but not clearly shown) that s-LN_vs

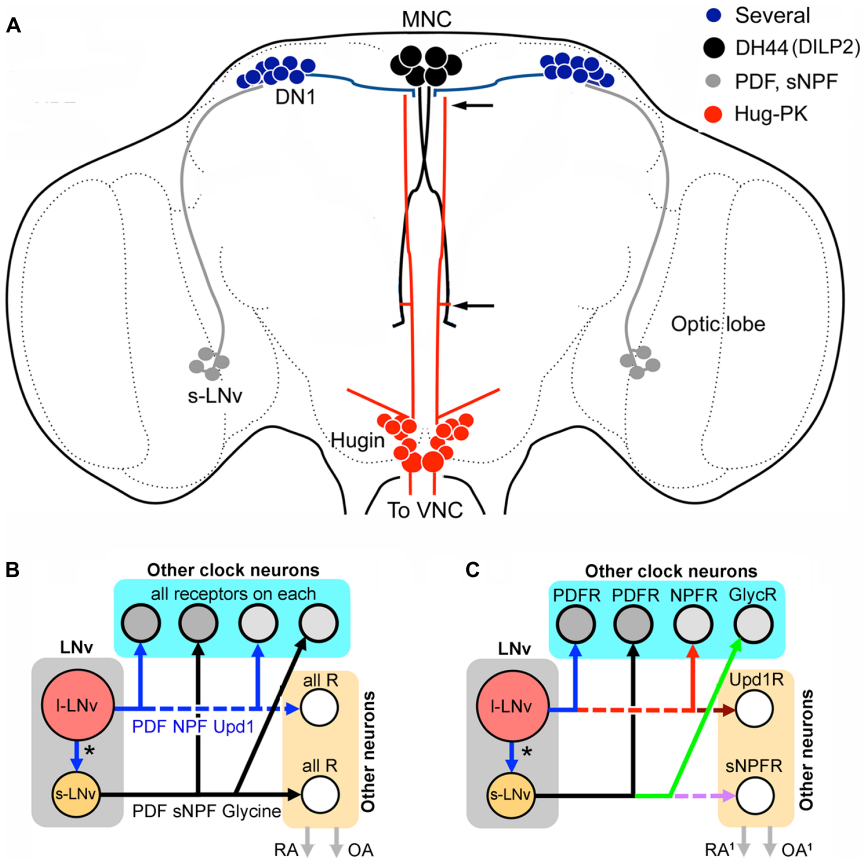

FIGURE 7 | Networks of peptide cotransmission in the clock circuits of *Drosophila*. **(A)** Neuronal circuit that regulates locomotor activity proposed in King et al. (2017). It was suggested that s-LN$_v$s signal to DN1s that in turn act on DH44-producing MNCs. These signal with DH44 to hugin cells in the SEG via the DH44-R1. Some of the hugin cells have axons that terminate in the VNC where they contact glutamatergic neurons in motor centers that generate locomotion. The two arrows indicate areas where interactions between MNCs and hugin cells can occur. It is not known which peptide/neurotransmitter of the s-LNvs and DN1s that signal in this pathway. For s-LNvs it could be PDF, sNPF or glycine and for DN1s there are several candidate peptides (see **Figure 5E**). Note that the MNCs also produce DILP2 (Ohhara et al., 2018). **(B)** The colocalized neuropeptides and other signaling molecules in the LN$_v$s may target different constellations of neurons within and outside the clock circuitry; these are represented by "other clock neurons" and "other neurons," respectively. The "other neurons" are effectors downstream to the clock circuits that regulate, e.g., rhythmic activity in behavior and physiology (RA), or neuronal systems that influence other behaviors such as reproduction, foraging and feeding (and indirectly metabolism), or produce systemic responses via hormone release (OA). In the simplest model, shown in **(B)**, all the target neurons for the large and small LN$_v$s express receptors for all of the signal molecules released. Thus, targets of s-LN$_v$s within and outside the clock circuit would all express receptors (R) for PDF, sNPF and glycine, and targets of l-LN$_v$s have receptors for PDF, NPF and Upd1 (unpaired-1). Each of the neurons would thus receive multiple complementary signals. **(C)** Hypothetically the target neurons of the LN$_v$s could be more diverse and express only subsets of the relevant receptors in different combinations. In this extreme example each target neuron type expresses only one receptor type and the downstream circuits could generate neuropeptide and signal substance-specific activity. Here, activity in, e.g., s-LN$_v$s would simultaneously generate a more diverse set of actions within and outside the network. These could include some actions that are targeted only to neurons outside the bona fide clock network (e.g., the Upd1 or sNPF actions in this example). The outputs, RA^1 and OA^1, could be more diversified.

signal with PDF to the DN1 neurons, which in turn use an unknown substance to activate the DH44-MNCs. The signal between the MNCs and Hugin neurons was shown to be DH44, presumably via its receptor DH44-R1 and these communicate with glutamatergic neurons in motor circuits possibly with the peptide hug-PK (King et al., 2017). This pathway has some room for additional or alternative signals from s-LN$_v$s (sNPF or glycine) and for DN1s there are several candidate peptides (see **Figure 5E**). It can be mentioned that also brain insulin producing cells (IPCs) are under modulation by DN1s giving rise to rhythmic action potential firing frequency in IPCs (Barber et al., 2016). This study suggests that IPCs, although they have cell autonomous nutritional inputs that also affect the firing rhythm, are under additional circuit regulation. Thus, IPC signaling that affects feeding and metabolism is under rhythmic clock control (Barber et al., 2016). It is not known which of the multiple substances in DN1s that modulate IPC activity.

Finally, it has been demonstrated that there is a link between the central clock and the peripheral clock in the fat body in *Drosophila*. Many gene transcripts cycle in the fat body, but some cytochrome P450 transcripts cycle independently of the fat body clock and are instead dependent on NPF expressing brain clock neurons, probably LNds (Erion et al., 2016). It is not clear how the signal from the NPF clock neurons reaches the fat

body, but it is likely to be via interactions with neurosecretory cells such as IPCs or other MNCs. It is also not entirely clear whether NPF is the only required signal from these clock neurons since NPF knockdown was less effective than silencing the NPF neurons (Erion et al., 2016). As shown above the NPF expressing LN_vs also produce ITP or PDF (see **Figure 5C**) and the LNds perhaps produce even further neuropeptides (**Figure 5E**).

Clearly, cotransmission plays a fundamental role in different parts of the clock circuitry and is of key importance for understanding the organization and logic of the regulatory hierarchy in the network. How does the clock network use multiple SMNs and neuropeptides for internal and external signaling? An attempt to summarize hypothetical cotransmission outputs from LNv clock neurons is shown in **Figures 7B,C**. In **Figure 7B** the simplest scheme assumes that all direct target neurons of each small or large LN_v express all the receptors for the released peptides, inferring that each target within the clock network, or outside, would be modulated by several substances. The scheme in **Figure 7C** displays the other extreme where each of the substances acts on different target neurons (with a corresponding receptor), thus producing divergent outputs that can generate specific effects in different parts of the network or outside. The outside network action is shown for example by DN1s interacting with IPCs and DH44 expressing MNCs. Another molecule that might target non-circuit neurons is Upd1 that may signal to orexigenic NPF non-clock neurons. This scheme would enable single clock neuron types to modulate both network properties and activity related to other behaviors such as feeding, metabolism and reproduction. Probably neither of the two schemes is fully correct. Even a scheme that is a hybrid of the two proposed ones would probably be subject to an additional possibility: the presence of given receptors may not predict signaling outcomes. The action of specific ligands on receptors in different cell types may result in responses that differ depending on cell type and context.

COTRANSMISSION IN THE OLFACTORY SYSTEM

Subpopulations of each of the components of the olfactory system, the olfactory sensory neurons (OSNs), local interneurons (LNs), and projection neurons (PNs) that carry sensory signals to higher brain centers, have all been shown to display colocalization of different combinations of neuropeptides and SMNs. Studies of olfactory sensory processing and odor-guided behavior have explored a few cases of cotransmission of neuropeptides and SMNs to reveal mechanisms of presynaptic facilitation or inhibition that regulates state-dependent food search, as discussed below.

Olfactory Sensory Neurons Colocalize Acetylcholine and sNPF or MIP

Sensory cells, including OSNs, in *Drosophila* utilize acetylcholine as their primary neurotransmitter (Buchner et al., 1986; Yasuyama and Salvaterra, 1999; Masse et al., 2009). A scheme of the neurons and SMNs and neuropeptides in the olfactory system is shown in **Figure 8A**.

It was found that a subset of the OSNs of the antennae and maxillary palps coexpress sNPF (Nässel et al., 2008). The sNPF expressing OSNs supply axon terminations to at least 13 of the approximately 50 glomeruli of the antennal lobe (Carlsson et al., 2010). Each glomerulus receives sensory input from one specific odor channel (olfactory receptor type) (Couto et al., 2005; Fishilevich and Vosshall, 2005) and, thus, a subset of these odor signals can be relayed with acetylcholine and modulated by an intrinsic neuromodulator, such as sNPF.

A specific role of sNPF in modulation of food odor detection was demonstrated in *Drosophila* (Root et al., 2011). A hungry fly displays vigorous food search (foraging behavior) and obviously pays more attention to food-related odors. This odor sensitivity is regulated by systemic insulin signaling, and an autocrine loop in the OSNs involving sNPF and its receptor sNPFR (**Figure 8B**). The insulin receptor (dInR) is expressed on OSNs and so are both sNPF and its receptor, sNPF-R (Root et al., 2011). In hungry flies circulating insulin (DILP) levels are low and expression of sNPF-R is high in OSNs and food odor stimulation triggers release of sNPF, which via action on the autoreceptor increases release of acetylcholine, the primary transmitter at the synapse with PNs. This potentiates the odor signal to higher brain centers and leads to increased food search (Root et al., 2011). After feeding the DILP levels increase in the circulation and activation of the dInR in OSNs in antennae causes an inhibition of transcription of the sNPFR and thus minimal autocrine sNPF signaling leading to decreased activation of PNs and decreased food search (Root et al., 2011). Thus, sNPF is a cotransmitter of acetylcholine that facilitates synaptic activation dependent on insulin signaling over an extended period until the fly has found and ingested food.

Another neuropeptide expressed in a small subpopulation of OSNs is MIP, and also the MIP/sex peptide receptor is expressed in the same cells (Hussain et al., 2016a,b). This is seen in female flies in OSNs expressing Ir41a/Ir76b ionotropic receptors that are sensitive to polyamines. Similar to sNPF the MIP peptide acts in an autocrine loop in OSNs to regulate polyamine attraction in mated flies, and sex peptide does not seem to be involved (Hussain et al., 2016a,b).

Local Interneurons of the Antennal Lobe Colocalize GABA and Tachykinin and Several Other Combinations

The LNs of the antennal lobe use acetylcholine, GABA and glutamate as neurotransmitters (Masse et al., 2009; Seki et al., 2010). As seen in **Table 2** there are LNs coexpressing different combinations of SMNs and neuropeptides: some GABAergic LNs produce TK (Ignell et al., 2009), some cholinergic LNs express MIP or AstA, furthermore TK is coexpressed with MIP or AstA, and MIP was found together with AstA in LNs (Carlsson et al., 2010).

The role of TK signaling has been analyzed in the olfactory system. TK from LNs acts on TK receptors (DTKR, *TkR99D*)

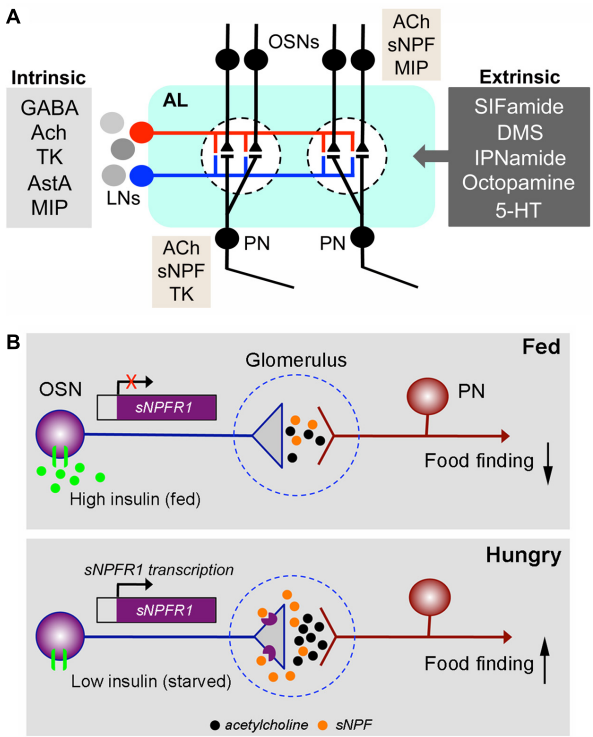

FIGURE 8 | Coexpression and neuromodulation in the olfactory system. **(A)** Neuromodulation in the *Drosophila* antennal lobe. The antennal lobe (AL) is shown highly schematically with only two glomeruli (dashed outlines). Inputs to the glomeruli are from OSNs in the antenna and labial palps. The OSNs synapse on PN that relay signals to higher brain centers (MBs and lateral horn). The OSNs and PNs are modulated by local neurons (LNs), which form intrinsic modulatory circuits and by extrinsic neurons that utilize several neurotransmitters/neuromodulators. The LNs are either GABAergic, cholinergic (Ach), or in some cases glutamatergic. The former two types are known to colocalize the neuropeptides tachykinin (TK), allatostatin-A (AstA) or myoinhibitory peptide (MIP) (Carlsson et al., 2010), whereas it is not known whether glutamatergic ones colocalize any peptide. The extrinsic neurons utilize SIFamide, dromyosuppressin (DMS), IPNamide (from precursor NPLP1), octopamine or serotonin (5-HT) (Dacks et al., 2006; Sinakevitch and Strausfeld, 2006; Roy et al., 2007; Carlsson et al., 2010). It is not known whether any of these extrinsic neurons colocalize other neurotransmitters/neuropeptides. Additionally, a subpopulation of the OSNs coexpress Ach and sNPF (Nässel et al., 2008; Carlsson et al., 2010) and in females some OSNs with Ir-type receptors coexpress MIP (Hussain et al., 2016a). Recent reports from single cell transcriptomics suggest that some PNs may express sNPF and others TK in addition to Ach (Croset et al., 2017). The general outline of this figure is redrawn from (Lizbinski and Dacks, 2017). **(B)** The OSNs are regulated presynaptically by insulin and sNPF. This figure, based on a paper by Root et al. (2011), shows an OSN synapsing on a PN in the antennal lobe of a fed *Drosophila* fly (top) and a hungry one (bottom). The synapse is located within a glomerulus. In the fed fly, the level of circulating insulin is high and the activated insulin receptor (dInR) on the OSN inhibits transcription of the sNPF receptor, sNPFR1. Thus, there is low expression of the sNPFR1 presynaptically on the OSN axon termination and signal transfer with acetylcholine at the synapse is weak. As a result, food search/finding is low. In the hungry (starved) fly, insulin levels are low and the transcription of sNPFR1 in the OSNs is activated. Consequently, presynaptic sNPFR1 expression increases and released sNPF activates the presynapse leading to enhanced release of acetylcholine and thus increased signaling in the synapse: food search/finding increases. This is an example of an autocrine presynaptic regulation. The figure is slightly altered from Nässel (2012), which was compiled from Root et al. (2011).

by suppressing calcium and synaptic transmission in the OSNs, thereby providing presynaptic inhibitory feedback (Ignell et al., 2009). It was later shown that also TK receptor expression in OSNs (with Or42b and Or85a receptors) is regulated by feeding-dependent DILP signaling (Ko et al., 2015). The coordinated action of the two peptides decreases synaptic outputs from Or42b OSNs (positive valence) and increases Or85a OSNs (negative valence) simultaneously, thereby diminishing the overall attractive value of food odors. During starvation, reduced insulin levels leads to upregulation of sNPFR and DTKR in their respective OSNs resulting in an increased attraction of food odors (Ko et al., 2015). Whereas sNPF facilitates cholinergic transmission in OSNs, it is not clear whether TK acts to modulate GABA transmission in LNs.

ANOTHER CASE OF PRESYNAPTIC FACILITATION: ACETYLCHOLINE AND sNPF IN KENYON CELLS OF THE MUSHROOM BODIES

The MBs are prominent paired neuropils in the protocerebrum of insects (see **Figure 1**) and known to be centers of olfactory learning and memory (Heisenberg, 2003). The MBs are formed by thousands of intrinsic neurons called Kenyon cells and various types of extrinsic neurons of efferent and afferent nature (Takemura et al., 2017). In *Drosophila* the MBs have been under intense investigation for many years (see Heisenberg, 2003; Davis, 2005; Perisse et al., 2013), but only recently was a SMN assigned to the Kenyon cells. Evidence was put forth for the production of acetylcholine by these cells and that this neurotransmitter mediates the output of the MB via nicotinic receptors on MB output neurons, MBONs, and is critical for learning (Barnstedt et al., 2016). It had been shown earlier that a major subpopulation of the Kenyon cells express sNPF (Johard et al., 2008), and a neuromodulator role of sNPF was indicated in a study of sugar reward olfactory memory (Knapek et al., 2013). Now it is known that sNPF potentiates the response to acetylcholine in MBONs suggesting that the peptide presynaptically facilitates the response to the fast neurotransmitter (Barnstedt et al., 2016) as shown earlier in the OSNs (Root et al., 2011). Since food-associated memory formation is enhanced by hunger (Krashes et al., 2009) it would be of interest to determine whether sNPF signaling in the MBs is regulated by the nutritional state of the fly, as was shown in the OSNs (Root et al., 2011).

COLOCALIZATION OF PEPTIDES IN INTESTINAL ENDOCRINE CELLS

Like the neurosecretory cells of the brain and VNC, the midgut endocrine cells in some cases coexpress peptide hormones that might act on different targets to orchestrate physiology and maintain homeostasis. However, functional aspects of coreleased hormones have not been investigated so far.

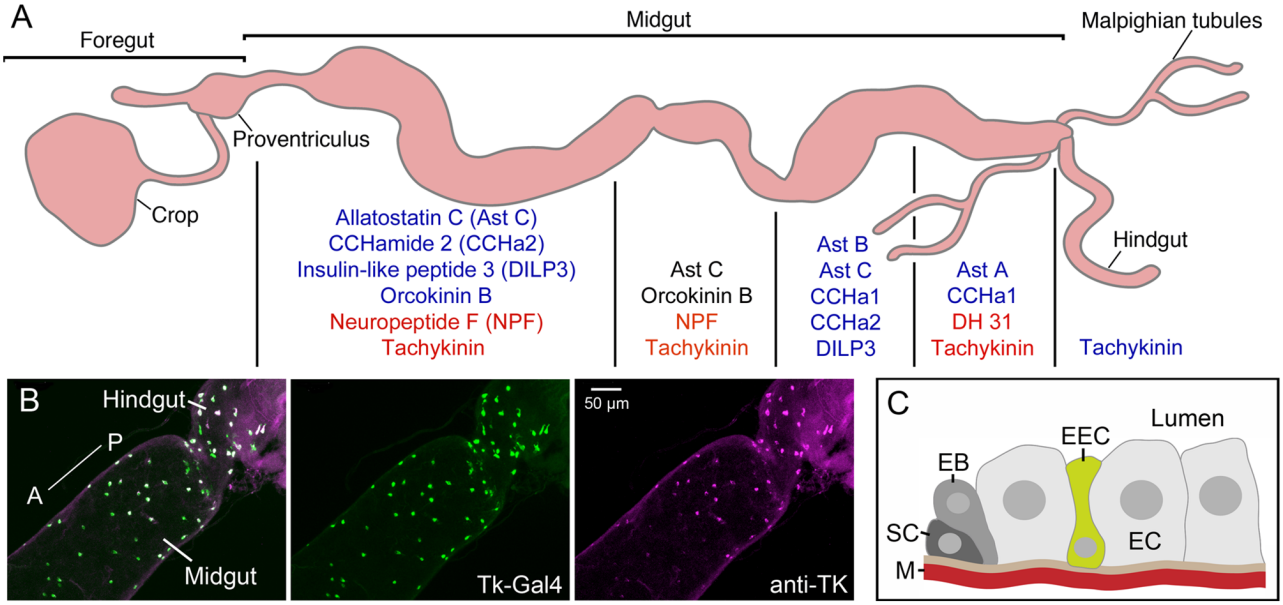

FIGURE 9 | Neuropeptides and peptide hormones in the *Drosophila* intestine. **(A)** In the midgut enteroendocrine cells (EECs) produce neuropeptides/peptide hormones in a region-specific manner. Four midgut regions are shown here [based on (Veenstra and Ida, 2014)]. Peptides produced are shown in blue text, and those that are colocalized in EECs in the different regions are shown in red or black text (NPF and tachykinin, AstC and orcokinin B, DH31 and tachykinin). DH31 is calcitonin-like diuretic hormone 31. There are also EECs producing tachykinin in the anterior hindgut. This figure is compiled from Veenstra et al. (2008); Veenstra and Ida (2014). **(B)** EECs in the posterior midgut and anterior hindgut produce tachykinin (TK) as demonstrated by Tk-Gal4 driven GFP (green) and antiserum to TK (magenta). From Liu et al. (2016b). **(C)** Schematic depiction of an EEC located between enterocytes (EC). The EECs may sense nutrients in the gut lumen and release peptide either locally in a paracrine fashion or into the circulation outside the muscle layer (M). The other cells shown are stem cells (SC) and enteroblasts (EB) that are responsible for renewal of the gut epithelium. This figure was redrawn from a figure in Lemaitre and Miguel-Aliaga (2013).

In the *Drosophila* midgut epithelium there are different types of endocrine cells, known as enteroendocrine cells, EECs (**Figure 9**). In adult flies 10 different neuropeptides/peptide hormones have been detected in such EECs (Veenstra et al., 2008; Veenstra and Ida, 2014) (see **Figure 9**). Additionally DILP3 is produced by intestinal muscle cells (Veenstra et al., 2008) and sparse bursicon expression was seen in some EECs (Scopelliti et al., 2014). In EECs of the anterior and middle midgut TK and NPF are colocalized, in the midportion orcokinin B and allatostatin C, and in the posterior end TK and DH31 (**Figure 9A**) (Veenstra et al., 2008; Veenstra and Ida, 2014; Chen et al., 2016a). Of note is that in the larval gut anteriorly located MIP producing EECs were shown to also express Cha, suggesting that they produce acetylcholine, which may act in paracrine signaling (LaJeunesse et al., 2010). Little is known about the function of gut-derived peptides in *Drosophila*. Data suggests that the EECs can release peptide hormones into the circulation, as well as use them for paracrine signaling (Winther and Nässel, 2001; Veenstra et al., 2008; Reiher et al., 2011; Li et al., 2013; Song et al., 2014; Wegener and Veenstra, 2015; Chen et al., 2016b; Liu and Jin, 2017).

It was demonstrated *in vitro* that intestines of locust and cockroach display depolarization-induced release of TK and that hemolymph levels of TK increased after starvation, suggesting that EECs can release peptide into the open circulation (Winther and Nässel, 2001). Other indirect evidence also supports the possibility of hormonal action of EEC-derived peptides (AstA and CCHamide2) on distant targets, such as the brain (Li et al., 2013; Chen et al., 2016b). One study has shown that TK from EECs acts in a paracrine fashion in the midgut of *Drosophila* to regulate local lipid production in the enterocytes, thereby contributing to lipid homeostasis systemically (Song et al., 2014). These authors also showed that EEC-derived TK does not affect behavior of the fly, indicating that the gut peptide does not affect neurons in the CNS. Possible roles of peptides colocalized with TK were not investigated in the same context. Further local functions of gut peptides have been suggested for other insects: modulation of gut peristalsis, secretion of digestive enzymes and regulation of ion transport (see LaJeunesse et al., 2010; Nässel and Winther, 2010; Reiher et al., 2011). It is noteworthy that some EECs (NPF- and DH31-producing) express gustatory receptors, such as for instance Gr43a, known to be a fructose sensor (Park et al., 2016). Thus, nutrient sensing could be cell autonomous in EECs and regulate peptide release.

To search for sites of action of EEC-derived gut peptides one can start by screening for expression of their cognate receptors in various tissues. Using available transcriptome databases Veenstra et al. (2008), Veenstra and Ida (2014) identified transcripts of receptors of all gut peptides except orcokinin B in the midgut, and AstA-R2 also in the hindgut. Most of these receptors are also expressed in the CNS, suggesting that the gut peptides theoretically could act in both these tissues. Many other targets of gut peptides are possible, since inter-organ communication

appears to be important aspect of maintaining homeostasis in the fly (Owusu-Ansah and Perrimon, 2015; Liu and Jin, 2017).

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

This review highlighted some neuronal and endocrine systems in the CNS and intestine of *Drosophila* where neuroactive substances are coexpressed. These include neurosecretory cells in the brain and VNC, chemosensory cells and interneurons of the olfactory system, nociceptive neurons, intrinsic neurons of the mushroom body, different clock neurons, as well as EECs of the intestine. However, in spite of these reports on cellular co-distribution of neuropeptides/neuropeptides and neuropeptides/SMNs in *Drosophila*, analysis of the functional relevance of cotransmission has lagged far behind. Furthermore, reports published so far on mapping of coexpression to specific neurons are likely to cover only a small fraction of the actual cases. This is suggestive from a few recent reports analyzing transcriptomes of single dissociated cells in the *Drosophila* brain where preliminary data already uncovered plenty of new combinations of colocalized substances (Abruzzi et al., 2017; Croset et al., 2017; Davie et al., 2017) shown in **Tables 3**, **4**. These novel data constitute a rich source for future systematic mapping of neuropeptides and SMNs to neurons and circuits in the brain of *Drosophila* by imaging techniques.

Several features of neuronal cotransmission mentioned in the introduction remain unexplored in *Drosophila*. For instance, it is not clear to what extent peptidergic neurons in general are connected to other neurons by traditional synapses and to what degree peptide signaling is paracrine (or parasynaptic). However, a recent analysis of early *Drosophila* larvae revealed synaptic connections between some identified sets of peptidergic neurons, including the hugin cells and IPCs (Schlegel et al., 2016), but evidence for volume transmission remains to be provided. Related to this, it is not known how far neuromodulators can diffuse within the insect CNS. In general these questions are more acute in *Drosophila* than in mammals since almost no mapping of neuropeptide/SMN receptors is available for insects and thus the spatial match between release sites and receptors is not known. Another aspect that remains to be investigated in *Drosophila* is the complex dynamics of cotransmission in modulation of network activity, matching that available for the stomatogastric ganglion in crustaceans (see Nusbaum et al., 2001, 2017; Marder and Bucher, 2007; Marder, 2012; Nusbaum and Blitz, 2012). Most *Drosophila* studies employ genetic tools to tamper with signaling components, and even when combined with dynamic calcium imaging these manipulations are commonly too crude to reveal relevant dynamic changes in the network properties or neuronal pathways. However, *Drosophila* has proven excellent for analysis of neuromodulation in single synapses in the olfactory system (Root et al., 2008; Ko et al., 2015). In the following I will discuss the few advances made in cotransmission in *Drosophila* and highlight some of the areas where further studies would be valuable.

Cotransmission Analyzed in *Drosophila*

Table 5 summarizes neurons and circuits where analysis of cotransmission has been employed in *Drosophila*. This Table also highlights that sNPF plays multiple roles in synaptic facilitation or other cotransmission in a multitude of circuits in the CNS, which is in line with its widespread distribution in large numbers of neurons of different types (Nässel et al., 2008). For instance in sets of mushroom body Kenyon cells and antennal OSNs synapsing in the antennal lobes it has been shown that acetylcholine and sNPF are colocalized and that the neuropeptide acts presynaptically to potentiate signaling with the SMN (Root et al., 2011; Barnstedt et al., 2016). Possibly this is a common role of this peptide in various neurons co-expressing SMNs and sNPF described earlier (Nässel et al., 2008) (see **Tables 2**, **4**). It would be of interest to test whether sNPF also acts presynaptically to regulate release of colocalized neuropeptides for instance in clock neurons or even neurosecretory cells. In the s-LN_v clock neurons, which colocalize PDF and sNPF, it has been demonstrated that these two peptides have distinct functions and target neurons. Thus, sNPF, but not PDF from s-LN_vs target the PTTH-producing neurosecretory cells in the brain to coordinate the central clock with that in the prothoracic gland and thereby time the ecdysone production and adult eclosion (Selcho et al., 2017). Another case is where s-LN_vs target DN1 clock neurons with sNPF, whereas they act on LNds, DN3s and themselves with PDF in a circuit that ensures phase shifts in the activity of clock neurons, as part of clock entrainment (Liang et al., 2017). So far it is not known whether sNPF and PDF from s-LN_vs also act together on any target neurons. A final example of sNPF action is in feedback regulation of the presynaptic sensory neurons by a set of DILP7/sNPF-expressing interneurons in a nociceptive pathway (Hu et al., 2017).

Neurosecretory cells producing two or more peptide hormones that have been studied so far seem to orchestrate physiology by sharing some target cells/tissues, but also appear to have some unique targets of the individual peptides. For instance IPCs of the brain produce four DILPs and two DSKs (**Figure 2A**) and these seem to be transcriptionally regulated individually and probably their release is also differentially controlled [summarized in (Nässel and Vanden Broeck, 2016)]. More importantly the individual DILPs of the IPCs appear to have distinct physiological roles (with some redundancy), although they share a single receptor tyrosine kinase, dInR (see Brogiolo et al., 2001; Grönke et al., 2010; Nässel and Vanden Broeck, 2016). Furthermore, there are two DSK receptors (GPCRs) and these cholecystokinin-like peptides have multiple functions [summarized in (Nässel and Winther, 2010; Nässel and Williams, 2014)]. In summary, the combined roles of DILPs and DSKs from IPCs seem to be to orchestrate satiety and post feeding physiology (Söderberg et al., 2012).

Another example is a set of LNCs that produce the peptides Crz, sNPF, and proctolin (**Figure 2A**). Experimental data suggest that sNPF regulates DILP signaling from the IPCs in the brain and thereby affects stress responses and metabolism (Kapan et al., 2012), whereas Crz appears to act primarily as a hormone in a systemic fashion to target Crz receptors in the fat body to regulate

TABLE 5 | Summary of established functional roles of colocalized neuropeptides/SMNs in *Drosophila*.

Neuron type[1]	Peptide/SMN[2]	Cotransmission/divergent roles	Reference
MB Kenyon cells	sNPF, ACh	Presynaptic facilitation (learning)	Barnstedt et al., 2016
OSNs (sensory)	sNPF, ACh	Presynaptic facilitation (olfaction)	Root et al., 2011
OSNs (sensory)	MIP, ACh	Presynaptic facilitation (olfaction)[3]	Hussain et al., 2016a
DP1 (interneuron)	sNPF, DILP7	Feedback facilitation (nociception)[4]	Hu et al., 2017
s-LNv (clock)	sNPF, PDF[5]	Different targets (activity phase shift)	Liang et al., 2017
s-LNv (clock)	sNPF, PDF[5,6]	Different targets (Ecdyson production)	Selcho et al., 2017
DLP (neurosecretory)	sNPF, Crz[5]	Different targets (CNS and systemic)	Kubrak et al., 2016
Ipc-1 (neurosecretory)	sNPF, TK[5]	Different targets (CNS and systemic)	Kahsai et al., 2010
IPC (neurosecretory)	DILPs, DSK[5]	Different targets (systemic)	Nässel and Vanden Broeck, 2016
ABLK (neurosecretory)	LK, DH44[5]	Different targets (systemic)	Zandawala et al., 2018

[1]In adult CNS except DP neurons which are in larvae. [2]Only the ones studied are listed. [3]In mated females. [4]DP neurons signal back to mechanosensory cells with sNPF, DILP7 plays no role in this feedback circuit. [5]These neurons/neuroendocrine cells use the colocalized peptides to target different neurons or tissues for different actions, to orchestrate function. [6]During final molt (adult eclosion).

responses to metabolic stress (Kubrak et al., 2016). This indicates that the same set of LNCs act on different targets in the CNS and periphery to orchestrate a systemic response.

A final example is a set of neurosecretory cells, ABLKs (**Figure 3A**), in abdominal ganglia that were shown to target Malpighian tubules with the diuretic hormones DH44 and LK to regulate secretion and stress responses, but also have other targets that affect feeding, and water retention in differential ways for the two peptides (Zandawala et al., 2018).

Cotransmission in *Drosophila* in the Future

Clearly, cotransmission in *Drosophila* is an open field of mostly uncharted territory. There is no clear choice of a general study system, since there are many different aspects of cotransmission to be investigated and each aspect may be best studied in a specific circuit or neuroendocrine system. Thus, I list a few systems as examples where advances could be made.

For analysis of cotransmission and roles of multiple neuromodulators in regulating network properties, as in the stomatogastric ganglion in crustaceans (see Nusbaum et al., 2017), there is unfortunately no easily accessible simple motor network in flies in which electrophysiological recordings accompanied by application of neuroactive substances and drugs could reveal dynamics of network modulation. However, progress is being made in larval *Drosophila* in a neuronal network regulating feeding and locomotion that is accessible to electrophysiology and optogenetics (Schoofs et al., 2014; Hückesfeld et al., 2015). This network includes the peptidergic Hugin cells of the SEG whose extensive connectome was described recently (Schlegel et al., 2016).

The anatomically well-described olfactory system is amenable to calcium imaging, electrophysiology, and application of neuroactive substances while stimulating the OSNs with odorants (Masse et al., 2009; Seki et al., 2010; Wilson, 2013) and is already utilized in analysis of cotransmission at the first synapse between OSNs and PNs (Root et al., 2008; Ko et al., 2015). There are many types of LNs in the antennal lobe, known to colocalize neuropeptides and SMNs and the concerted actions of for instance GABA and TK or acetylcholine and MIP in shaping olfactory information passing on to higher centers remain a future challenge. Also the novel discovery that LNs may signal with both acetylcholine and two different neuropeptides, sNPF and TK (Croset et al., 2017) adds another layer to plasticity in olfactory signaling to the MBs and lateral horn worthy of investigation. Similarly, the mechanisms behind sNPF facilitation of acetylcholine transmission in mushroom circuitry (Barnstedt et al., 2016), especially possible nutrient-state dependence during olfactory learning, merits further study.

Clearly the clock circuitry is another promising system for analysis of the function of colocalized neuropeptides and SMNs due to the wealth of data on network properties and roles of individual neuron groups. Existing data suggests extensive involvement of neuropeptides in different parts of the network (Johard et al., 2009; Schlichting et al., 2016; Liang et al., 2017), whereas the role of SMNs is relatively uncharted and cotransmission with the two remains to be investigated. Another interesting possibility is that various clock neurons utilize their colocalized signaling substances to target different follower neurons. This has already been indicated for the s-LN_vs that signal with sNPF and PDF to different neurons of the circuit (Liang et al., 2017). Likewise it is suggestive, but not proven, that Upd1 from l-LN_vs, that also colocalize PDF and NPF, target non-clock neurons (Beshel et al., 2017). The genetic manipulations usually performed are relatively crude, even when using temporarily controlled manipulations, and may miss subtle dynamics of cotransmission. Thus, there is a need to combine with analysis of synaptic signaling and neuromodulation, such as performed in the crustacean stomatogastric system.

Finally, the complex endocrinology of *Drosophila* with extensive interorgan communication to orchestrate behavior, maintain homeostasis, or regulate developmental processes remains an important research field with much promise for the future (Rajan and Perrimon, 2011; Owusu-Ansah and Perrimon, 2015). Neurosecretory cells are relatively easy to access for analysis of hormonal roles of multiple peptides (see Söderberg et al., 2012; Zandawala et al., 2018). It is feasible to knock down

colocalized neuropeptides or their receptors in target tissues, individually or several together, and analyze systemic effects on the organism.

In conclusion analysis of cotransmission in *Drosophila* is still in its infancy and hopefully this review will convince the reader that *Drosophila* is a promising model organism to employ for future functional studies of colocalized neuroactive substances.

AUTHOR CONTRIBUTIONS

DN conceived and wrote this review.

FUNDING

The author's research was funded by the Swedish Research Council (Vetenskapsrådet; 2015-04626).

ACKNOWLEDGMENTS

I thank Paul H. Taghert for valuable comments on the section on clock circuits and Meet Zandawala for very helpful suggestions on an earlier version of the manuscript. I am also grateful to former and present colleagues for their important contributions to some of the original research presented in this review.

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular Neuroscience

PERSPECTIVE
published: 21 August 2018
doi: 10.3389/fncel.2018.00272

Costorage of High Molecular Weight Neurotransmitters in Large Dense Core Vesicles of Mammalian Neurons

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It is today widely accepted that several types of high molecular weight (MW) neurotransmitters produced by neurons are synthesized at the cell body, selectively stored within large dense core vesicles (LDCVs) and anterogradely transported to terminals where they elicit their biological role(s). Among these molecules there are neuropeptides and neurotrophic factors, the main focus of this perspective article. I here first provide a brief resume of the state of art on neuronal secretion, with primary emphasis on the molecular composition and mechanism(s) of filling and release of LDCVs. Then, I discuss the perspectives and future directions of research in the field as regarding the synthesis and storage of multiple high MW transmitters in LDCVs and the possibility that a selective sorting of LDCVs occurs along different neuronal processes and/or their branches. I also consider the ongoing discussion that diverse types of neurons may contain LDCVs with different sets of integral proteins or dial in a different fashion with LDCVs containing the same cargo. In addition, I provide original data on the size of LDCVs in rat dorsal root ganglion neurons and their central terminals in the spinal cord after immunogold labeling for calcitonin gene-related peptide (CGRP), neuropeptide K, substance P, neurokinin A or somatostatin. These data corroborate the idea that, similarly to endocrine cells, LDCVs undergo a process of maturation which involves a homotypic fusion followed by a reduction in size and condensation of cargo. They also give support to the conjecture that release at terminals occurs by cavicapture, a process of partial fusion of the vesicle with the axolemma, accompanied by depletion of cargo and diminution of size.

Keywords: coexistence, co-localization, co-storage, large granular vesicles, neuropeptide, neurotransmission, release, small synaptic vesicles

OPEN ACCESS

Edited by:
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Reviewed by:
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Received: *10 July 2018*
Accepted: *03 August 2018*
Published: *21 August 2018*

Citation:
Merighi A (2018) Costorage of High Molecular Weight Neurotransmitters in Large Dense Core Vesicles of Mammalian Neurons. Front. Cell. Neurosci. 12:272. doi: 10.3389/fncel.2018.00272

STATE OF ART ON HIGH MOLECULAR WEIGHT NEUROTRANSMITTER LOCALIZATION AND FUNCTION

Neuronal Secretion and Types of Secretory Vesicles in Neurons

Differently from other cells, neurons display at least three different types of secretory vesicles, each showing distinctive features as regarding their secretion and biogenesis. Biosynthetic activity in neurons is very intense and, for a substantial part, devoted to synthesize and assembly these vesicles. Such an intensive activity is testified by the abundance of rough endoplasmic reticulum (RER) and the existence of large Golgi complexes in neuronal perikarya. Secretory vesicles are produced along the regulated secretory pathway and store soluble proteins, peptides or low

molecular weight (MW) neurotransmitters. Very recent studies have demonstrated that they may also contain small ribonucleic acids (sRNAs), at least in the electric organ of *Torpedo californica* and in mouse synapses (Kim et al., 2015; Li et al., 2015, 2017; Gümürdü et al., 2017). Once assembled, secretory vesicles are actively transported to specific subcellular domains for extracellular delivery in response to appropriate signals. The typical large (75–100 nm) dense core vesicles (LDCVs) in neurons, which mainly concern this perspective article, contain proteins and/or peptides (**Figures 1A,B**). It may be useful to recall here that LDCVs were originally defined in non-neuronal cell types, where they can be much larger than those found in neurons. This likely is important for understanding the differences between peptide hormone release and neuropeptide release (see below). Proteinaceous molecules contained in neuronal LDCVs are synthesized in the cell body, generally as larger precursors that are commonly referred to as pre-pro-peptides, packaged into LDCVs to be transported to processes and, eventually, delivered into the extracellular space (Merighi, 2017). However, it remains unclear where, along their long journey from cell body to terminals, maturation of LDCVs' neurotransmitter proteins takes place.

Biochemical studies on undifferentiated PC12 cells, which are devoid of axons, indicate that the maturation of LDCVs is accompanied by sorting non-regulated secretory proteins, including the SNARE proteins, from immature vesicles through the recruitment of clathrin coats, a process that is considered essential for the maturation of LDCVs (see Morvan and Tooze, 2008). In parallel, several observations on the processing of pre-pro-peptides in hypothalamic neurons and their neurohypophyseal axon terminals support that a post-translational cleavage of vasopressin, oxytocin and their neurophysins occurs during axonal transport (e.g., Gainer et al., 1977a,b). These latter studies did not take into consideration the aforementioned modifications of the proteins of the LDCVs' membrane. However, it was more recently demonstrated that, in cultured trigeminal ganglion neurons, the calcitonin gene-related peptide (CGRP) occurs together with three SNAREs and synaptotagmin in LDCVs and that SNARE proteins (SNAP25, syntaxin one and the synaptobrevin isoforms) were implicated in the exocytosis from LDCVs (Meng et al., 2007). Even more recently, SNAP25, and synaptobrevin isoform 2, as well as SNAP47, were demonstrated to mediate the axonal release of brain-derived neurotrophic factor (BDNF) from cortical neurons (Shimojo et al., 2015). Therefore, it appears that also in *bona fide* neurons SNAREs may be constituent of the mature LDCVs, although it remains to be established whether post-translational axonal cleavage of pre-pro-neurotransmitter proteins is a general rule or rather a peculiarity of the hypothalamic neurosecretory neurons.

In nerve terminals, small (40–50 nm) synaptic vesicles (SSVs) or small clear core vesicles are definitely more numerous than LDCVs and store and deliver small neurotransmitters such as acetylcholine, glutamate (**Figures 1A,B**), glycine, and gamma amino butyric acid (GABA). Finally, biogenic amines are differently packaged in neuronal processes within the central and peripheral nervous systems (see Hökfelt, 2010). In the latter, biogenic amines are stored in either small (40–60 nm diameter) dense core vesicles (SDCVs) or irregularly shaped LDCVs, depending on the particular population of neurons. Conversely, brain dopamine vesicles do not fall in any of the described classes because they are 70 nm in diameter and clear. At the transmission electron microscope (TEM), SSVs undergoing anterograde axonal transport appear as tubule-vesicular structures of 50 nm diameter and variable length (Tsukita and Ishikawa, 1980). These morphological observations, together with the results of numerous biochemical studies, led to the conclusion that SSVs are locally assembled at synapses and that their protein components are reconstituted as complete synaptic vesicles in the early endosome compartment (Takamori, 2009). Thus, SSVs are characteristically recycled several times and locally filled with neurotransmitter at synapses, for reuse following a series of exocytotic and endocytotic events (Lou, 2018).

Differently from SSVs, LDCVs are depleted after secretion, whereas SDCVs containing noradrenaline are filled with the neurotransmitter during axonal transport, a fact that explains the presence of their characteristic dense core in TEM images (Zhang et al., 2011).

Assembly, Filling and Release of Neuronal LDCVs

Integral Proteins of LDCVs

Independently from their cargo, LDCVs express a series of integral proteins that, biochemically, make them a relatively homogeneous population of vesicles. Among these are the chromogranins (Bartolomucci et al., 2011), which are important regulators of cargo sorting in LDCVs biogenesis, although very recent work in mouse hippocampal neurons has demonstrated that they are not indispensable for LDCVs' exocytosis (Dominguez et al., 2018).

LDCVs' Cargo

The cargo of LDCVs consists of proteinaceous materials. For the most, these proteins are of small size and currently referred to as neuropeptides (**Figures 1A,B**). In its original definition, a neuropeptide is a small protein molecule contained in neurons, composed of up to a hundred amino acids. As mentioned, neuropeptides are usually produced as large, inactive precursors, which are then enzymatically cleaved to yield the biologically active peptides. Commonly, precursors contain several molecules of the same neuropeptide and/or more or less structurally related compounds. Storage of neuropeptides and their precursors in LDCVs was first shown in the 80s of the last century with TEM, but, in more recent times, LDCVs were demonstrated to be loaded also with bigger molecules such as BDNF or the glial-derived neurotropic factor (GDNF) and their pro-peptides. There are numerous examples of costorage of multiple peptides within individual LDCVs in various areas of the central and peripheral nervous systems (Merighi, 2017).

As LDCVs mature, the pro-peptides herein contained undergo proteolysis to become active. This maturation process

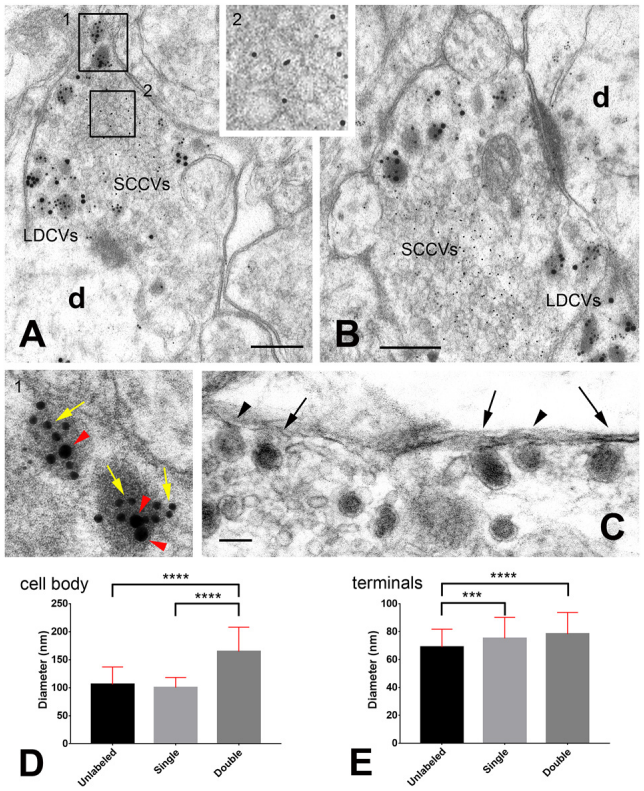

FIGURE 1 | Storage of neuropeptides in rat dorsal root ganglion (DRG) neurons. **(A,B)** Primary afferent terminals in the spinal cord dorsal horn after triple immunogold labeling with antibodies against glutamate (5 nm gold particles), calcitonin gene-related peptide (CGRP; 10 nm gold particles indicated by the yellow arrows in the insert) and substance P (20 nm gold particles indicated by the red arrow heads in the insert). For details on antibodies and labeling methods see Merighi et al. (1991). The two rectangles in **(A)** are shown at higher magnifications in the inserts. **(C)** A particular of an axon terminal in the rat dorsal horn after slice incubation in 75 mM K^+ to stimulate LDCVs' exocytosis. Tissue has been processed with the Tannic Acid Ringer Incubation (TARI)-method (Buma et al., 1984). Fixation with tannic acid prior to conventional glutaraldehyde + osmium post-fixation powerfully intensifies the electron density of the substances secreted into the extracellular space. As the cargo of LDCVs is immediately fixed by tannic acid once there, it remains in close apposition to the terminal membrane that appears thicker than after conventional transmission electron microscope (TEM) fixation. Note that three LDCVs in proximity of the axolemma (arrows) display a very electrondense matrix, whereas two other vesicles (arrow heads) display some dissolution of their membranes and are lighter. These images may represent different stages in the process of cavicapture by which neuropeptides may be released at terminals (see also **Figure 2**). **(D,E)** Diameter (mean ± SD) of unlabeled, single- and double-labeled LDCVs in the neuronal cell body **(D)** and central terminals **(E)** of rat DRG neurons. Note the increase in size of double-labeled LDCVs compared to unlabeled or single-labeled LDCVs in DRGs, likely as a consequence of new cargo addition. Note also that in terminals unlabeled vesicles are smaller than single- and/or double-labeled LDCVs, which, instead, display similar sizes. This observation supports the idea that unlabeled vesicles in terminals may have been depleted of their cargo after cavicapture. Statistics was performed with the GraphPad Prism 7 software. Normality was assessed using the D'Agostino & Pearson normality test. Means were compared using the Kruskal-Wallis non-parametric test followed by Dunn's multiple comparison. # LDCVs: 346 (cell body), 523 (terminals). ***$P = 0.0004$; ****$P < 0.0001$ (two-tailed). Abbreviations: d = dendrite; LDCVs = large dense core vesicles; SSVs = small clear core vesicles. Bars: **(A,B)** = 200 nm; **(C)** = 100 nm.

usually starts in the trans-Golgi network (TGN) and continues in the secretory vesicles themselves (Kögel and Gerdes, 2009). The specific molecular signals that drive the packaging and aggregation of secreted proteins or their characteristic integral membrane proteins into LDCVs within the TGN are starting to be unraveled. Among these, one should recall the WD40 domain protein EIPR-1 and the endosome-associated recycling protein (EARP) complex (Topalidou et al., 2016). LDCVs emerge as immature vesicles from the TGN of the Golgi complex (**Figure 2**), and it was recently suggested that the conserved coiled-coil protein CCCP-1 intervenes in the homotypic fusion of immature LDCVs during the course of their maturation (Cattin-Ortolá et al., 2017).

Release of Cargo From LDCVs

Neuronal LDCVs are not fully equivalent to non-neuronal LDCVs, a point that merits attention in discussing current knowledge on release of cargo from these two classes of LDCVs. Studies of endocrine and exocrine cells with 300–1000 nm LDCVs have often implicated F-actin (and sometimes myosin) in release, because it apparently takes work to move these large vesicles and extrude their contents. In contrast, experiments on synaptic neuropeptide vesicles with cytochalasin and mycalolide B (an F-actin/dynactin disruptor) showed that F-actin does not affect LDCV mobilization, anterograde transport, synaptic capture or evoked synaptic neuropeptide release (Shakiryanova et al., 2005; Cavolo et al., 2015, 2016).

Mechanisms by which LDCV and SDCVs release their cargo include exocytosis, kiss and run or cavicapture (Merighi, 2017). Exocytosis is a relatively slow process that requires complete fusion of the vesicle to the plasma membrane; kiss and run or cavicapture are faster and need the formation of a transient fusion pore (TFP) between the LDCV and the plasma membranes. TFP mechanisms permit a fast transfer of amine neurotransmitters from the inside of SDCVs to the extracellular space. Of these mechanisms, neuropeptide kiss and run was recently demonstrated to occur at nerve terminals in *Drosophila* (Wong et al., 2015) and in mammalian DRG neurons (Wang et al., 2017). It seems also possible that neuropeptides and larger proteins can escape from LDCVs by cavicapture, during which an expansion of the TFP triggers a partial release of large-size neurotransmitters (**Figures 1C**, **2**).

As a rule, individual neurons are capable to produce several high MW transmitters of proteinaceous nature and store them in LDCVs. In mammals, ultrastructural demonstration was provided that e.g., two different neuropeptides and a growth factor with transmitter functions could be co-stored in individual LDCVs (Salio et al., 2007, 2014). Thus, there are at least two main possibilities depicting the modalities of release of these molecules (**Figure 2**). From one side, all co-stored high MW transmitter could be released together. At opposite, individual molecules could be liberated singularly or in different combinations. A further issue of complexity derives from the very peculiar organization of neurons, which form more or less intricate arborizations of their processes, the latter, in turn, existing in at least two functionally different types, i.e., axons and dendrites.

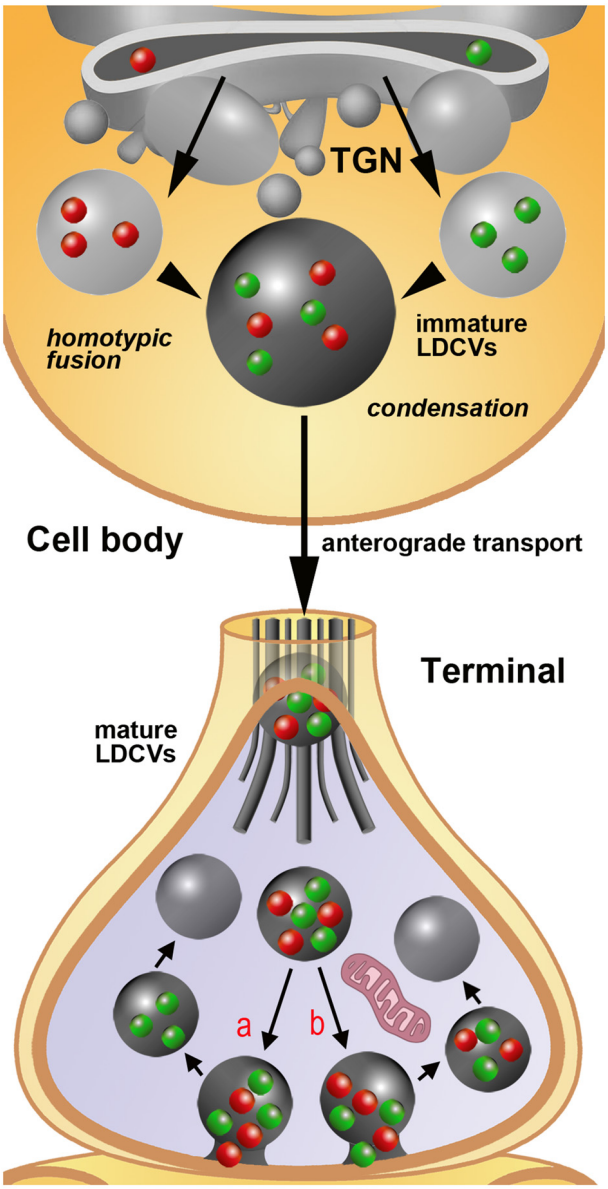

FIGURE 2 | Schematic representation of the process of assembly, filling and release of LDCVs as extrapolated from immunogold staining studies on rat primary sensory neurons in DRGs. For simplicity, only the synthesis of two neuropeptides/proteins (red and green spheres) is depicted. The sizes of immature and mature LDCVs containing no cargo (negative after immunogold labeling), only one of the two molecules (single-labeled) or both molecules (double-labeled) are represented in accordance with the quantitative data reported in **Figures 1D,E**. In the example, immature LDCVs budding from the trans-golgi network (TGN) contain either one *or* the other neuropeptide and undergo a process of homotypic fusion to give rise to a larger LDCV. This vesicle stores both peptides, but still may be regarded as immature on the basis of its very large size. It subsequently undergoes a process of condensation with a reduction in size that is completed once the axon terminal is reached by anterograde transport. In terminals, LDCVs are smaller and very likely undergo a process of cavicapture to release their cargo (see also the insert #1 of **Figures 1A,C**). This process may, in theory, yield to a selective: (a) or a non-selective (b) release of the co-stored neuropeptides. Irrespectively of this possibility, individual LDCVs may endure several cycles of

(*Continued*)

FIGURE 2 | Continued
cavicapture until they are fully depleted of their cargo. Quantitative analysis (**Figure 1E**) demonstrates that empty (unlabeled) LDCVs are smaller than those containing only one (and thus single-labeled) of the two co-stored peptides. To make the figure easier, LDCVs are represented without their outer membrane, which is instead clearly visible in tissues subjected to fixation with tannic acid (**Figure 1C**). For the same reason, SSVs are not rendered in the terminal.

FUTURE DIRECTIONS AND PERSPECTIVES

Neuropeptides and other proteins that may be synthesized by neurons are known to be physiologically very important, but also play a substantial role in many pathological conditions. However, the biogenesis of LDCVs and the mechanisms governing their molecular composition still remain to be unraveled in full (Hummer et al., 2017).There are, in my opinion, two main lines of research to be pursued in the future for a better understanding of the significance of costorage of high MW transmitters in neurons. The first should primarily investigate the synthesis, storage and maturation of LDCVs, the second their targeting to neuronal processes with an attention to possible differences among specific neuronal populations. Information on both issues is substantially missing, as, at present, investigations have been, for the most, carried out on simple organisms such as *Caenorhabditis elegans* or *Aplysia californica* or performed in mammalian secretory cells other than neurons, such as e.g., the adrenal chromaffin cells. In addition, the relatively small number of investigations in mammalian neurons has basically been carried out on isolated primary cells or cell lines *in vitro*, with few remarkable exceptions discussed below.

Synthesis and Storage of Multiple High MW Transmitters in LDCVs

Localization of immature LDCVs in neurons remains a difficult task to be performed. It still is unclear which morphological and/or biochemical differences, if any, exist between LDCVs at different stages of maturity. Ultrastructurally, it appears that immature vesicles in chromaffin cells are of heterogeneous size, but all have a dense core. In rat PC 12 cells, the dense core of immature secretory granules (80 nm) is smaller than that of mature granules (114 nm), and on these observation it was hypothesized that one or more immature granules fuse together during maturation (Tooze et al., 1991; Tooze, 1991). Other investigations, again conducted in cells other than neurons, have shown that maturation of LDCVs accompanies with condensation of the matrix and, at least theoretically, a reduction in size that, however, could be compensated by homotypic fusion of immature vesicles stemming from the TGN (Kögel and Gerdes, 2009). Studies in mouse chromaffin cells have shown that the neuronal adaptor protein 3 (AP-3), a vesicle-coat protein that in neurons intervenes in transmitter release, is localized to the TGN and hypothesized that AP-3 is selectively expressed in immature

LDCVs (Grabner et al., 2006). More recently, a genome editing study on HID-1 knockout PC12 cells has proposed that the protein, originally demonstrated to be implicated in neuropeptide sorting and secretion in *Caenorhabditis elegans*, influences the early steps in LDCV biogenesis by controlling the formation of their dense core at the TGN (Hummer et al., 2017). Thus vesicle size, expression of neuronal AP-3 and/or HID-1 could be regarded as markers of immature LDCVs in endocrine cells, but this remains to be established in full for neurons.

Pioneering work carried out with the use of multiple immunogold labeling methods more than 25 years ago led to establishing that multiple neuropeptides could be co-stored within individual LDCVs in the cell body and processes of certain primary sensory neurons (for review see Merighi, 2017). More recent work has shown that neuropeptide co-storage also occurs in neurons of the central nervous system (Salio et al., 2007). As neurons normally appear to be producin g more than a single neuropeptide/transmitter protein (Merighi, 2017), it seems highly possible that these proteinaceous molecules are not selectively packaged into different mature LDCVs, but rather form a mix in individual vesicles once they are ready to be transported along axons. I have here analyzed the size of unlabeled, single-labeled and double-labeled LDCVs in the cell bodies of rat dorsal root ganglion (DRG) neurons using different combinations of antibodies against several sensory neuropeptides (CGRP, the tachykinins neuropeptide K, substance P and neurokinin A or somatostatin) with different double immunogold labeling techniques (Merighi and Polak, 1993). It is of interest that in these neurons there is no difference in size between unlabeled and single-labeled LDCVs, whereas double-labeled vesicles are larger (**Figure 1D**). This observation is consistent with the idea that LDCVs containing just one component of the peptide mix are immature and that the increase in size of mature double-labeled vesicles is a consequence of homotypic fusion with other immature LCDVs containing the second peptide (**Figure 2**), as shown in endocrine cells (Kögel and Gerdes, 2009). As we have previously demonstrated that mature LDCVs in the central terminals of the DRG neurons may contain a mix of three (and likely even more) neuropeptides/larger proteinaceous transmitters (Salio et al., 2007, 2014), the data herein reported are strongly indicative of the possibility that homotypic fusion may be a general phenomenon through which LDCVs mature before being transported to terminals. Co-stored proteinaceous transmitters occur in LDCVs in remarkably constant ratios. We have e.g., demonstrated that substance P, CGRP and BDNF occur in a stoichiometric ratio of 0.7 BDNF:1 CGRP:1 substance P in neurons of DRGs and central nucleus of amygdala (Salio et al., 2007). Therefore, it seems reasonable that such a ratio is attained at the level of protein synthesis, before individual molecules are directed to the TGN (**Figure 2**).

The main functional implication of co-storage of bioactive molecules within LDCVs is that the neuropeptides and/or the other high MW transmitter herein contained may be released together and probably act together in determining the response of target cells. It would be interesting to investigate whether or not these co-stored molecules are indeed released in concert, or if some sort of mechanisms would permit a selective release according to functional needs. Under this perspective, the relative rate of individual peptide dissolution from the LDCV core (matrix) might be important, since it is critical for the speed of peptide secretion *in vitro* (for a recent review see Merighi, 2017). I have very recently discussed the possibility that two functionally antagonist subpopulations of peptidergic DRG neurons exchange their information to regulate nociception (Merighi, 2018). These two populations of neurons contained a mix of peptides with either BDNF (Merighi et al., 2008) or GDNF (Salio et al., 2014). It appeared highly probable that the two neurotrophic factors come into play only in particular functional conditions, e.g., when the sensory system was overstimulated under inflammatory conditions. In the future, it would be interesting to check whether these molecules can indeed undergo some sort of selective release, as this would represent an additional regulatory mechanism to finely tune nociception.

Are LDCVs Selectively Targeted to Different Neuronal Processes?

Whether or not LDCVs are selectively targeted along different neuronal processes and/or their branches remains to be established in full. Our immunogold studies onto the DRG neurons and their central and peripheral projections were indicative of a lack of selectivity, as the same combination of peptides, e.g., substance P and CGRP, is detected at both peripheral (Gulbenkian et al., 1986) and central (Merighi et al., 1991) axonal projections. However, we did not investigate this issue in full, and, to the best of my knowledge, it still stands as an open question.

At the central projections of the peptidergic DRG neurons in spinal cord there is a very large fraction (virtually all?) of the total population of LDCVs containing coexisting neuropeptides/protein transmitters compared to the very limited pool of these vesicles in the cell body of these neurons. Another issue which deserves future investigations is the occurrence and significance of unlabeled LDCVs at axon terminals. I have often interpreted this as a false negative observation, as: *1*. There is a general consensus, also based onto light microscopic and transcriptomics studies, that *all* DRG peptidergic neurons contain their main peptide marker CGRP (Amara et al., 1985). Therefore a lack of CGRP immunoreactivity would be difficult to explain in these neurons; *2*. Antigenicity in post-embedding immunogold labeling techniques on plastic sections (which are surface reactions) may often fall below the limits of sensitivity of these procedures (Merighi et al., 1991). To obtain additional cues on this issue, here I have calculated the size of LDCVs in the central terminals of the rat DRG neurons after CGRP+substance P or CGRP+somatostatin double labeling of the dorsal horn (**Figure 1E**). Remarkably, and differently from what is observed at the cell body (**Figure 1D**), double- and single-labeled LDCVs are larger than unlabeled vesicles. These observations give support to the idea that peptides are indeed released from these

vesicles through cavicapture (**Figure 1C**), a process by which individual LDCVs do not disappear after totally flat themselves against the neurolemma as in the well know regular exocytosis of SSVs (Rutter and Tsuboi, 2004)—see also the multi-media annex in Merighi, 2017. If this interpretation is correct, unlabeled LDCVs in the central terminals of the DRG neurons could be regarded as depleted of their cargo and thus not as technical artifacts (**Figure 2**). This conclusion, as mentioned above, is strongly based on the notion that CGRP is expressed in *all* peptidergic neurons in DRGs. Therefore, to generalize it to different (or all) types of LDCV-containing terminals would require to identify a general peptide marker for each neuronal population, to exclude that unlabeled LDCVs, in terminals other that those investigated here, contain a neuropeptide or other cargo that has simply not been assayed.

It is also of interest that when the mean sizes (±SD) of unlabeled, single- and double-labeled LDCVs are compared between the cell body and central terminals of the DRG neurons, those in perikarya are larger than in terminals for all the three groups (unlabeled LDCVs: cell body 105.8 ± 31.21, terminals 68.91 ± 12.8, $P < 0.0001$; single labeled LDCVs: cell body 100.3 ± 17.93, terminals 75.13 ± 15.09, $P < 0.0001$; double labeled LDCVs: cell body 164.5 ± 43.54, terminals 78.26 ± 15.44, $P < 0.0001$; Mann-Whitney test; #LDCVs = 878). This observation is in accordance with data on endocrine cells where a condensation of LDCVs was demonstrated in parallel with maturation (Kögel and Gerdes, 2009).

Other Sources of Complexity Still Await to Be Cleared

It is of interest that chromogranin B and phogrin, another integral protein of LDCVs in endocrine cells, have been reported to be specifically contained in LDCVs from excitatory and inhibitory hippocampal neurons, respectively (Ramírez-Franco et al., 2016). This observation deserves further investigations as it opens the yet unforeseen possibility that integral proteins of LDCVs may be different in diverse types of neurons.

Also, a study on the cellular processing of neuropeptide Y (NPY) has demonstrated that differential trafficking of immunoreactive LDCVs occurs in hippocampal vs. hypothalamic neurons (Ramamoorthy et al., 2011). If these observations will be extended to other neuropeptides or proteinaceous cargo of LDCVs, further complexity will be added, as trafficking of neuropeptide-containing LDCVs could be specific for different populations of neurons, and perhaps not determined entirely by the characteristics of the particular peptide *per se*.

ETHICS STATEMENT

The experimental procedures described in this paper were approved by the Ethics Committee of the University of Turin. This study was carried out according to current EU Recommendations on the Care and Use of Experimental Animals.

AUTHOR CONTRIBUTIONS

AM conceived and performed the experiments, analyzed data, prepared the figures and wrote the manuscript.

FUNDING

The experimental work described in this article was supported by intramural funding from the Department of Veterinary Sciences, University of Turin (Italy).

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular Neuroscience

REVIEW
published: 10 October 2018
doi: 10.3389/fncel.2018.00342

Protons as Messengers of Intercellular Communication in the Nervous System

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In this review, evidence demonstrating that protons (H^+) constitute a complex, regulated intercellular signaling mechanisms are presented. Given that pH is a strictly regulated variable in multicellular organisms, localized extracellular pH changes may constitute significant signals of cellular processes that occur in a cell or a group of cells. Several studies have demonstrated that the low pH of synaptic vesicles implies that neurotransmitter release is always accompanied by the co-release of H^+ into the synaptic cleft, leading to transient extracellular pH shifts. Also, evidence has accumulated indicating that extracellular H^+ concentration regulation is complex and implies a source of protons in a network of transporters, ion exchangers, and buffer capacity of the media that may finally establish the extracellular proton concentration. The activation of membrane transporters, increased production of CO_2 and of metabolites, such as lactate, produce significant extracellular pH shifts in nano- and micro-domains in the central nervous system (CNS), constituting a reliable signal for intercellular communication. The acid sensing ion channels (ASIC) function as specific signal sensors of proton signaling mechanism, detecting subtle variations of extracellular H^+ in a range varying from pH 5 to 8. The main question in relation to this signaling system is whether it is only synaptically restricted, or a volume modulator of neuron excitability. This signaling system may have evolved from a metabolic activity detection mechanism to a highly localized extracellular proton dependent communication mechanism. In this study, evidence showing the mechanisms of regulation of extracellular pH shifts and of the ASICs and its function in modulating the excitability in various systems is reviewed, including data and its role in synaptic neurotransmission, volume transmission and even segregated neurotransmission, leading to a reliable extracellular signaling mechanism.

Keywords: ASIC, vestibule labyrinth, cochlea, amygdala, fear

OPEN ACCESS

Edited by:
Miguel Angel Morales, Universidad Nacional Autónoma de México, Mexico

Reviewed by:
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Pere Garriga, Universitat Politecnica de Catalunya, Spain

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Received: *26 June 2018*
Accepted: *14 September 2018*
Published: *10 October 2018*

Citation:
Soto E, Ortega-Ramírez A and Vega R (2018) Protons as Messengers of Intercellular Communication in the Nervous System. Front. Cell. Neurosci. 12:342. doi: 10.3389/fncel.2018.00342

Abbreviations: $\Delta\mu H^+$, Electrochemical gradient; ΔpH, H^+ concentration gradient; $\Delta\psi$, Electrical potential; AE, Anion Exchanger; $AMPA_R$, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; ASD, Autism spectrum disorders; ASIC, Acid Sensing Ionic Channels; CAs, Carbonic anhydrase enzyme family; CHO, Chinese Hamster Ovary; CNS, Central nervous system; DEG, Degenerins; DRG, Dorsal root ganglion neurons; ENaC, Epithelial sodium channel; EPSC, Excitatory postsynaptic current; GABA, Gamma aminobutyric acid; GMQ, 2-guanidine-4-methylquinazoline; H^+, Protons; HNSFE, Human nervous system function emulation; Hv1, Voltage-gated proton channel; KA_R, Kainate receptors; Kir, Inward rectifier K^+ channels; MCT, Monocarboxylate transporters; MNTB, Medial nucleus of the trapezoid body; MSN, Medium spinal neurons; NAc, Nucleus accumbens; NCBTs, Na^+-coupled HCO3 transporters; NHE, Na^+/H^+ exchangers; NMDAR, N-methyl-D-aspartate receptors; pHe, Extracellular pH; pHi, Intracellular pH; PTZ, Pentylenetetrazole; SGNs, Spiral ganglion neurons; SIDS, Sudden infant death syndrome; SV, Synaptic vesicles; TASK1, TASK2, Two-pore domain K^+ channel; TM1 and TM2, Transmembrane segments 1 and 2; TREK1, TWIK-related K^+ channel 1; TRPV1, Transient Receptor Potential-1; TWIK, Tandem of P-domain in a weak inwardly rectifying K^+ cannel; V-ATPase, Vacuolar H^+-ATPase; VGAT, Vesicular GABA transporters; VGCC, Voltage gated calcium channels; VGLUT, Vesicular glutamate transporters.

INTRODUCTION

We first review evidence related to extracellular proton production and of its regulation and control mechanisms, then the structure and functions of the Acid Sensing Ion Channels (ASIC) are discussed, along with other extracellular pH sensing molecules, and finally evidence of the role of proton signaling mechanism in specific synaptic transmission, volume neurotransmission, and segregated specific proton production are reviewed.

Extracellular pH is a highly controlled variable, with many regulatory processes maintaining it within a restricted value, which in vertebrates is close to neutrality. The proper function of organisms very much depends on pH homeostasis and at the systems level, its variations are minimal, otherwise catastrophic failure of organism occurs. The highly controlled extracellular H^+ concentration allows for its small local variations to be "read" as specific signals. Somehow during evolution, cells develop mechanisms for the sensing of pH variations produced by activity in its neighboring cells. The driving force for evolution of these mechanisms most likely takes place during the transition to multicellular organisms, thus constituting a signaling system. Proton signaling is very much like the Ca^{2+} signaling mechanisms (Carafoli, 2005), but mainly act on extracellular mediums. Evidence has accumulated showing that H^+ may accumulate in nano- or micro-domains and that they may operate as intercellular messengers (Beg et al., 2008). In higher organisms, proton-mediated signaling has been found to work in concert with classical neurotransmission, mediating various processes such as fear conditioned learning, retinal cell activation, inhibition of convulsive crisis, and in transduction and sensory coding in various systems (Li and Xu, 2011).

Buildup of extracellular proton concentration may be the consequence of metabolic activity, thus releasing protons in a constitutively unregulated form, or H^+, may also be co-released with classical neurotransmitters in a regulated form. Extracellular H^+ concentration increase may also be produced by a specific transport mechanism acting together with the buffer capacity of the media. There is no evidence of regulated independent H^+ release in any synapse. However, it has been found that protons fulfill most of the criteria in order to be considered a neurotransmitter; including release in the synapse, postsynaptic receptors, mechanisms to remove them from synaptic cleft, exogenous application resembling normal system activation, agonists resembling normal activation of the system, and antagonists possibly blocking the postsynaptic response (Du et al., 2017). A problem of classifying protons as neurotransmitters is related to the fact that its regulated release is always a co-release with classical neurotransmitters, which results as a byproduct of neurotransmitter transport mechanisms into the synaptic vesicles. However, regulated extracellular medium acidification by means of the activation of transporters and exchange molecules may lead to a very restricted proton accumulation. Therefore, it seems appropriate to consider the system formed by H^+ as a messenger and its specific receptors the ASIC as an extracellular signaling mechanism, which may modulate various neuronal processes, and have a salient role in the pathophysiology of various diseases of the central nervous system (CNS). Some of these processes, which we have reviewed, are related to metabolic buildup of extracellular proton concentration; the fear response in the amygdala, the inhibitory neuron activation in convulsive crisis and the motor response in hypoxia, among others. In these cases, a mass of neurons leads to an increase in extracellular H^+ concentration, which activates ASICs, expressed either at the synaptic or extrasynaptic level. The extrasynaptic activation of receptors has been shown for various neurotransmitters, such as dopamine, serotonin and cannabinoids (Del-Bel and De-Miguel, 2018), leading to the concept of volume transmission, which is a form of communication mediated by extracellular diffusion of transmitter substances through extracellular space (Fuxe et al., 2007).

Restricted actions of H^+ have been shown in the lateral amygdala (Du et al., 2014) and in the nucleus accumbens, where protons contribute to the excitatory postsynaptic current (EPSC) (Kreple et al., 2014). To define the extent at which proton concentration in synaptic like nano-domains may activate the ASICs, a construct in HEK293T cells was devised, showing that H^+ current passing through light activated *Archaerodopsin-3* or voltage-gated proton channel (Hv1) can activate closely coupled ASIC channels and induce its activation in closely located "sniffer" cells (Zeng et al., 2015). Modeling of this system showed that proton currents may lead to a pH change of almost 4 units (from 7.4 to 4 in a solution with 10 mM HEPES) and of 0.6 (from 7.4 to 6.8 units in a solution with 22 mM $NaHCO_3$) within 10–100 nm (Zeng et al., 2015). These results demonstrate that extracellular H^+ concentration changes in nano-domains can activate the ASICs. Thus, evidence for restricted signaling by protons is feasible and evidence has been obtained both from native and heterologous expression systems.

PROTON HOMEOSTASIS AND PROTON ACCUMULATION

Over time, organisms have had to adapt to diverse environments, redefining their characteristics; such as cellular pH regulation, cell volume, and maintaining ionic homeostasis to survive, reproduce and preserve their species, some of which have been able to evolve in extreme conditions up to the present (Rothschild and Mancinelli, 2001). Although environmental variables are critical for the evolution of species, it is known that the regulation of extracellular pH (pHe) and intracellular pH (pHi) is essential for life, since it is related to enzymatic processes, ionic modulation, and nutrient homeostasis. There is also evidence that the structuring of the genetic code occurred in an acidic environment, therefore, a large spectrum of membrane proteins with highly specialized functions for the preservation of cellular pH homeostasis have emerged during evolution (Brett et al., 2005; Di Giulio, 2005; Daniel et al., 2006).

To maintain the pHe within the physiological limits (7.3–7.4 in higher vertebrates) (Casey et al., 2010), there must be a balance between the contribution of the production of H^+ and the buffering or elimination of H^+. The mobile buffer systems

that regulate pHe in the CNS include the bicarbonate/carbonic acid system (HCO_3^-/H_2CO_3), hemoglobin, plasma proteins and phosphates, of which the most significant is the HCO_3^-/H_2CO_3 (about 75% of the total of buffer capacity of the blood) (Chesler, 2003). In addition to the mobile buffer systems, there are several cytoplasmic transporters that carry protons through the membrane in order to maintain pHi (pH 7.2) and pHe values within physiological limits (Casey et al., 2010). Metabolic reactions, protein catabolism and organic acids can produce intense intracellular acidification, which is why most H^+ transporters are responsible for alkalizing the cytosol, extruding protons, or capturing them in intracellular vesicles and organelles. The H^+ transporters present in vertebrate cells include: Na^+/H^+ exchangers (NHE), HCO_3^- transporters, Vacuolar H^+-ATPase (V-ATPase), monocarboxylic acid transporters and the carbonic anhydrase enzyme family (CAs), among others (**Table 1**; Obara et al., 2008 for reviews see: Chesler, 2003; Verma et al., 2015; Zhao et al., 2016).

Homeostasis of pHi and pHe is crucial in the CNS, since it is related to neuronal excitability and neurotransmission. In the brain, neuronal activity causes local and transient pH changes during physiological processes; neuronal activity can induce a transient and localized pH fluctuation at synaptic cleft that varies from 0.2 to 0.6 units, depending on stimulation protocol (Zeng et al., 2015). The loading of neurotransmitters in the synaptic vesicles occurs due to the action of the V-ATPase, so the synaptic vesicles have an acidic pH (pH Δ 5.2–5.7) (Storozhuk et al., 2016). During neurotransmission, vesicular content is released into synaptic space, co-releasing neurotransmitters and protons, and thus producing a brief but intense acidification followed by a slow alkalization (Sinning and Hübner, 2013).

In neurons, the main acid is the AE3 chloride-bicarbonate exchanger, an electroneutral exchanger that extrudes one $HCO3^-$ by one Cl^- (Ruffin et al., 2014). The main acid extruders are the Na^+-H^+ exchangers (mainly NHE1, NHE3, and NHE5 in the CNS) which exchange one Na^+ for one H^+ (Donowitz et al., 2013) and the Na^+-coupled HCO_3- transporters (NCBTs) (Parker and Boron, 2013). Usually NHEs exchange an intracellular H^+ for an extracellular Na^+ (dissipating the inward gradient for Na^+), restoring pHi after an acid load. The Na^+ -dependent exchangers (Na^+-driven Cl^-/HCO_3^-) are highly expressed in the cerebellum, cerebral cortex, thalamus and hippocampus, they remove intracellular Cl^- in exchange for extracellular Na^+ and HCO_3^-, a process which implies bicarbonate influx and proton efflux (Alvadia et al., 2017). In mice the disruption of Na^+-driven Cl-/HCO3- increased the seizure threshold (Sinning and Hübner, 2013; Zhao et al., 2016; **Figure 1**).

The transport systems have a primarily homeostatic function related to the maintenance of intracellular pH. Its role in proton signaling processes depends on their colocalization with pH-sensitive ion channels in membrane micro-regions. Therefore, in the study of the expression of transporters, it is essential to define its location in membrane micro-regions. For example, action potential firing in cultured hippocampal

TABLE 1 | Transporters and enzymes involved in H^+ extrusion and loading in the CNS.

Transporter	Isoforms	Distribution	Function	Reference
Na^+/H^+ exchanger	NHE1-NHE9	All are expressed in mammalian CNS cells. NHE1-NHE5 plasmalemmal localization, NHE6 y NHE7 intracellular localization	NHE catalyzes the exchange of one extracellular sodium ion for one intracellular proton	Slepkov et al., 2007; Donowitz et al., 2013
Bicarbonate transporters				
$Na^+/HCO3^-$ cotransporters	NBCe1-2, NBCn1-2, NDCBE	Brain, choroid plexus, and meninges	Mediate cotransport of Na^+ and base (HCO_3^- and/or CO_3^{2-}) may act as acid extruder or loaders	Obara et al., 2008; Parker and Boron, 2013
Anion exchangers	AE1-AE4	Brain, retina, salivary glands, mature erythrocytes, and immature cultured oligodendrocytes	Generally act as acid loaders, extruding HCO_3^- in exchange for Cl^- influx.	Obara et al., 2008; Zhao et al., 2016
Na^+-driven $Cl^-/HCO3^-$ exchanger	NCBE, NDCBE	Cerebral cortex, cerebellum, medulla, thalamus, hippocampus	Removes extracellular Na^+ in exchange for intracellular Cl^-. This process is associated with HCO_3^- influx and H^+ efflux.	Sinning and Hübner, 2013; Zhao et al., 2016
Vacuolar type proton ATPase	V-ATPase	Astrocytes and neurons	Using the ATP hydrolysis derived energy, transports protons from cytoplasm into either the lumen of single membrane organelles, or extracellular space	Obara et al., 2008; Casey et al., 2010
Monocarboxylic acid transporters	MCT1–MCT4	Blood vessels, astrocytes, neurons	Cotransport of one monocarboxylate anion (lactate, pyruvate, acetoacetate and/or b-hydroxybutyrate) with one proton	Obara et al., 2008
Carbonic anhydrases	CA I, II, III, VII, XIII, IV, IX, XII, XIV, XV, and VI	Nervous tissue of different species of mammals (intra and/or extracellular location)	Catalyze the inter conversion of CO_2 and H_2O and the dissociated ions of carbonic acid (i.e., bicarbonate and protons)	Obara et al., 2008: Sinning and Hübner, 2013

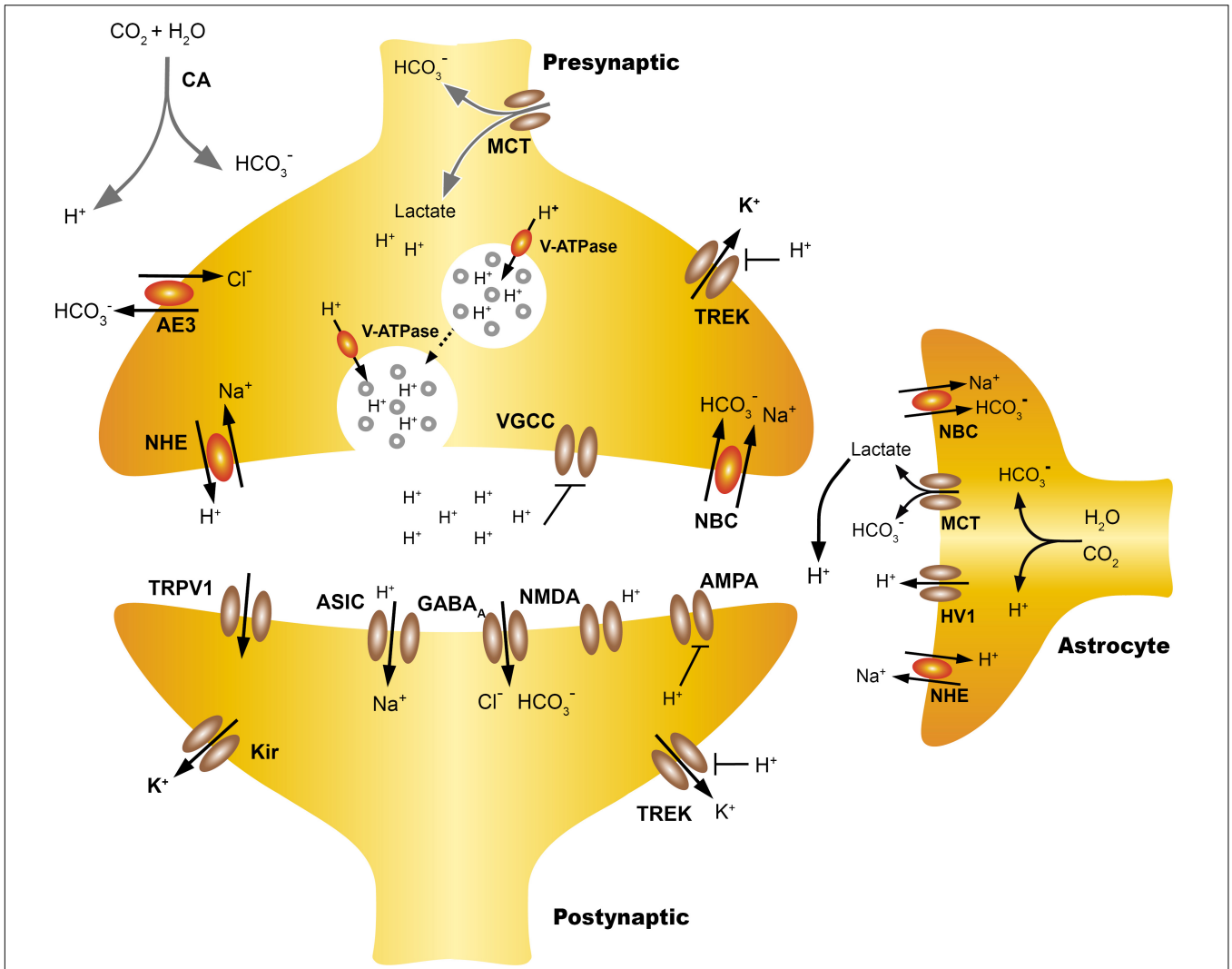

FIGURE 1 | Extracellular proton homeostasis. Presynaptic cells express pumps and transporters that contribute to control pHi, including monocarboxylate transporters (MCT), anion exchanger (AE), Na^+ -H^+ exchangers (NHE), coupled sodium bicarbonate transporters (NBC/NCBTs) and the V-ATPase. During neurotransmission, the extracellular acidification of the synaptic cleft can take place with both rapid or slow kinetics. Synaptic vesicles co-release protons and neurotransmitters, also the transient incorporation of V-ATPase in the plasma membrane contribute to the extrusion of protons, producing rapid acidification of the synaptic cleft. Also intensive activity increases the energy demand of astrocytes, which increases the production of lactate and CO_2, this can diffuse freely while the lactate is transported from the astrocyte to the extracellular space by MCT, which leads to a slow extracellular acidification. Lactate may also be taken from the presynaptic neuron as a source of energy. TWIK-related K^+ channels (TREK) are modulated both by pHe and pHi, the intracellular H^+ increase the open probability of TREK-1, hyperpolarizing the cells while extracellular H^+ inhibits TREK-1 and also voltage gated calcium channels (VGCC). The NBC transporters are widely expressed in neurons and the astrocytes and their action by introducing HCO_3 from the extracellular mediums, producing gradual extracellular acidification. Acid pHe can also activate postsynaptic channels like ASIC or TRPV1 while reducing the open probability of NMDAR. The regulation and actions of pHi and pHe at the synapse are discussed in more detail in Proton Homeostasis and Proton Accumulation.

neurons induces the activation of glutamate N-methyl-D-aspartate receptors ($NMDA_R$) that may recruit NHE5 to the dendritic membrane surface, where it will contribute to synaptic cleft acidification and suppression of dendritic spine growth (Diering et al., 2011). The knock-down of NHE5, or overexpression of a dominant-negative mutant, causes dendritic spine overgrowth (Diering and Numata, 2014). Of most interest was the recent demonstration that links the NHE9 coding gen -Slc9a9- to autism spectrum disorders (ASD). The elimination of NHE9 in mice produced an ASD-like behavior and provides the field with a new mouse model of ASDs (Yang et al., 2016).

In relation to the role of H^+ as synaptic co-transmitters, the V-ATPase plays a pivotal role in transporting protons from the cytoplasm into synaptic vesicles using energy from ATP hydrolysis. Also, synaptic vesicle fusion during neurotransmitter release transiently incorporates V-ATPase into the synaptic membrane, where it contributes to the acidification of synaptic cleft (Casey et al., 2010). Regardless of the neurotransmitter, synaptic vesicles (SV) express the V-ATPase, its activity produces

a concentration gradient of H^+ (ΔpH), and an electrical potential ($\Delta\psi$) in the membrane of the SV. The electrochemical gradient ($\Delta\mu H^+$) is used by the vesicle transporters to charge neurotransmitters in the SV. Although most neurotransmitters use V-ATPase derived gradients, there are differences in intravesicular H^+ concentrations and transport mechanisms. For example, glutamatergic SV exhibit higher acid luminal vesicular pH (pH ∼5.8) than GABAergic SV (∼6.4) (Eriksen et al., 2016; Farsi et al., 2016). The loading of glutamate depends on the vesicular glutamate transporters (VGLUT), which function as a glutamate/proton exchanger associated with a channel-like chloride conductance (Martineau et al., 2017). The Cl^- conductance accounts for the Cl^- dependence of VGLUT activity (Egashira et al., 2016; Martineau et al., 2017). In contrast, Gamma aminobutyric acid (GABA) loading in SV is done by vesicular GABA transporters (VGAT), these require $\Delta\mu H^+$ for optimal activity. VGAT operates as a GABA/H^+ antiporter, with no other ions participating in the transport (Farsi et al., 2016).

ASICs

The ASICs are chemically gated ion channels that are voltage-insensitive, cation-selective, (mostly permeable to Na^+) and non-specifically blocked by amiloride, they belong to an evolutionary old channel family, the Epithelial Sodium Channel/Degenerins (ENac/DEG) (Krishtal and Pidoplichko, 1980; Krishtal and Pidoplichko, 1981; Waldmann et al., 1997; Kellenberger and Schild, 2002; nicely reviewed by Hanukoglu, 2017). There are at least seven isoforms of the ASICs (1a, 1b, 2a, 2b, 3, 4,and 5) derived from five *ACCN1-5* genes (HUGO Gene Nomenclature Committee). They are widely expressed in the peripheral and central nervous system as well as other tissues. Different studies have shown that activation of these channels is linked to various physiological processes, such as pain sensing, auditory and visual processing, fear conditioning, drug addiction, epilepsy ending, and in pathological processes such as anxiety, ischemia and multiple sclerosis (Pignataro et al., 2007; Xiong et al., 2008; Wemmie et al., 2013).

Typically, ASIC currents show a peak current followed by complete or partial desensitization, depending on the subunit composition of the channel (Hesselager et al., 2004). Functional ASICs are formed by trimeric proteins. Studies of homomeric channels show that ASIC isoforms have important differences in the affinity for protons, ASIC3 is the most sensitive unit with a pH_{50} of 6.4, and the least sensitive is the ASIC2a with a pH_{50} of 4.5. (ASIC1b = 6.1; ASIC 1a 5.8 and the ASIC4 and ASIC2b did not form functional homomeric channels) (**Table 2**). Thus, functionally, the ASICs span about four units range of pH sensitivity (from about 4 to 8). The ASIC subunits also differ in their current kinetics. ASICs activate within <5 ms in the ASIC3 to 6–14 ms for the ASIC2a (Bässler et al., 2001; Li et al., 2010), and the current desensitizes with variable kinetics. The desensitization and inactivation coefficient (ratio of the current at the end of desensitization versus peak current) in the continual presence of protons significantly defines the functional consequences of ASIC activation (**Figure 2**). The ASIC1a and 1b current almost completely desensitizes, while the ASIC2a slowly desensitizes and ASIC3 quickly desensitizes, although a sustained component is exhibited, which is always about 0.3 of the peak current (Gründer and Pusch, 2015). Thus, the total current carried by ASIC3 is much larger than that of ASIC1, and its activation in neurons induces a sustained depolarization and large Na^+ inflow. The properties of heteromers are unpredictable from those of homomers. In the CNS, the ASIC1a seems to be the most prominently expressed of the ASICs, although ASIC2a and ASIC2b are also significantly expressed in some regions of the brain, and ASIC3 and ASIC4 have a restricted expression (Wu J. et al., 2016). ASIC2a and ASIC2b interact with ASIC1a to form heteromeric channels, shifting the pH sensitivity and desensitization kinetics of acid gated currents (Askwith et al., 2004; Hesselager et al., 2004). ASIC2a interacts with PSD 95 protein and was shown to contribute to the transport of ASIC1a containing channels in dendritic spines (Zha et al., 2009). Although the desensitization process of ASICs casts doubt on their potential to follow rapid neuronal signaling, there is evidence that activation by small pH changes produced practically no desensitization and recovery seems to be very fast in relation to neuronal activity (MacLean and Jayaraman, 2016). An excellent study of the ASIC response to rapid pH changes showed that currents from ASIC1a homomers and ASIC1a/2a heteromers may deactivate with very fast time constants (1a ≅ 0.7 – 1a/2a ≅ 0.3 ms), and that unusually slow desensitization rate (1a ≅ 700 – 1a/2a ≅ 780 ms) endows these receptor channels with the capability of following fast trains of stimuli during long lasting periods (1ms at 50 Hz during 2 s), suggesting that they may sustain postsynaptic responses when other receptors desensitize (MacLean and Jayaraman, 2016). In native ASICs in the dorsal root ganglion (DRG), neuron deactivation was ≈ 0.33 ms (MacLean and Jayaraman, 2016), although currents showed a higher variability of their desensitization kinetics due to the expression of ASIC1a, ASIC2a, and ASIC3 heteromeric channels (Kusama et al., 2013). Moreover, recovery from desensitization and deactivation kinetics of ASICs was dependent on pH, with a significant reduction of kinetics with acidic pH. Deactivation of the ASIC1a/2a changes from an extremely fast <1 ms deactivation at pH 8 to a slow >300 ms deactivation at pH 7.0 (MacLean and Jayaraman, 2017). This implies that charge transfer in ASICs will be dependent on the extracellular pH, which makes ASICs very unique among ligand-gated channels.

Among the ASICs, the ASIC5 is the lesser known. It is phylogenetically between the ASIC and the ENaCs, although it has a 30% homology with ASICs, it is not activated by protons but by bile acids, leading to the denomination of BASIC (Lefèvre et al., 2014). It was shown that channel activates by membrane-active substances, suggesting that BASIC is sensitive to changes in the membrane structure (Schmidt et al., 2014). In the brain, ASIC5 is restrictively expressed in interneurons in the granular layer in a subset of Unipolar Brush Cells of the ventral uvula and nodulus in the cerebellum, where its specific function is thought to be critical and distinctive (Boiko et al., 2014).

There are also important pharmacological differences between the ASICs (Osmakov et al., 2014). The ASIC1a is particularly sensitive to Hi1a and PcTx1 toxins, the ASIC3 is blocked

TABLE 2 | Subunits, distribution and functions of ASICs.

Gene	Subunit	pH_{50}	Distribution	Physiology	Reference
ACCN2	ASIC1a	5.8	CNS/PNS	Synaptic plasticity, learning and memory, fear conditioning, visual transduction, visceral mechano-reception, primary muscle hyperalgesia, apoptosis, chondroprotection and bone resorption	Wemmie et al., 2003; Hesselager et al., 2004; Ettaiche et al., 2006; Weng et al., 2007; Walder et al., 2011; Li et al., 2014
	ASIC1b	6.1	PNS		Gründer et al., 2000; Hesselager et al., 2004; Ugawa et al., 2006
ACCN1	ASIC2a	4.5	CNS/PNS	Visual transduction, detection of sour taste, mechanosensation, arterial baroreceptor reflex	Hesselager et al., 2004; Ettaiche et al., 2006; Ortega-Ramírez et al., 2017.
	ASIC2b	NA	CNS/PNS	Integrity of retina, modulator of ASIC1a, ASIC1b, ASIC2a, and ASIC3 currents	Ettaiche et al., 2006; Sherwood et al., 2011
ACCN3	ASIC3	6.4	CNS/PNS	Chemoreception], skin mechanosensory, auditory and visual processing, mechanosensory of the intestinal tract	Hesselager et al., 2004; Ettaiche et al., 2009; Lin et al., 2016; Ortega-Ramírez et al., 2017
ACCN4	ASIC4	NA	CNS/PNS	Modulate the amount of functional ASICs into the plasma membrane and as a regulator of pain	Donier et al., 2008

ACCN, Amiloride-Sensitive Cation Channel; CNS: Central Nervous System; PNS, Peripheral Nervous System; pH50, Half-maximum pH of activation.

by APETx2, while ASIC2 is positively modulated by MitTx and by ApTx2 (Cristofori-Armstrong and Rash, 2017). The PcTx is mainly inhibitory in ASIC1a while it seems to potentiate the current in ASIC1b homomers and ASIC1a/ASIC2a heteromeric channels in a state-dependent manner. This action was also shown in ASIC currents recorded in rat cortical neurons (Chen et al., 2006; Liu et al., 2018). Thus, pharmacological specificity contributes to defining the role of specific subunits in certain CNS processes (Wu J. et al., 2016). The desensitization rate and tachyphylaxis are modulated by extracellular anions. Extracellular Cl^- slowed desensitization and increased tachyphylaxis in a dose-dependent form both in native hippocampal ASICs and in transfected ASIC1a channels (Kusama et al., 2010); in ASIC2a and ASIC3, anions also modulate the kinetics of desensitization and the pH dependence of the activation (Kusama et al., 2013). Interestingly, it has been shown that ASIC currents may be modulated by GABA(A) receptor currents in hippocampal neurons in DRG neurons and in HEK293 expression cells, suggesting that these two ion channels are within a microdomain where they may functionally interact. GABA receptors ($GABA_R$) activation decrease the response of ASICs to pHe changes and ASIC1a activation also modifies the kinetics of GABA(A) receptor (Chen et al., 2011; Zhao et al., 2014). Also, other endogenous molecules such as spermine, agmatine, arachidonic acid, serotonin, dynorphins, and histamine may modulate the ASICs (Wemmie et al., 2013; Nagaeva et al., 2016; Wang et al., 2016; Ortega-Ramírez et al., 2017).

The ASIC subunits are formed by two transmembrane segments (TM1 and TM2) with its C and N terminal, located intracellularly and joined by a large extracellular loop. In the CNS, ASIC1a, ASIC2a, and ASIC2b, which are arranged in homo- and hetero-trimeric complexes, form most ASIC channels. The ASICs have been found to be evenly distributed in neurons, although concentrated in synaptic regions and anchored to postsynaptic density scaffolding proteins (Zha et al., 2009). The very fast gating kinetics of the ASICs and the Ca^{2+} blocking action led to the proposal that the ASIC3 channel opening was triggered because H^+, displacing Ca^{2+}, relieves the blockage of the channel (Immke and McCleskey, 2003). Recent evidence shows that a ring of rat ASIC3 glutamates, located above the channel gate, modulates proton sensitivity and contributes to the Ca^{2+} block site. Mutations of this site reduce Ca^{2+} block of the channel, making it similar to ASIC1a (Zuo et al., 2018). In chicken ASIC1a, the most thoroughly studied of the ASICs, the gating of the channel induces a displacement of the TM2 segment, opening the pore like a diaphragm and allowing ions to pass through a selectivity filter formed by G-A-S motifs from each of three adjacent subunits (Baconguis et al., 2014). The selectivity for monovalent cations of the filter is $Li^+ \approx Na^+ > K^+ Rb^+ > Cs^+$ (Yang and Palmer, 2014). The permeability of the ASIC1a to Ca^{2+} has been found to be higher than other ASICs which are nearly impermeable to Ca^{2+} (Yermolaieva et al., 2004); but reports of the permeability ratio PNa/PCa for ASIC1a are conflicting, since a large variability ranging from 2.5 to 18.5 has been found (Bässler et al., 2001; Chu et al., 2002; Zhang and Canessa, 2002; Canessa, 2015). In cells transfected with ASIC1a, as well as heteromeric ASIC1a and ASIC2a and ASIC2b, it was found that PNa/PCa for ASIC1a was 1.8, for ASIC2a/1a it was 25.5 and for ASIC2b/1a it was 4.1 (Sherwood et al., 2011).

There is still a question about how cations reach the extracellular vestibule of the ASIC channel (Yang et al., 2018). The channel pore profile is formed by three interconnected vestibules forming a pathway for cations to reach the extracellular vestibule and cross the membrane when the channel opens

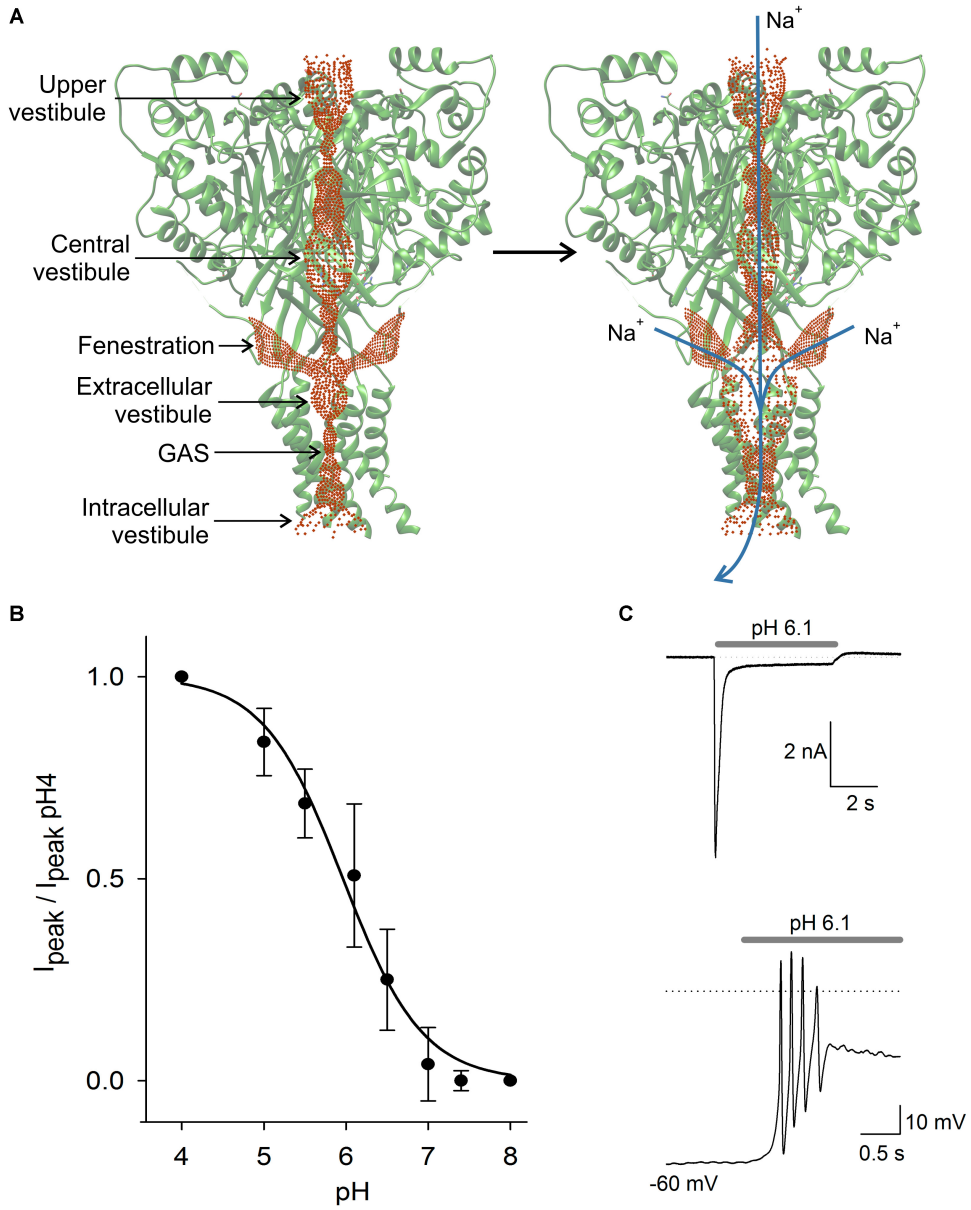

FIGURE 2 | ASIC structure and properties. In **(A)** scheme of the ASIC channel trimer in the closed and open states. Current is activated by H^+ and carried by Na^+ and in lower proportion by Ca^{2+}. Activation of the ASIC led to a significant expansion of the central pore, due to a complex modification of the channel structure. The three lateral fenestrations would significantly contribute to ion passage into the extracellular vestibule. In **(B)** pH dependence of ASIC activation in DRG neurons. The current showed typical sigmoidal pH dependence with a pH_{50} of 6.1. In **(C)** typical ASIC current in voltage clamp from a DRG neuron produced by pH 6.1 solution perfusion. Current reached a peak and then desensitized during the first second to a plateau of sustained current. In the lower panel in current clamp condition, the perfusion of pH 6.1 to a DRG neuron induced a series of action potentials followed by a large sustained depolarization, coinciding with the recording in voltage clamp.

(**Figure 2**; Yoder et al., 2018). The extracellular vestibule has three large fenestrations from which cations most likely enter (Gründer and Chen, 2010). Gating of the channel produces an expansion of the extracellular vestibule and reduction of lateral fenestrations (Yoder et al., 2018). Molecules interacting with the fenestration will act as partial blockers of the current, and molecules interacting with the central pore may produce a similar effect. Alas, the definition of the full ion permeability path, or of the relative contribution of the lateral fenestration, or the central pore, seems relevant to determine the action mechanism of molecules binding in the ASICs.

One significant question that has been put forward is whether or not the protons are the only and sufficient endogenous ligand for ASIC activation. The idea that proton activation of ASICs is a byproduct, and that a real endogenous activator is a "large neurotransmitter like" substance has been tested

(Yu et al., 2010). However, until now, protons remain as the sole and most potent endogenous direct agonist of ASICs. The recent definition of the gating mechanism of chicken ASIC1a supports the idea that protons interacting with the acid pocket are the agonists for ASIC activation (Jasti et al., 2007; Gonzales et al., 2009; Vullo et al., 2017; Yoder et al., 2018). However, some elements out of the physiological range of ASIC2a pH_{50} activation of about 4.5–4.9 (Hesselager et al., 2004) suggest that something else will activate this channel. There are various endogenous modulators and partial agonists of the ASICs, but none known to physiologically activate the ASICs (Ortega-Ramírez et al., 2017). The 2-guanidine-4-methylquinazoline (GMQ) modulates ASIC3 at pH < 7.4 through a binding site distinct from the proton sensor (Yu et al., 2010). The MitTx, which is a viper toxin, evolved as a cytotoxin that, by maintaining the ASIC1a and 1b in the open state, produces cell damage (Bohlen et al., 2011). MitTx also modulates ASIC2a pH sensitivity. Recently, lindoldhamine, an alkaloid from *Laurus nobilis,* was shown to activate the ASIC3 in a proton-independent form and to act as a positive allosteric modulator of human and rat ASIC3 channels (Osmakov et al., 2018).

MEMBRANE SENSORS FOR pH. OTHER THAN ASICs

Extracellular pH changes modulate diverse cellular processes such as neuronal excitability, neurotransmitter release and postsynaptic responses, because pHe modulates the activity of different neuronal ion channels apart from the specific H^+ sensing channels, including voltage-dependent Ca^{2+}, K^+, and Na^+ channels, glutamate and $GABA_R$, and Transient Receptor Potential-1 (TRPV1), among others (Chesler, 2003; Chiacchiaretta et al., 2017). The questions that arise are how pH influences channel activity, and what the physiological relevance of this channel modulation is. The slight acidification of the synaptic cleft modulates voltage-gated Ca^{2+} and Na^+ channels in two ways, first, protons alter charged amino acids near the pore, thus reducing channel conductance. The H^+ shifts the voltage dependence to more positive potentials, and in the case of Na^+ channels, an alkaline medium lightly enhances the current (Tombaugh and Somjen, 1996). The sensibility of Ca^{2+} channels to pHe constitutes a significant element of proton signaling in the CNS, implicated both in vision and auditory function as described in the following section.

The two-pore domain K^+ channels are essential for stabilizing the resting potential in most neurons, their activation produces a time- and voltage-independent K^+ background current. These channels are usually inhibited by acidosis TASK 1 (two-pore domain K^+ channel 1), TASK3, TASK2, TWIK (tandem of P-domain in a weak inwardly rectifying K^+ cannel) and TREK1 (TWIK-related K^+ channel 1) and potentiated by extracellular alkalosis, except for TREK2 channels that are activated by a small pH drop (within 7.2 to 7.4) (Ehling et al., 2015). The physiological importance of pHe regulation of these channels continues to increase, it has been shown that under acid pHe conditions, the TASK and TWIK channels can even change their ionic selectivity and become permeable to Na^+ (Ma L. et al., 2012). Additionally, the family of inward rectifier K^+ channels (Kir) contributes to the leak K^+ conductance in neurons and Kir conductance decreases with the acidification of the extracellular mediums, contributing to neuron depolarization (Coetzee et al., 1999).

Ionotropic neurotransmitter receptors, including GABA(A) receptor and $NMDA_R$, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors ($AMPA_R$), and Kainate receptors (KA_R), are also modulated by pHe. The glutamate receptors are involved in neuronal development, synaptic plasticity, memory formation, and excitatory synaptic transmission (Traynelis et al., 2010). It has been reported that small drops in the pHe (pH 6.9–7.3) could reduce $NMDA_R$ activity, except for recombinant NMDA N1/N3A that is strongly enhanced by acidification (Traynelis and Cull-Candy, 1990; Cummings and Popescu, 2016; **Figure 3**). Mutagenesis analysis indicates that critical residues for gaiting in these receptors regulate the pHe sensitivity of $NMDA_R$, reducing their open probability in acid pHe (Low et al., 2003; Dravid et al., 2007). The effect of pHe on KA_R is voltage-independent

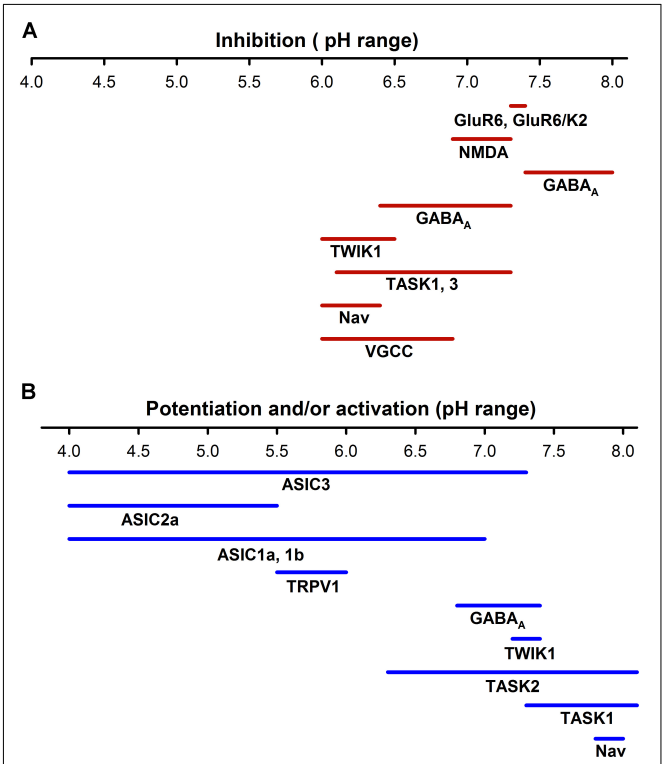

FIGURE 3 | Sensitivity to pH of different neuronal ion channels. **(A)** Inhibition by pH of different neural ionic channels span from a pH of about 6.0 to 8.0, which indicates that at normal pH (7.4) a certain percentage of receptors such as KAR, AMPAR, or NMDAR are partially blocked. **(B)** ASICs are activated from a range of pHs from <7.8 to 4.0; in contrast the TRPV1 channels are much less sensitive to pH. Other channels such as voltage -gated K^+, Na^+, and Ca^{2+} channels can increase their activity at more alkaline pHs. The potentiation or activation of different channels has a much higher range (pH 4–8.0) than inhibition.

and subunit dependent. The KA_R consists of 5 subunits (GluR5, GluR6, GluR5, K1, and K2) that combine in homo or heteromeric channels. Almost all kainate receptors are inhibited by protons, with the exception of the heteromeric GluR6/KA1 receptor, which is expressed in presynaptic neurons and potentiated by acid pH. At pH 7.3–7.4, homomeric GluR6 and heteromeric GluR6/KA2 are inhibited at Δ 20–25%, while GluR6/KA1 is enhanced to around 30% (Mott et al., 2003). In contrast, $AMPA_R$ are much less extracellular proton sensitive (half-maximal inhibition at 6.1) and inhibition is due to enhanced desensitization of the $AMPA_R$ (Ihle and Patneau, 2000).

The activation of ionotrpic GABA(A) receptor produce an inward Cl^- current. However, GABA(A) also seems to conduce bicarbonate from intra to extracellular space that leads to hyperpolarization of the postsynaptic neurons and alkalization of synaptic cleft (Ma B.F. et al., 2012). Reported regulation of GABA(A) by pHe at the postsynaptic level is highly variable. In hypothalamic neurons, acid pHe (∼6.4) inhibits GABA(A) current elicited by 10 μM GABA to 66.7 ± 6.8 %, whereas in alkaline pHe (8.4) increases to 212 ± 32.5% (Chen and Huang, 2014). In contrast, GABA(A) receptors in cultured cerebellar granule cells reduce their activity in alkaline pHe and are enhanced in acid pHe (Dietrich and Morad, 2010). The variability in the response of this receptor to pHe changes may be accounted for by the extremely variable experimental conditions in which they have been studied (experiments been carried out in native and recombinant GABA(A) at different concentrations of GABA and the presence or absence of buffers, such as HEPES).

Extracellular concentration of H^+ may also activate some channels like the TREK2 K^+ channels described above, and the TRPV1. The TRPV1 are non-selective cation channels activated by voltage, heat (>43°C), low pH (<6) and by several endogenous ligands (capsaicin, anandamide, and other endovanilloids). They are expressed throughout the CNS mainly in cortex, hippocampus, dentate gyrus, hypothalamus and superior colliculus (Toth et al., 2005). It has been suggested that they contribute to complex brain functions such as addiction, cognition and mood (Gibson et al., 2008; Tian et al., 2010, 2018; You et al., 2012). TRPV1 also localizes to synapses, and it has been proposed that it can modulate neurotransmission, synaptic plasticity and neuronal survival (Ho et al., 2012; Martins et al., 2014). In the dentate gyrus and nucleus accumbens, postsynaptic activation of TRPV1 from anandamide causes long term depression (Chavez et al., 2010; Grueter et al., 2010). TRPV1 are also implicated in neurodegeneration, in mesencephalic neuronal cultures and cortical microglia, and over-activation of TRPV1 raises intracellular Ca^{2+}, producing mitochondrial damage and apoptosis (Kim et al., 2006).

The various proton sensitive channels, along with proton transporter activity, exchangers and buffer capacity always constitute a confusion variable in experiments that analyze the role of ASICs in the proton signaling mechanism in the nervous system. Their potential role and activation and inhibition by protons should always be considered an alternative explanation and a variable that should be controlled to ascertain the ASIC role in pHe actions.

EVIDENCE OF THE ROLE OF H^+ IN SYNAPTIC TRANSMISSION

Since the discovery of pHe sensitive responses in neurons, it was speculated that a proton concentration rise in extracellular mediums may activate a specific signaling system (Krishtal et al., 1987). The first solid evidence indicating the role of protons as an intercellular synaptic messenger was derived from experiments in *C. Elegans* in which it was shown that a H^+ concentration rise by PBO-4 (a putative Na^+/H^+ ion exchanger) expressed in the lateral membrane of the intestine is enough to induce intestinal muscle contraction (Beg et al., 2008). Oscillatory transepithelial proton concentration regulates rhythmic behavior of the defecator program of *C. Elegans* (Pfeiffer et al., 2008). Further evidence showing the role of protons in synaptic transmission was obtained in the vertebrate retina, where it was shown that protons are the elusive mediator of lateral inhibition between horizontal cells and photoreceptors. Proposed mechanisms of lateral inhibition include GABA, protons, or an ephaptic mechanism (Kramer and Davenport, 2015). However, protons acting on Ca^{2+} channels have gained support as the mechanism that account for lateral inhibition, in fact, the use of genetically encoded pH sensors showed that L-type Ca^{2+} channels at the synaptic cleft are the sensors for protons (Wang et al., 2014). In the cone to horizontal neurons, synaptic cleft acidification implies the Na^+-H^+ exchangers as the main source of protons, and activity of HCO_3^- transport in the horizontal cells will produce alkalization during light-evoked photoreceptor hyperpolarization (Warren et al., 2016). Notably in this case, similar to that of *C. elegans*, acidification is most probably mediated by the activity of an exchanger mechanism, specifically an NHE whose identity is not yet defined. It is worth noting that horizontal cells in the retina use GABA as a neurotransmitter, therefore in this case there is a segregation of GABA release and protons, both functioning in the same synapse.

Regarding the ASIC expression in the retina, the ASIC1a was found in cone photoreceptors, horizontal cells, some amacrine, and bipolar cells, and in the ganglion cell layer. Knockdown of ASIC1a or its blockade by PcTx1 decreased the photopic a- and b-waves and oscillatory potentials of the electroretinogram (Ettaiche et al., 2006). The ASIC3 is also expressed in the rod inner segment of photoreceptors, in horizontal cells, and some amacrine cells and in ganglion neurons. In early life (2–3 months) ASIC3 knockout mice show an increase in scotopic electroretinogram, but older mates (8 months) show a significant reduction in electroretinogram a- and b-waves, and disorganization of retina with degenerations of rod inner segments (Ettaiche et al., 2009).

In the auditory and vestibular system, in mice, the cochlear spiral ganglion neurons (SGNs) elicit a proton-gated ionic current that may be relevant in the response to high intensity

auditory stimuli, since knockout of ASIC2 (including the ASIC2a and ASIC2b) exhibits increased resistance to noise-induced temporary threshold shifts, indicating a function of ASIC2 in hearing and the potentially harmful effects of acidosis (Peng et al., 2004). The SGNs and the organ of Corti of mice express ASIC3, and knockout of ASIC3 developed early hearing loss at about four months of age (Hildebrand et al., 2004). The ASIC1b subunit was detected in SGNs and at the insertion point of the stereocilia into the cuticular plate in the outer hair cells of the cochlea (Ugawa et al., 2006). In the rat vestibule, ASIC1b, and 4 were cloned and cDNA amplified (Gründer et al., 2000; Bässler et al., 2001). The ASICs have been shown to be expressed both in the rodent vestibular and cochlear afferent neurons (Mercado et al., 2006; González-Garrido et al., 2015). Expression of ASIC1a and ASIC2a was found in small vestibular ganglion neurons and afferent fibers in the utricle and crista stroma of the rat. The ASIC2b, ASIC3, and ASIC4 were expressed to a lesser extent (Mercado et al., 2006). The discharge of the vestibular system primary afferent neurons is highly sensitive to external pH changes and ASIC antagonists, such as amiloride and acetylsalicylic acid. These factors significantly reduced the vestibular-nerve discharge, corroborating that ASICs participate in the establishment of the afferent-resting discharge (Mercado et al., 2006; Vega et al., 2009). FMRF-amide was demonstrated to be present in calix ending synapses in the vestibular neuroepithelia, and FMRFamide perfusion increased the activity of the afferent neurons of the semicircular canal, indicating that ASIC currents are tonically active in resting condition (Mercado et al., 2012). In fact, it has been demonstrated that low pH perfusion may be enough to activate the action potential discharge in vestibular and cochlear afferent neurons. Based on these results, it has been proposed that ASICs mediate a synaptic input to cochlear and vestibular afferent neurons (Soto et al., 2014). In adult zebra fish, ASIC1 and ASIC4 were also found to be expressed in the hair cells of neuromasts, and ASIC2 in the afferent neurons, indicating the potential role for these ion channels in mechanosensation and postransductional sensory processing of movement information (Abbate et al., 2016).

Paired recordings of afferent neurons and hair cells in bullfrog amphibian papillae showed that presynaptic Ca^{2+} current has a sag after the activation, which coincides with neurotransmitter release from hair cells. The sag in the current was shown to be produced by proton accumulation within the basolateral region of the cell where most of the Ca^{2+} channels are located, thus constituting a negative feedback system (Cho and von Gersdorff, 2014). This shows that a pH drop in synaptic endings can activate the ASICs which contribute to the EPSC, but at the same time contribute to presynaptic Ca^{2+} current decreases, limiting transmitter release. It has also been found that a drop of the pHe decreases K^+ currents in isolated hair cells from the rat semicircular canals (Almanza et al., 2008). Therefore, depending on the balance, timing of the ASIC activation, and the decrease of the Ca^{2+} and the K^+ currents, the acidification of synaptic cleft in the hair cell systems may boost the postsynaptic response and restrict the release time of neurotransmitters (**Figure 4**; Almanza et al., 2008). In the sensory neuroepithelium of the lagena obtained from the turtle, the activation of hair cells may induce pH changes in the basal side of neurosensory epithelia, and modeling data indicates that vesicle release may account for the pH drop in this microdomain (Highstein et al., 2014).

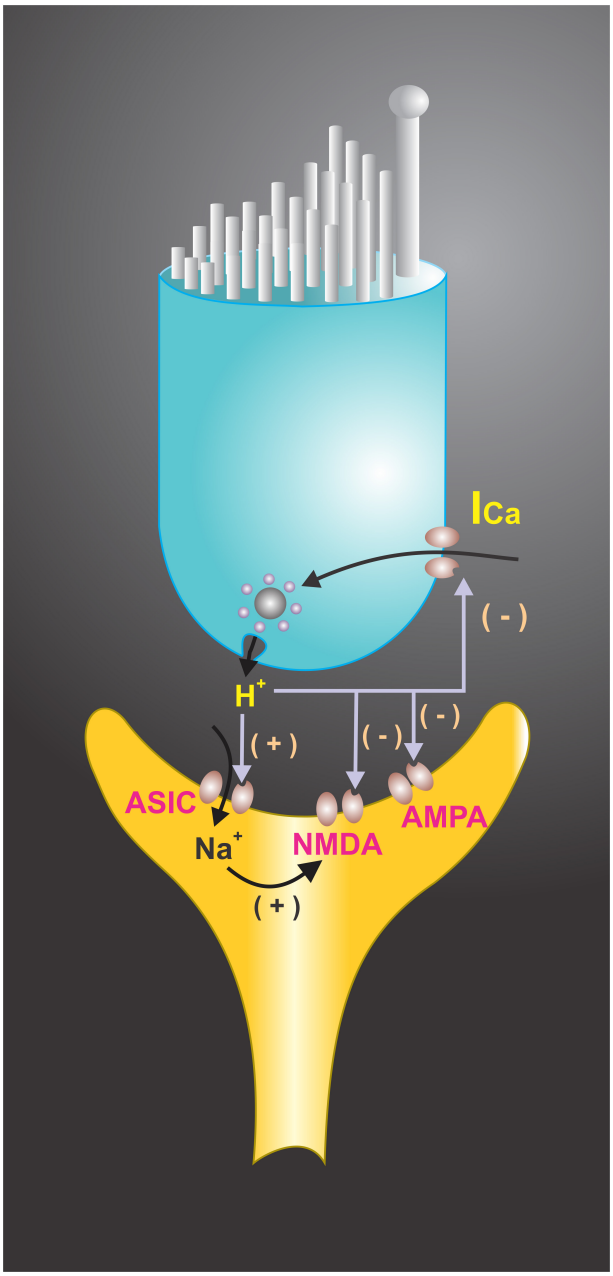

FIGURE 4 | Extracellular protons have been shown to modulate voltage-activated ionic channels in hair cell receptoneural junctions. Presynaptic K^+ and Ca^{2+} currents are modulated by H^+, suggesting that they may function as a synaptic feedback mechanism in hair cells. A shift in the voltage dependence of the Ca^{2+} current to a more positive membrane potential was achieved at pH < 6.8. Extracellular pH also modulates the NMDA and AMPA receptors response to afferent transmitters and interacts with ASICs located at the synaptic endings, contributing to EPSC. The end result of H^+ interactions with ionic channels may boost the postsynaptic response and restrict the release of neurotransmitters.

It is worth noting that the rapid deactivation kinetics and slow desensitization of the ASIC currents endow ASIC mediated responses with the capability of following high frequencies without any loss of response. Recombinant ASIC1a homomers and ASIC1a/2a heteromers, as well as native ASICs of DRG neurons, follow trains of brief pH 8.0 to 5.0 stimuli at high frequencies (> 50 Hz) without any loss of response amplitude or kinetic characteristics. Compared to glutamate evoked responses, they show a capacity for high-frequency signaling when other receptors desensitize (MacLean and Jayaraman, 2016). This makes ASICs ideal candidates for high frequency responses needed for sensory coding in cochlear afferent neurons (Fettiplace, 2017).

Interestingly, it has been shown that accumulation of protons due to hypoxia, and activation of anaerobic mechanisms in the inner ear may finally induce an activation of the vestibular afferent neurons, expressing ASICs and the induction of movement. This mechanism may be part of the processes which induce a person to move when there is a hypoxic condition. The role of this mechanism in Sudden Infant Death Syndrome (SIDS) has been considered as potentially relevant (Allen et al., 2013; Ramirez et al., 2016). Also, a high expression level of NHE in the brain stem is associated with an increase in incidence of deaths by SIDS (Wiemann et al., 2008). This last mechanism will be related to the pH modulation of the excitability of respiratory control neurons.

A significant role of H^+ as co-transmitter signaling is found in the auditory pathway. The postsynaptic neurons of the medial nucleus of the trapezoid body (MNTB) at the mouse calyx of Held synapse express functional homomeric ASIC1a channels that can be activated by protons co-released from synaptic vesicles (González-Inchauspe et al., 2017). Currents evoked by acid pHe perfusion were blocked by PcTx1 and in ASIC1a$^{-/-}$ mice. Most relevant is the fact that postsynaptic potentials produced by presynaptic stimulation are of a magnitude sufficient to evoke action potentials in postsynaptic neurons of the MNTB in absence of glutamate receptor activation. High frequency stimulation of presynaptic terminals leads to Ca^{2+} increase in MNTB neurons. The lack of functional ASICs during high frequency stimulation enhances short-term depression of glutamatergic EPSCs. These results demonstrate that presynaptic co-release of protons modulate synaptic transmission by activating ASIC1a at the calyx of Held-MNTB synapse (González-Inchauspe et al., 2017).

In other sensory systems, such as pain pathways, ASICs are widely expressed both in the peripheral and central nervous systems (Deval et al., 2010; Wemmie et al., 2013). ASIC3 is highly expressed in DRG neurons, mediating pain associated with a decrease of pHe in processes such as inflammation, ischemia and cancer. The ASIC3 also mediates the mechanical hyperalgesia associated with muscle inflammation (Sluka et al., 2003, 2007), although most of the DRG neurons (including cutaneous afferents) express ASICs that probably contribute to pain processing in various modalities (Ortega-Ramírez et al., 2017). In the brain, intracerebroventricular injections of PcTx1 attenuate acute pain responses, as well as pain behavior in chronic inflammatory and neuropathic pain models (Mazzuca et al., 2007), Also, mambalgin-1, an ASIC1a blocker, attenuates pain behavior due to the inhibition of heteromeric ASIC1a/2a in the spinal dorsal horn neurons in an opioid-independent form (Baron et al., 2008). Moreover, suppression of ASIC1a attenuates both mechanical and thermal hypersensitivity induced by peripheral inflammation. The role of ASICs in pain processing is further supported by the high level of expression of ASIC1a in multiple brain regions associated with pain, such as the ventral and dorsal regions of periaqueductal gray matter (Wemmie et al., 2003). These results show that ASICs are essential for pain system activation at spinal and supraspinal levels.

Expression and activation of ASICs in brain areas involved in motor behavior and sensitivity to various psychostimulants such as cocaine, morphine and amphetamines have suggested that ASICs play an important role in addictive behavior (Suman et al., 2010; Jiang et al., 2013). Chronic exposure to cocaine increases the expression of ASIC1 and ASIC2 in the striatum (both caudate putamen and nucleus accumbens -NAc-) (Zhang et al., 2009), and overexpression of ASIC1a in the NAc reduces the self-administration of cocaine in rats. The suppression of ASIC1a increases the conditioned place preference produced by cocaine and morphine (Kreple et al., 2014). At the synaptic level, EPSC in medium spinal neurons (MSN) in the NAc was increased when the buffer capacity in the extracellular medium was reduced, in contrast, the use of amiloride (an unspecific ASIC antagonist) reduces EPSC. ASIC1a knockout mice showed alterations in dendritic spine morphology and frequency of EPSC, suggesting that ASIC1a can regulate excitatory synaptic transmission in the NAc, supporting the hypothesis that H^+ co-release with glutamate significantly contributes to synaptic input by activation of the ASICs and contributes to a decrease in addictive behavior (Kreple et al., 2014).

In the lateral amygdala, it was found that ASIC1 expression is significantly higher than in other areas of the CNS, and in ASIC1 knockout mice the H^+ evoked currents of amygdala neurons and also fear conditioning were undetectable (Wemmie et al., 2003). The rise in the extracellular proton concentration, secondarily to sustained activation of neurons, is at the basis of fear response and fear conditioning in lateral amygdala pyramidal neurons, contributing to EPSC (Du et al., 2014). The neurons are initially activated by glutamatergic input, so, the effect is due to a positive feedback between pHe neuron activity and ASICs activation. Other pHe-related mechanisms, such as membrane pump expression and abundance of mobile buffer in the media, along with aerobic capability of the neurons, would have a significant role in the acidification of extracellular media. The reaction between CO_2 and water catalyzed by carbonic anhydrase generates large H^+ concentration changes, in fact, inhaling CO_2 can trigger panic attacks (Coryell et al., 2006), most likely because of the activation of ASIC currents in amygdala. Buffering pH attenuated fear behavior, and directly reduced pH with amygdala microinjections, reproduced the effect of CO_2 (Ziemann et al., 2009). Interestingly, the effect mediated by pHe changes secondary to carbonic acid has been introduced in the computerized human nervous system function emulation (HNSFE) technology, which uses CO_2 sensors to emulate the response of ASICs allowed to produce

fearful emotional responses and complex avoidant behavior of an android (Frenger, 2010, 2017).

In the case of a convulsive crisis, the overactivation of neurons increases the extracellular H^+ concentration and the activation of ASIC1a and ASIC3, whose expression in GABAergic inhibitory interneurons is larger than that in excitatory neurons, contributing to ending seizures. The kainate-induced seizures were longer and more severe in ASIC1 knockout mice. Consistent with the proposal that ASICs participate in ending seizures, the loss of ASIC1a also reduced postictal depression (Ziemann et al., 2008). Although ASIC3 brain expression is considered low, it was found to be expressed in inhibitory GABAergic interneurons and glial cells. A block of ASIC3 by APETx2 in pilocarpine- or pentylenetetrazole (PTZ)-induced seizures shortened the latency and increased the incidence of seizures (Cao et al., 2014). Thus, the increase in extra pH leads to increased activity, mainly in inhibitory neurons, finally limiting the neuronal discharge by the release of inhibitory neurotransmitter. The ending of a convulsive crisis has been a mystery in clinical neurosciences. It was always thought that some metabolic mechanism was critical for ending crisis, but no exact mechanism was devised until the ASIC was discovered. However, an opposite effect of ASICs in epilepsy has also been found, ASIC2a overexpression resulted in increased hippocampal seizure susceptibility (Wu H. et al., 2016). In fact, amiloride delays the onset of pilocarpine-induced seizures in rats (N'Gouemo, 2008). Brain hypometabolism is a common finding in patients and in animal models of epilepsy, hippocampal glucose hypometabolism elevates ASIC2a expression by suppressing Transcription factor CP2 expression, which further enhances the excitability of CA1 pyramidal neurons and seizure susceptibility in patients with temporal lobe epilepsy (Zhang et al., 2017). Zhang et al. (2017) have proposed that before seizure onset, increased ASIC2a expression could increase neuronal excitability. As large quantities of lactic and glutamic acid are released during seizures, extracellular H^+ accumulation activates ASIC1a and ASIC3, causing GABA release from interneurons and ending the seizure.

CONCLUSION AND PERSPECTIVES

In the near future, we will get information and develop a whole picture of networks of functional interactions among membrane proteins including receptors, transporters and ionic channels. A channelome picture of the cell at the micro- and nano-domains will allow us to understand channel function and its network and mutual modulation, coupled ionic fluxes, membrane potential and osmotic and diffusional forces, interacting all together to determine cell excitability and cell communication.

Proton signaling implies a paradigm shift in relation to neurotransmission and neuromodulation in the CNS. Metabolic activity and the hydrolysis of ATP produce a constitutive and non-regulated release of protons in the extracellular space, which constitute a form of volume neurotransmission and, as described above, may play a key role in neuron excitability regulation. Examples of H^+ mediated signaling indicate a restricted co-transmitter role for protons at the synaptic level; there is also evidence of H^+ and GABA transmitter segregation in horizontal neurons; and a modulatory role of H^+ of metabolic origin also exists, but until now there is no evidence, in vertebrates, to show that there is a regulated Ca^{2+} dependent release of protons in synaptic endings; protons at synaptic endings are always co-released with some neurotransmitter. However, release and regulation of extracellular protons implies a complexity which seems to go farther than the standard neurotransmission and modulation concepts in the nervous system, constituting a new paradigm in cell signaling mechanisms.

AUTHOR CONTRIBUTIONS

ES conceived the article and its structure. ES, AO-R, and RV worked together to write the article.

FUNDING

This work was supported by a grant from Consejo Nacional de Ciencia y Tecnología de México (CONACyT) grant 1544 (Fronteras de la Ciencia) to ES, and BUAP-VIEP grant Cuerpos Académicos BUAP-CA-119 to ES.

ACKNOWLEDGMENTS

Authors wish to thank Prof. Robert Simpson for proofreading of the English manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular Neuroscience

ORIGINAL RESEARCH
published: 13 November 2018
doi: 10.3389/fncel.2018.00411

Sympathetic Hyperactivity and Age Affect Segregation and Expression of Neurotransmitters

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Sympathetic neurons of the rat superior cervical ganglion (SCG) can segregate their neurotransmitters and co-transmitters to separate varicosities of single axons. We have shown that transmitter segregation is a plastic phenomenon and that it is correlated with the strength of synaptic transmission. Here, we determined whether sympathetic dysfunction occurring in stress and hypertension was correlated with plastic changes of neurotransmitter segregation. We characterized the expression of the markers, L-glutamic acid decarboxylase of 67 kDa (GAD67) and vesicular acetylcholine (ACh) transporter (VAChT) in the SCG of cold stressed and spontaneously hypertensive rats (SHR). Considering that the SCG comprises a heterogeneous neuronal population, we explored whether the expression and segregation of neurotransmitters would also have an intraganglionic heterogeneous distribution in ganglia of stressed and hypertensive rats. Furthermore, since hypertension in SHR is detected around 8–10 weeks, we evaluated expression and segregation of ACh and GABA in adult hypertensive (12-week old (wo)) and young pre-hypertensive (6-wo) SHR. We found an increase in segregation of ACh and GABA with no change in transmitter expression in ganglia of stressed animals. In contrast, in SHR, there was an increase in GABA expression, although segregation did not vary. Segregation showed a caudo-rostral gradient in controls but not in the ganglia of stressed animals. GABA expression showed a rostro-caudal gradient in adult SHR, which was not present in young 6-wo rats. In young SHR, ACh increased and, unexpectedly, segregation of ACh and GABA was higher than in adults. Data suggest that ACh and GABA segregation increases in acute sympathetic hyperactivity like stress, but does not vary in chronic hyperactivity such as in hypertension. Changes in segregation are age-dependent and might be involved in the mechanisms underlying stress and hypertension.

Keywords: co-transmission, sympathetic ganglia, stress, hypertension, SHR, acetylcholine, GABA

OPEN ACCESS

Edited by:
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Reviewed by:
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Received: *02 August 2018*
Accepted: *22 October 2018*
Published: *13 November 2018*

Citation:
Merino-Jiménez C, Miguel F, Feria Pliego JA, Zetina Rosales ME, Cifuentes F and Morales MA (2018) Sympathetic Hyperactivity and Age Affect Segregation and Expression of Neurotransmitters. Front. Cell. Neurosci. 12:411. doi: 10.3389/fncel.2018.00411

INTRODUCTION

The sympathetic nervous system (SNS) regulates many functions including blood pressure, cardiac contractility, intestinal motility and exocrine gland secretion, among others. To achieve these functions, the SNS requires a proper balance in content and cellular distribution of its neurotransmitters. It is known that peripheral efferent sympathetic activity is sustained by the effect of a main transmitter, acetylcholine (ACh) at preganglionic and norepinephrine (NE)

at postganglionic level, and by the action of two or more concurrently released co-transmitters. This property of releasing more than one transmitter it is known as neuronal co-release and allows neurons to use more than one neurotransmitter to convey signals across the synaptic cleft (Burnstock, 1976; Kupfermann, 1991). Initially, it was believed that co-release was carried out by the co-storage of the same set of neurotransmitters at all presynaptic endings (Chan-Palay and Palay, 1984; Burnstock, 1990). However, later evidence showed that neurons are able to segregate their neurotransmitters, and independently store and release them at distinct terminals, allowing neurons to exert each transmitter function separately (Fisher et al., 1988; Hattori et al., 1991; Morales et al., 1995; Sámano et al., 2012; Zhang et al., 2015). Like other neurons, sympathetic ones have this capability to segregate their neurotransmitters (Morales et al., 1995; Chanthaphavong et al., 2003; Sámano et al., 2006, 2009; Vega et al., 2010, 2016).

Neurotransmitter segregation is a plastic phenomenon that depends on neuronal requirements (Sámano et al., 2012). For instance, we demonstrated that in rat sympathetic neurons, both *in vitro* and *in vivo*, neurotransmitter segregation is modulated by neurotrophic factors (Vega et al., 2010, 2016). Additionally, we found a likely functional role of segregation in the rat superior cervical ganglia (SCG), we detected a correlation between the level of segregation of ACh and GABA, and the strength of synaptic transmission, where more segregation correlates with stronger synaptic transmission (Elinos et al., 2016). Considering this evidence regarding the plasticity of neurotransmitter segregation, and its likely functional roles, here we explored whether certain physio-pathologic conditions coursing with sympathetic dysfunction, like stress and hypertension, also correlate with changes in the segregation of neurotransmitters in the rat SCG.

It has been reported that in stress and hypertension there is an increase in sympathetic activity (Guyenet, 2006; Lambert and Lambert, 2011). Stress is defined as a state of disharmony, or threatened homeostasis (Chrousos and Gold, 1992). It is worth to note that stress is not always noxious, stress can be also associated to eustress, i.e., a condition perceived as pleasant or exciting, that could be positive stimulus to emotional and intellectual growth and development (Selye, 1950). Stress increases the rate and force of cardiac contraction, respiratory frequency, metabolism and blood flow, in response to an increase in sympathetic nervous activity (Jansen et al., 1995; Kvetnansky et al., 2013). It is known that different stressors induce different patterns of activation of the SNS (Ibrahim et al., 2015). Thus, stress in addition to altering the synthesis of catecholamines in the sympato-adrenomedular system, affects the function of the sympato-neural system, which innervates most of the organs (Goldstein and Kopin, 2008; Lambert and Lambert, 2011; Ibrahim et al., 2015). Sympathetic ganglia like other structures of the sympato-neural system undergo changes in stress conditions; for example, immobilization stress elevates tyrosine hydroxylase (TH) and neuropeptide Y (NPY) mRNAs in rat SCG (Nankova et al., 1996); and stress induced by exposure to a cold environment increases TH activity in sympathetic ganglia (Ulus and Wurtman, 1979; Kvetnansky and Sabban, 1993).

Enhanced sympathetic nerve activity has been detected and implicated in the pathophysiology of hypertension, either in animal models and hypertensive patients (Lundin and Thorén, 1982; Magee and Schofield, 1992, 1994; Mancia et al., 1999; Guyenet, 2006). For instance, it has been shown that essential-hypertensive patients display plasma NE values greater than those of normotensive individuals (Mancia et al., 1999). Furthermore, microneurographic approaches showed that sympathetic nerve traffic increases progressively from the normotensive to the moderately and more severe essential-hypertensive state (Schlaich et al., 2004).

We hypothesized that in stress and hypertension, SNS overactivity might change the distribution of ganglionic neurotransmitters in the SCG of the rat, to cope with sympathetic dysfunction. To explore this hypothesis, we investigated the expression and segregation of ACh and GABA, by immunostaining of vesicular ACh transporter (VAChT) and L-glutamic acid decarboxylase of 67 kDa (GAD67), in animals subjected to cold stress, and in spontaneously hypertensive rats (SHR). Considering the presence of sympathetic overactivity, we evaluated GABA, an inhibitory neurotransmitter, whose level of expression could have changed to counteract the sympathetic hyperfunction. The neuronal population of the rat SCG is diverse, and can be grouped into rostral and caudal based on their regional distribution (Dail and Barton, 1983; Flett and Bell, 1991; Elinos et al., 2016). Since the rostral and caudal neurons might be differentially involved in sympathetic overactivity, we asked whether changes in expression and segregation of ACh and GABA could be different in each ganglionic region. Finally, considering that in SHR, hypertension is detected around the age of 8–10 weeks (Lee et al., 1991; Li et al., 2012), we determined the expression and segregation of sympathetic transmitters at two ages, in pre-hypertensive young 6-week old (wo) and in hypertensive adult 12-wo SHR. We found an increase in the segregation of ACh and GABA under stress conditions, with no changes in the level expression of these neurotransmitters, while in hypertension, segregation did not vary, but GABA expression increase in SHR at both ages. Unexpectedly, we found greater segregation and higher GABA and ACh content in young SHR and control normotensive Wistar Kyoto (WKy) rats, compared to adults.

MATERIALS AND METHODS

Animals

Experimental procedures were conducted according to the ethical guidelines for the use of laboratory animals of the National Academy of Sciences of the United Sates and approved by our Institutional Committee for the Care and Use of Animals in the Laboratory. To study the distribution of ganglionic neurotransmitters in the SCG of the rat in response to hypertension we used SHR adult and young male rats, and as a control normotensive rats of the same strain, the WKy. For the stress model, we use male Wistar rats between 8- and 9-wo (240–260 g), because all our previous data in segregation and expression of ACh and GABA have been observed in this strain.

Stress Induction by Prolonged Exposure to Cold

Male Wistar rats were divided into two groups. One group was subjected to stress by keeping them at a temperature of 5°C for 24 h during a period of 5 days under controlled conditions of 12 h light-dark cycles with food and water *ad libitum*. The second group was used as the control. The SCG and spinal cord were dissected out immediately after stress induction. The stress induction by cold was confirmed by measuring body weight and the expression level of TH protein and mRNA in the adrenal medulla (AM).

RNA Extraction and RT-PCR Analyses

For this assay, we used 11 stressed and eight control rats. After stress induction, the AM was removed and immediately frozen in liquid nitrogen. In each animal, total RNA was isolated using Trizol following the manufacturer's instructions (Invitrogen, Life Technologies). Total RNA (1.5 μg) was reverse transcribed using SuperScript II reverse transcriptase (Life Technologies) and 500 ng of random primers (Promega, Madison, WI, USA). For TH mRNA 2 μl of complementary DNA (cDNA) was amplified by PCR Master Mix (Promega) using oligonucleotides designed to amplify a fragment of 646 bp (nt 720–1365) from TH, F (5′GAAGGGCCTCTATGCTACCCA) and R (5′TGGGCGCTGGATACGAGA) based on the TH mRNA of *Rattus norvegicus* (NM 012740.3). For actin mRNA analyses, oligonucleotides designed to amplify a fragment of 236 bp (nt 451–686) from actin, F (5′GAGACCTTCAACACCCC) and R (5′GTGGTGGTGAAGCTGTAGCC) based on the actin mRNA of *Rattus norvegicus* (NM 031144.3). The cycle conditions used for both fragments were: 20 cycles at 94°C for 30 s, 63°C for 30 s and 72°C for 30 s. Amplified products were analyzed by electrophoresis in agarose gels pre-stained with ethidium bromide. The gels were digitalized using Typhoon FLA 9500 scanner (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). Band intensity was analyzed using ImageJ software (US National Institutes of Health).

Western Blot Assay

For protein assay we used five stressed and four control rats. After stress induction, the AM was removed and homogenized in lysis buffer (in mM: 150 NaCl, 1 EDTA, 0.5 DTT, 20 Tris, 0.5% Triton X-100, pH 8.0) containing 1% SDS and 1× proteinase inhibitor cocktail (Roche Applied Sciences, Indianapolis, IN, USA). Protein concentration was determined by Bradford method. Then, 15 μg of protein were resolved on 12.5% polyacrylamide-SDS gels and transferred onto PVDF (Immobilon-P, Millipore, Billerica, MA, USA) membranes. The membranes were blocked with 5% non-fat dry milk for 2 h and then incubated overnight at 4°C with mouse anti-TH polyclonal antibody (Millipore; 1:15,000) and goat anti-actin polyclonal antibody (Santa Cruz; 1:1,000). Immunoreactive bands were detected with anti-mouse (Millipore; 1:10,000) and anti-goat (Bethyl; 1:10,000) horseradish peroxidase-conjugated secondary antibodies, using Immobilon Western Chemiluminescent HRP substrate (Millipore). Antibody dilutions and rinses were carried out in PBS-Tween 5%.

Histological Procedures for Decentralization of SCG and Retrograde Labeling of Sympathetic Preganglionic Neurons

In a set of rats, we decentralized one of the SCG by transecting the sympathetic cervical trunk (SCT) 3–5 mm caudal to the ganglion. Seven days later the ganglion was dissected, removed and processed for immunocytochemistry. To retrograde labeling and immunostaining cell bodies of SPN, in other groups of rats, the SCT of one side was cut 10–12 mm from the spinal cord (SC) and the Fluoro-Gold (FG) tracer (Fluorochrome, LLC, Denver, CO, USA) was applied at the distal end. Three days later SC was dissected, removed and processed for immunolabeling. Rats were anesthetized with sodium pentobarbital (125 mg/kg i.p.), and transcardiacally perfused with 100 ml of ice-cold phosphate buffered saline (0.01 M PBS, pH 7.4) for 3 min and then with 250 ml of ice-cold fixative solution (2% paraformaldehyde, 0.18% picric acid in 0.1 M PBS, pH 7.4), 100 ml for 3 min and the remaining 150 ml for 40 min. One of the SCG and a SC segment (from C8 to T3) were quickly dissected and postfixed overnight in the same fixative solution and cryoprotected in sucrose solution (10%–30% w/v). Longitudinal sections of SCG and horizontal or transverse sections of SC were cut at 12 μm thickness on a cryostat (LEICA CM1520) at −20°C and recovered on Superfrost plus gelatin-coated slides (Electron Microscopy Sciences, Halfied, PA, USA). For ganglia, longitudinal slices were randomly sampled from 40 to 45 sections cut along its z-axis, while for SC, slices containing the intermediolateral nuclei were processed for double immunolabeling.

Immunocytochemistry Assays

The tissue sections were washed once with PBS, permeabilized and blocked with 10% bovine serum albumin (BSA), 0.3% Triton X-100 for 2 h. Primary antibodies, goat polyclonal anti-choline acetyl transferase (ChAT, the enzyme responsible for the synthesis of ACh) and anti-VAChT, and mouse monoclonal anti-L-GAD67 (the enzyme responsible for the synthesis of GABA), were incubated overnight in a humid atmosphere at room temperature. Specific conditions used for each antibody are given in **Table 1**. Tissue sections were washed twice for 15 min each in 0.1 M PBS, 0.3% Triton X-100 and then incubated for 2 h with the appropriate secondary antibody (**Table 1**). After incubation the tissue sections were washed again and finally mounted on slides with fluorescence mounting medium (Dako, Santa Clara, CA, USA).

Image Acquisition and Analysis

From a selected slice, immunofluorescence tile images (35–40, depending on the size of the section) were sequentially acquired with a Nikon A1R+ laser scanning confocal head coupled to an

TABLE 1 | Antibodies used for immunohistochemistry.

Antiserum	Type of antibody	Conjugate to	Dilution	Source	Catalog number
Primary					
ChAT (human)	Goat polyclonal		1:200 (IF)	Millipore, Chemicon, MA, USA	AB144P
VAChT (rat)	Goat polyclonal		1:400 (IF)	Immunostar, Inc.	24286
GAD67 (synthetic)	Mouse polyclonal		1:200 (IF)	Millipore, Chemicon, MA, USA	MAB5406
TH (rat)	Mouse polyclonal		1:15,000 (WB)	Millipore, Chemicon, MA, USA	MAB318
Secondary					
α goat IgG	Donkey	Alexa 488	1:1,000 (IF)	Jackson ImmunoResearch Lab, Inc., ME, USA	705-545-147
α mouse IgG	Donkey	Alexa 594	1:750 (IF)	Jackson ImmunoResearch Lab, Inc., ME, USA	715-585-150

Eclipse Ti-E inverted microscope (Nikon Corporation, Tokyo, Japan) equipped with a XYZ motorized stage (TI-S-ER, Nikon). Tile imaging was done with a CFI Plan Apo lambda 40× (N.A. 0.95), using 1.4 mW of 488 nm and 0.7 mW of 561 nm laser power, pinhole aperture of 38.31 μm, GaAsP detectors and image stitching set at 17% overlapping, all controlled through NIS Elements C software v5.00 (Nikon).

Using the Metamorph image analysis system (v. 7.5.6; Universal Imaging Corporation, Molecular Devices, Downingtown, PA, USA), we removed out of focus blur by means of deconvolution functions. We then identified the specific labels by selecting puncta optical density (OD) that surpassed the negative staining background level (i.e., puncta OD > background mean + 2 SD). We assessed the number of overlapping pixels for each marker in double-labeled varicosities in the complete ganglion section and their regions (rostral and caudal). The area occupied by VAChT and GAD67 was expressed as a percentage of the whole section area. The co-occurrence of the two labels was expressed as the ratio of the percentage of fibers co-expressing the two labels relative to the percentage of fibers expressing only one of them. For the SPN cells, we counted the number of all immnunostained cell bodies and determined the percent of GAD67 immunoreactive cells that co-stain for ChAT.

Blood Pressure Measurement

Systolic blood pressure (SBP) was measured prior to experiments with an indirect tail-cuff apparatus. Adult SHR had SBP over 160 mm Hg, while control WKy rats and young 6-wo SHR had SBP <120 mm Hg.

Statistics

Results obtained from independent experiments were expressed as mean ± SEM. The differences between pairs of groups of different experimental conditions were evaluated with an unpaired Student's *t*-test. For analyzing segregation and expression of neurotransmitters along the ganglionic regions, we employed one-way ANOVA using Tukey's multiple comparison test. To evaluate if age is an important factor that changes the expression and distribution of GAD67 and VAChT in hypertension, we performed two-way ANOVA followed by Bonferroni post-tests. *P*-values < 0.05 were considered statistically significant for the quantification of immunofluorescence density and segregation values.

RESULTS

Stress Model

Cold-Stress Enhanced Segregation of ACh-GABA in SCG

To assess the presence of stress and consequently of sympathetic hyperactivity in cold exposed rats, we determined body weight and the expression of TH in the AM. Rats exposed to cold despite they consumed the same amount of food as control rats, lost 5%–10% of their weight (10.0 ± 2.3 g) in contrast to control rats that gained 22.0 ± 3.3 g ($P < 0.05$) over the same period. In agreement with previous reports (Ulus and Wurtman, 1979; Kvetnansky and Sabban, 1993; Liu et al., 2005), we found that cold stress elevated significantly both TH mRNA and TH content in rat AM; TH mRNA increased 17.6% and TH protein 1.6-fold ($P < 0.05$; **Figure 1**).

To study segregation of neurotransmitters in sympathetic ganglia, we explored expression and co-occurrence of VAChT and GAD67 in ganglionic varicosities originating from the

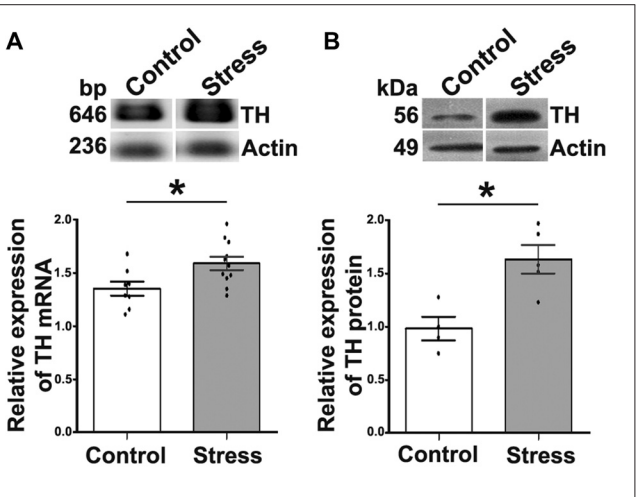

FIGURE 1 | Cold-stress increased expression of tyrosine hydroxylase (TH) mRNA and protein in adrenal medulla (AM). **(A)** Conventional RT-PCR analysis performed on total RNA obtained from AM showing an increase (17.5%) in TH mRNA after 5 days of cold-stress. RT-PCR assay was performed for the amplification of TH mRNA fragment of 646 bp. **(B)** Western blot (WB) analysis of TH protein (56 kDa) expression showing an increase (1.6-fold) after cold-stress. Graphs show quantification of TH relative to actin, mean ± SEM and the significance level. Actin was used as the housekeeping gene for RT-PCR and as the loading control for WB assays. *$P < 0.05$.

cell bodies of SPN that, in turn, were labeled for ChAT and GAD67. Although both isoforms of GAD, GAD65 and GAD67, contribute to GABA synthesis, we chose GAD67 because GAD65 has been found in mouse SCG (Ito et al., 2005), while in rat SCG, GAD67 has been used (Ito et al., 2007; Elinos et al., 2016). In fact, we tried antibodies directed to each isoforms and we got better labeling to GAD67. The other and more important reason to choose GAD67 was for its major role in regulation of synaptic plasticity (Lau and Murthy, 2012). We retrograde-labeled SPN cell bodies with FG, we found 15–20 cells per animal, all were ChAT positive and some of them (4–5) were positive to GAD67, these GAD67 immunostained cells were always positive to ChAT (**Figures 2A,B**). In the SCG of control rats approximately half of GAD67-containing varicosities lack VAChT, and there is a caudo-rostral gradient of segregation of VAChT-GAD67 (Elinos et al., 2016). We found that in the SCG of cold stressed rats segregation of GAD67-VAChT increased from 50.1 ± 3.6% (control) to 67.8 ± 4.4% ($P < 0.05$; **Figure 3**), and also found that the caudo-rostral gradient of segregation of VAChT-GAD67 was not present (rostral 63.7 ± 5.7%, caudal 72.4 ± 2.0%; $P > 0.05$; **Figure 3B**). Change in segregation induced by stress is mainly due to a considerable and significant increase of segregation in the rostral area, which overcomes any regional intraganglionic differences.

To confirm that the immunolabeled varicosities studied have a preganglionic origin, we denervated the SCG of four rats by transecting the preganglionic sympathetic trunk 3–5 mm caudal to the ganglia. After 7 days of denervation, we found a complete lack of GAD67- and VAChT-containing varicosities confirming their preganglionic origin (**Supplementary Figure S1**).

Expression of GAD67 and VAChT Did Not Change in the SCG of Cold Stressed Rats

To explain the changes in segregation in cold stressed rats, we quantified GAD67 and VAChT, and found that content of both markers was not affected by cold stress. However, neurotransmitter segregation increased (see above) suggesting that they were redistributed. The density of GAD67 was 0.053 ± 0.003% in controls vs. 0.064 ± 0.005% in stressed animals ($P > 0.05$; **Figures 4A,B**), whereas VAChT density in controls was 1.09 ± 0.05%, which was not different to animals subjected to stress (1.23 ± 0.03%, $P > 0.05$; **Figures 4A,B**). Unlike controls, ganglion of rats subjected to cold stress did not show a rostro-caudal gradient of GAD67 expression (**Figures 4A,B**; see Elinos et al., 2016), instead they showed uniform expression of the enzyme throughout the ganglion (0.07 ± 0.01% in rostral and 0.06 ± 0.01% in caudal; $P > 0.05$; **Figures 4A,B**). VAChT-IR showed regional differences in controls but not in animals subjected to stress.

Hypertension Model

Segregation of ACh-GABA Was Similar in SHR and WKy

We confirmed the presence of sympathetic hyperactivity in SHR by assessing the basal sympathetic ganglionic transmission. We measured compound action potentials (CAP) in the postganglionic nerve evoked by supramaximal stimulation applied in the preganglionic nerve. We found that CAP amplitude in ganglia from SHR was higher than from WKy rats, (Vmax was 2.7 ± 0.2 mV in SHR vs. 1.7 ± 0.1 mV in WKy; $P < 0.001$).

Like in control rats, GAD67 and ChAT co-occurred in preganglionic cell bodies of SHR animals at the two age groups tested, i.e., 12- and 6-wo animals (**Figures 2C,D**). We found a clear segregation of VAChT and GAD67 in the preganglionic varicosities within the SCG in SHR and normotensive WKy

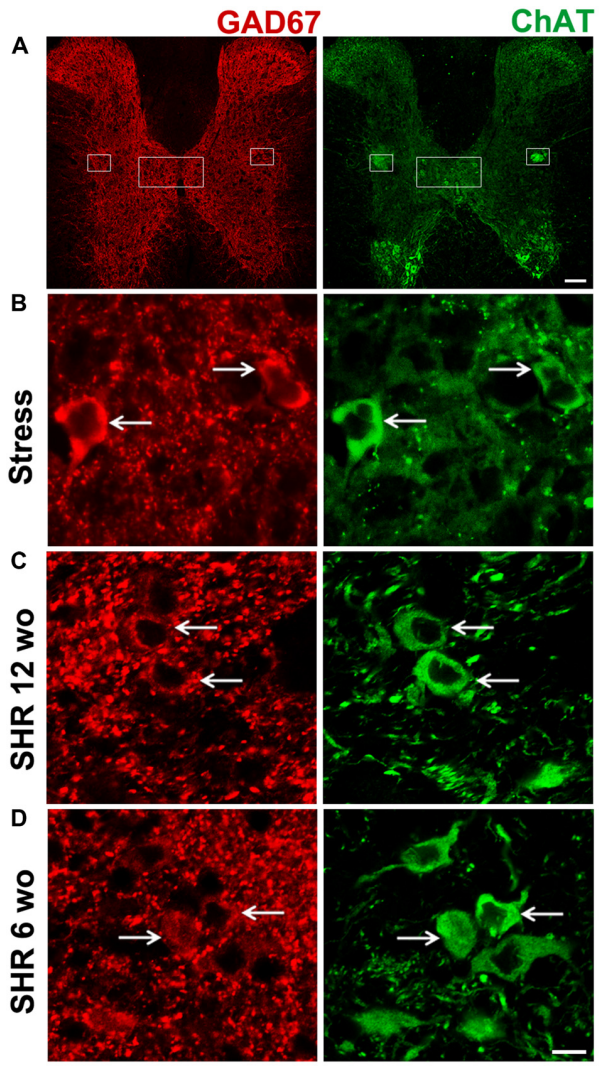

FIGURE 2 | All sympathetic preganglionic neuronal cell bodies positive for L-glutamic acid decarboxylase of 67 kDa (GAD67), co-expressed ChAT. Double immunostaining in sections of spinal cord for GAD67 (red) and ChAT (green). **(A)** Low magnification of transverse section of spinal cord depicting the whole gray region, the sympathetic nuclei are shown with boxes. **(B)** Micrograph of transverse section of spinal cord in cold-stressed rats ($n = 4$). **(C)** Micrograph of transverse section of spinal cord in spontaneously hypertensive rats (SHR) adult 12-week old (wo) rats ($n = 4$). **(D)** Micrograph of longitudinal section of spinal cord in SHR young 6-wo rats ($n = 3$). GAD67-IR cell bodies co-expressing ChAT are shown with arrows. Scale bar = 100 μm **(A)**, 10 μm **(B–D)**.

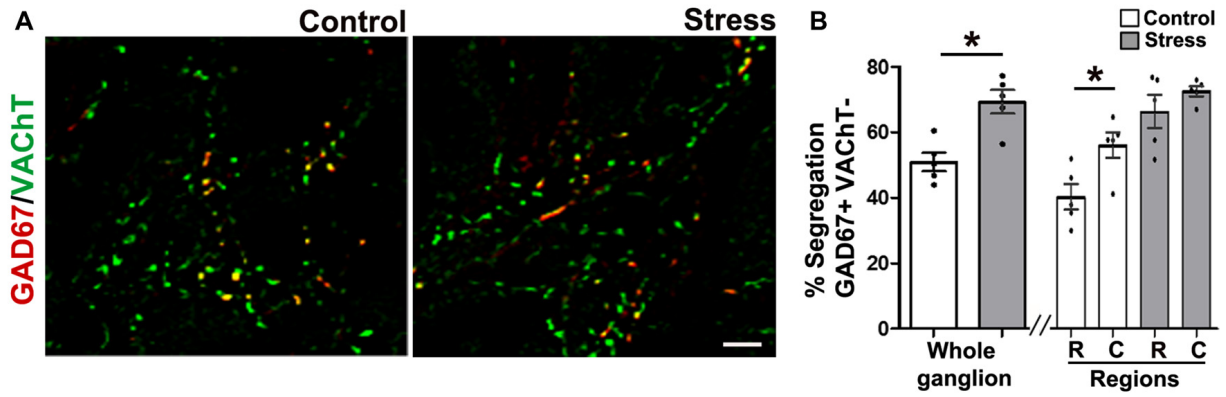

FIGURE 3 | Cold-stress increased segregation of acetylcholine (ACh) and GAD67. **(A)** Merged images showing the immunolabeling of GAD67 (red), vesicular ACh transporter (VAChT; green) and co-occurrence of both labels (yellow) in superior cervical ganglion (SCG) from control and cold-stressed rats. **(B)** Graphs showing percent segregation of VAChT/GAD67 in SCG of stressed rats with respect to control. Stress increased the percentage of segregation in the whole ganglion; 67.8 ± 4.4% compared to 50.1 ± 3.6% in control rats ($P < 0.05$). The caudo-rostral ganglionic regionalization of VAChT/GAD67 segregation found in control animals (rostral 37.1 ± 3.4%, caudal 55.4 ± 5.1%; $P < 0.05$) was no longer present in stressed rats (rostral 63.7 ± 5.7%, caudal 72.4 ± 2.0%; $P > 0.05$). Scale bar = 10 μm; $n = 5$. *$P < 0.05$.

rats. The level of segregation of VAChT-GAD67 did not vary between SHR and WKy rats regardless of the age analyzed. Thus, in adult 12-wo SHR, we found that 43.5 ± 5.4% of GAD67-containing preganglionic varicosities lacked VAChT, whereas in WKy rats the level of segregation was 40.8 ± 3.1% ($P > 0.05$; **Figures 5A,B**). In young animals, we detected same levels of segregation, 68.3 ± 1.1% in SHR and 68.9 ± 1.4% in Wky ($P > 0.05$; **Figures 5A,B**). Contrary to our findings in control animals, we did not find a caudo-rostral gradient of segregation for VAChT-GAD67 in SHR or WKy rats (**Figure 5B**).

Denervation of ganglia, removed all VAChT and GAD67-containing varicosities, indicating a preganglionic origin (**Supplementary Figure S1**).

GABA Expression Was Augmented in SCG of Adult and Young SHR. ACh Increased Only in Young SHR Rats

We found that GAD67 increased in the SCG of adult and young SHR. In adult rats, we detected 4–5 times more GAD67-containing varicosities in SHR than in WKy, thus, GAD67 density was 0.13 ± 0.03% in SHR vs. 0.03 ± 0.01% in WKy ($P < 0.01$; **Figures 6A,B**). In the same way, in young animals, GAD67 was detected in 0.40 ± 0.01% of varicosities in SHR and in 0.150 ± 0.004% of WKy rats ($P < 0.001$; **Figures 6A,B**). Regarding regionalization of GAD67 expression, it is worth emphasizing that while adult SHR GAD67 showed a rostro-caudal gradient (0.10 ± 0.03%, rostral vs. 0.03 ± 0.01% caudal; $P < 0.05$; **Figure 6B**), adult WKy showed no gradient (0.02 ± 0.01%, rostral and 0.010 ± 0.003% caudal; $P > 0.05$; **Figure 6B**). In turn, in young SHR and WKy rats this rostro-caudal gradient was not present. VAChT expression was similar in adult SHR and WKy rats, and showed a rostro-caudal distribution in both normotensive and hypertensive conditions (0.70 ± 0.02%, rostral and 0.30 ± 0.02% caudal in SHR; $P < 0.001$; and 0.60 ± 0.01%; rostral and 0.40 ± 0.01% caudal in WKy; $P < 0.001$; **Figure 6D**). In young animals, in contrast to adult rats, expression of VAChT was higher in SHR (1.14 ± 0.06% compared with WKy rats 0.75 ± 0.05%; $P < 0.01$; **Figures 6C,D**). The rostro-caudal distribution of VAChT-IR was absent in young animals (**Figure 6D**).

Segregation of VAChT and GABA Was Greater in Young Animals

It is noteworthy that, although, segregation did not vary between SHR and WKy rats, as stated above, it varied depending on the age. In SCG from young 6-wo SHR the percentage of GAD67-containing varicosities lacked VAChT, 68.3 ± 1.1% was significantly greater than in 12-wo adult SHR animals, 43.5 ± 5.4% ($P < 0.001$; **Figures 5A,B**).

DISCUSSION

Data presented here show that under both sympathetic overfunction conditions, cold stress and hypertension, the expression and segregation of neurotransmitters in the rat SCG varied differentially depending on the type of overfunction. In cold stressed rats, segregation of ACh and GABA increased without changes in their expression levels. The opposite was found in hypertensive animals, sympathetic overactivity coursed with a higher presence of GABA, but without any changes in the segregation level of ACh and GABA. Additionally, we unexpectedly found that young animals (6-wo) showed a higher level of ACh-GABA segregation than adult ones (12-wo).

We previously demonstrated that in sympathetic ganglia, both *in vitro* and *in vivo*, segregation of neurotransmitters is a plastic phenomenon, which is modulated by neurotrophic factors and neurotrophins. Thus, addition of ciliary neurotrophic factor (CNTF) increases the degree of segregation of NE and NPY

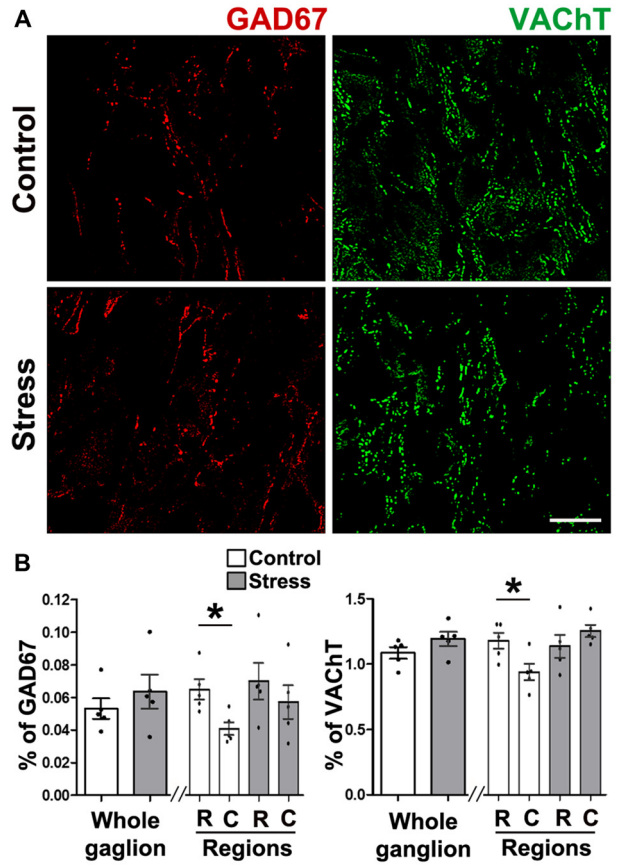

FIGURE 4 | Expression of GAD67 and VAChT in SCG was not modified by cold-stress. **(A)** GAD67 showed similar presence in control and stressed rats (0.053 ± 0.003% in control and 0.064 ± 0.005% in stress; $P > 0.05$). GAD67 showed a rostro-caudal gradient in control rats (0.07 ± 0.01% rostral vs. 0.04 ± 0.01% caudal; $P < 0.05$) that was not present in stressed rats (0.07 ± 0.01% rostral vs. 0.06 ± 0.01% caudal; $P > 0.05$). **(B)** VAChT in SCG from control and stressed rats showed no differences in whole ganglia (1.09 ± 0.05% in control and 1.23 ± 0.03% in stress; $P > 0.05$), or in ganglionic regions in control rats (1.20 ± 0.07% rostral vs. 0.93 ± 0.08% caudal; $P < 0.05$) and in stress (1.20 ± 0.07% rostral vs. 1.28 ± 0.05% caudal; $P > 0.05$). Scale bar = 25 μm; $n = 5$. *$P < 0.05$.

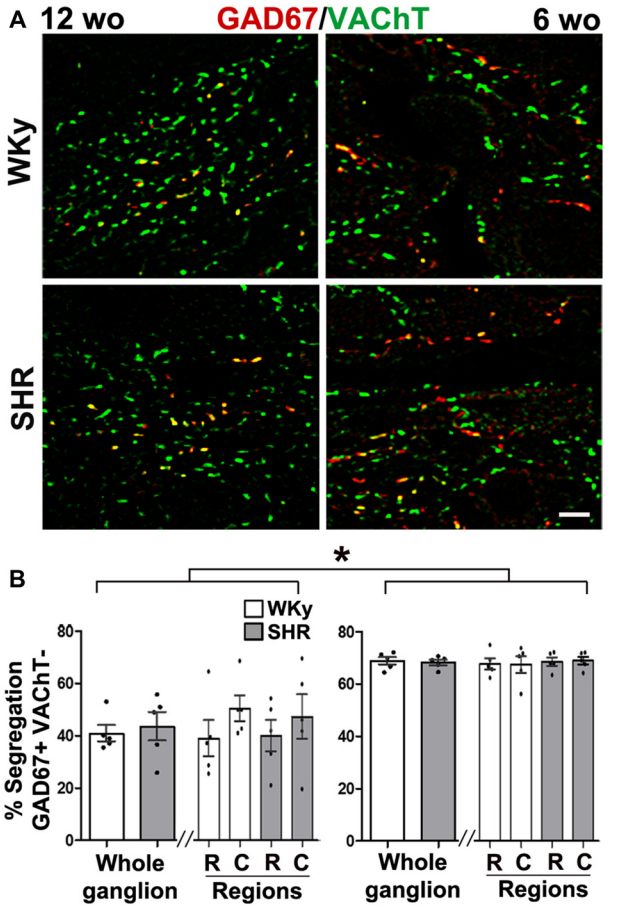

FIGURE 5 | Hypertension did not modify segregation of GAD67/VAChT in SCG from SHR of adult or young rats. However, segregation was larger in young than in adult SHR rats. **(A)** Merged images showing the co-occurrence (yellow) of two labels: GAD67 (red) and VAChT (green) in 12- and 6- wo SHR and WKy rats. **(B)** Regional distribution of the level of segregation of GAD67/VAChT in 12- and 6-wo SHR and Wistar Kyoto (WKy) rats. Percent of ACh and GABA segregation in the whole ganglion was larger in young than in adult SHR and WKy rats (68.3 ± 1.1% at 6 wo and 43.5 ± 5.4% at 12 wo; $P < 0.001$) for SHR and (68.9 ± 1.4% at 6 wo and 40.8 ± 3.1% at 12 wo; $P < 0.001$) for WKy. This larger segregation in young than in adult rats was also found at intraganglionic regional level, in SHR rostral (68.6 ± 1.6% at 6 wo and 40.0 ± 6.0% at 12 wo; $P < 0.001$) caudal (69.0 ± 1.5% at 6 wo and 47.2 ± 8.5% at 12 wo; $P < 0.01$); while in WKy rostral (67.8 ± 2.1% at 6 wo and 40.0 ± 6.9% at 12 wo; $P < 0.001$) caudal (67.6 ± 3.2% at 6 wo and 50.3 ± 4.9% at 12 wo; $P < 0.001$). Scale bar = 10 μm; $n = 5$ for all groups. *$P < 0.01$.

in varicosities of sympathetic ganglionic neurons co-cultured with cardiomyocytes (Vega et al., 2010). Furthermore, the neurotrophin, nerve growth factor (NGF) changes the segregation of ACh and methionine-enkephalin (mENK) in preganglionic sympathetic neuronal varicosities *in vivo* (Vega et al., 2016). Considering these evidences, we explored whether plastic changes in the segregation of neurotransmitters can also occur in physio-pathologic conditions, like stress and hypertension, conditions that course with augmented sympathetic activity.

It is known that stress involves the activation of central sympathetic nuclei and peripheral structures. Generally, the grade of contribution of sympathetic ganglia in stress is assessed by the level of catecholamines released into ganglionic neuronal targets (Kvetnansky et al., 2013). However, since the entire sympathetic axis is involved in stress it is expected that other sympathetic structures, like preganglionic neurons, may be compromised as well. In fact, it has been proposed that SPN increase ACh release during stress (Ulus and Wurtman, 1979). Our study focused on characterizing the cellular distribution of two SPN neurotransmitters, ACh and GABA, in the SCG of cold stressed rats. We found that there was no change in presynaptic ACh or GABA content, but a likely redistribution of them, which increased the segregation of ACh from GABA-containing varicosities. This finding suggests that sympathetic hyperactivity occurring in stress is directly correlated with the level of

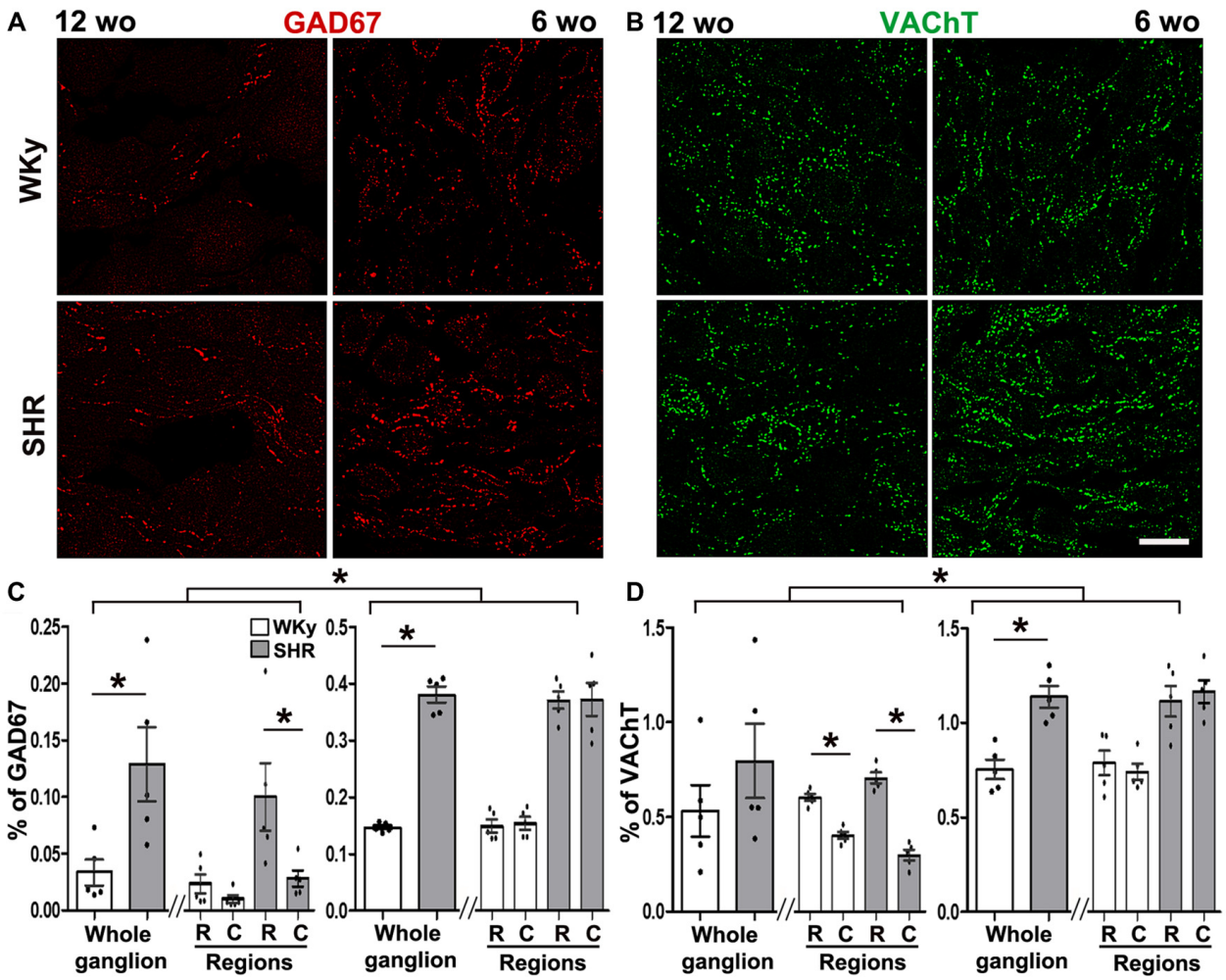

FIGURE 6 | Hypertension increased GAD67 expression in 12- and 6-wo rats and VAChT expression in 6-wo animals. **(A)** Immunostaining of SCG for GAD67 at 12- and 6-wo SHR and WKy rats. **(B)** GAD67-containing varicose fibers increased in adult and young SHR rats in the whole ganglion (0.12 ± 0.03% in SHR and 0.03 ± 0.01% in WKy at 12 wo; $P < 0.01$; in young animals: 0.37 ± 0.01% in SHR and 0.140 ± 0.003% in WKy; $P < 0.001$). At 12 wo in SHR rostral expression of GAD67 was larger than in caudal region (rostral 0.10 ± 0.02%; caudal 0. 020 ± 0.007%; $P < 0.05$). **(C)** Immunostaining of SCG for VAChT in 12 and 6 wo in SHR and WKy rats. **(D)** Although there were no changes in VAChT expression between SHR and WKY at 12 wo (0.79 ± 0.19%, in SHR; 0.52 ± 0.13%, in WKy, $P > 0.05$), VAChT expression was greater in SHR at 6 wo (1.13 ± 0.05% in SHR and 0.75 ± 0.05%, in WKy; $P < 0.01$). At 12 wo, there was a rostro-caudal gradient in VAChT expression in both strands SHR (rostral 0.70 ± 0.02%; caudal 0.29 ± 0.02%; $P < 0.001$); WKy (rostral 0.60 ± 0.01%; caudal 0.39 ± 0.01%; $P < 0.001$). At 6 wo, there was no regional distribution in VAChT expression SHR (1.11 ± 0.08% rostral and 1.16 ± 0.06% caudal, $P > 0.05$); WKy (0.78 ± 0.06% rostral and 0.74 ± 0.04% caudal, $P > 0.05$). Scale bar = 25 μm; $n = 5$ for all groups. *$P < 0.05$.

segregation. Thus, we postulate that an increase in segregation of ACh and GABA may lead to a stronger transmission in sympathetic ganglia, resulting from less GABA inhibitory modulation. In line with this hypothesis in a recent work where we explored possible intraganglionic regional differences in the level of ACh and GABA segregation, we found larger segregation levels in the caudal region of SCG, which correlates with more potent sympathetic transmission (Elinos et al., 2016). As we suggested in that work, it seems that inhibitory modulation of GABA on cholinergic transmission is less effective when GABA is released alone from separate boutons than when both neurotransmitters are co-localized and co-released from the same bouton. In other systems some functional implications of segregation have been reported, for example in microcultures of single dopaminergic neurons, dopamine and glutamate are segregated in two types of varicosities: a dopaminergic varicosity, which is involved in volume transmission, and a glutamatergic one that mediates rapid excitatory synaptic transmission (Sulzer et al., 1998). In the retina, it has been proposed that ACh and GABA, segregated and released from different presynaptic endings of single processes of starburst amacrine cells, are used to process independently different visual tasks (Lee et al., 2010).

Considering the differential level of ACh and GABA expression, and the segregation detected in the rostral and caudal regions of the SCG of control rats, we explored whether effects

of cold-stress on expression and segregation of ACh and GABA are regionally distributed. We found that cold-stress increased segregation preferentially in the rostral region. This increased segregation would reduce GABA modulatory inhibition. This results in strengthening of ganglionic transmission, which may contribute to the stress-induced sympathetic overfunction.

Regarding hypertension, we found that the segregation of neurotransmitters in sympathetic ganglia of SHR was not modified indicating that ganglionic segregation is not affected by the sympathetic overfunction underlying hypertension. Instead, we detected a significant increase in GABA presence. We also detected stronger synaptic transmission in SHR which can explain sympathetic overfunction in this pathological condition. Therefore, the enhancement of GABA expression would seem contradictory to this assumption, as one would expect a reduction in the presence and function of GABA-inhibition. A plausible explanation for this apparent contradiction is that GABA increased in an attempt to counteract the already established enhancement of sympathetic transmission coursing with hypertension.

According to the contradictory findings in segregation and expression levels of ACh and GABA detected in the two conditions of sympathetic overactivity, stress and hypertension, and considering that cold-stress is a short-lasting condition of sympathetic hyperfunction, whereas hypertension represents a long-lasting overactivity, it is possible to speculate that in stress the SNS responds by increasing segregation of ACh and GABA, which results in an enhancement of sympathetic activity. While in hypertension by increasing GABA content the SNS tries to overcome the already established sympathetic overactivity.

In view of our previous report of regional differences in the strength of ganglionic transmission, and the heterogeneous distribution of GABA and GABA-A receptors in the SCG of adult (10–12-wo) Wistar rats (Elinos et al., 2016), we investigated possible differences in expression of GABA and ACh in rostral and caudal SCG regions of SHR. We found a rostro-caudal gradient in GAD67 and VAChT expression in the SCG of adult SHR that contrasted with young 6-wo animals, where this gradient was not present. The regional changes in neurotransmitter presence in ganglia could be related to the different target organs innervated by rostral and caudal neurons.

Notwithstanding that in hypertension segregation of VAChT and GAD67 did not vary, we discovered an age-dependent level of segregation both in SHR and WKy rats, segregation was larger at 6-wo. We also found higher GAD67 and VAChT expression in 6-wo animals, in contrast to adult 12-wo SHR, where only GAD67 increased. In other regions, like the spinal cord, a developmental sequence of GABA excitatory/inhibitory effects has been reported, e.g., in spinal cord this switch occurs during the first postnatal week (Ben-Ari, 2014). In ganglia, we know that at 6-wo, GABA already shows an inhibitory effect (unpublished observation). Data of more segregation in young animals suggest that at early age neurotransmitters involved in co-release are largely located in separate axon terminals, and as the animals get older neurons modify expression and intracellular distribution of their neurotransmitters favoring a greater co-storage of mediators in the same boutons. Finally, the higher level of segregation at early age would favor independent release of GABA, which may exert other functions than synaptic cholinergic inhibition, like modulation of development, as it was suggested elsewhere (Wolff et al., 1987). As the animals get older the developmental functions of GABA are no longer required, therefore the number of GABA-containing varicosities lacking of VAChT would reduce, resulting in less segregation.

According to the data presented here we propose that in the SNS of the rat, the content and intracellular distribution of transmitters might contribute in setting the activity strength of this system. Segregation and expression of neurotransmitters can be differentially modulated in physio-pathological conditions that course with sympathetic hyperactivity, like stress and hypertension. In stress, sympathetic neurons respond by increasing segregation of ACh and GABA, which might result in enhancement of sympathetic activity that characterizes stress. While in hypertension, by increasing GABA content, neurons try to overcome the already established sympathetic overactivity. Moreover, we detected that segregation and expression of transmitters was different between sympathetic neurons of young and adult subjects.

AUTHOR CONTRIBUTIONS

MM and FC conceived and designed the research. CM-J, FM, MZR and JP performed the experiments. CM-J prepared figures. CM-J, MM and FC analyzed and interpreted the results. MM and FC edited and revised the manuscript. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work.

FUNDING

This work and a postdoctoral fellowship to CM-J were supported by Consejo Nacional de Ciencia y Tecnología (CONACYT) Mexico (Grant #128332).

ACKNOWLEDGMENTS

We thank Dr. Miguel Tapia, Microscopy Unit, Instituto de Investigaciones Biomédicas, UNAM, for his assistance in confocal microscopy and to Mr. Pedro Medina for his technical assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fncel.2018.00411/full#supplementary-material

FIGURE S1 | Denervation of SCG removed practically all immunostaining for GAD67 and VAChT. Micrographs of GAD67 **(A)** and VAChT **(B)** immunostaining in intact and denervated SCG.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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published: 29 August 2018
doi: 10.3389/fncir.2018.00070

Direct Glutamatergic Signaling From Midbrain Dopaminergic Neurons Onto Pyramidal Prefrontal Cortex Neurons

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The dopaminergic neurons of the ventral tegmental area (VTA) have been identified with the ability to co-release dopamine and glutamate. This ability was first documented in the nucleus accumbens but showed to be absent in the dorsal striatum. Recently the ability to release glutamate from a subpopulation of the VTA dopaminergic neurons has been shown to control the prefrontal cortex (PFC) excitation through the exclusive innervation of GABAergic fast spiking interneurons. Here, using an optogenetic approach, we expand this view by presenting that the VTA dopaminergic neurons do not only innervate interneurons but also pyramidal PFC neurons. This finding opens the range of possibilities for the VTA dopaminergic neurons to modulate the activity of PFC.

Keywords: co-release, dopamine, VTA, glutamate, prefrontal cortex

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Received: *01 March 2018*
Accepted: *07 August 2018*
Published: *29 August 2018*

Citation:
Pérez-López JL, Contreras-López R, Ramírez-Jarquín JO and Tecuapetla F (2018) Direct Glutamatergic Signaling From Midbrain Dopaminergic Neurons Onto Pyramidal Prefrontal Cortex Neurons. Front. Neural Circuits 12:70. doi: 10.3389/fncir.2018.00070

INTRODUCTION

The first data documenting the co-release of dopamine and glutamate by dopaminergic neurons dates back to 1998, when it was shown in cell cultures that dopaminergic neurons from the midbrain had the ability to release glutamate at their axonal terminals (Sulzer et al., 1998; Joyce and Rayport, 2000). Later on, the discussion of whether the co-release of glutamate by ventral tegmental area (VTA) dopaminergic neurons occurred *in vivo* was supported by VTA extracellular stimulation in brain slices and *in vivo* (Chuhma et al., 2004; Lavin et al., 2005), and by the description of VTA dopaminergic neurons containing the vesicular glutamate transporter type 2 (VGluT2; Dal Bo et al., 2008; Mendez et al., 2008). However, due to the nature of extracellular stimulation (it can activate fibers of passage), the selective activation of the VTA dopaminergic axons only came from the optogenetic activation of VTA axons expressing Channelrhodopsin-2 (ChR2; Stuber et al., 2010; Tecuapetla et al., 2010). In these experiments photo-activation of VTA dopaminergic axons in the nucleus accumbens evoked the release of glutamate in adult animals. Soon after, it was demonstrated that the selective deletion of VGluT2 in dopaminergic neurons eliminated the ability of VTA dopaminergic neurons to release glutamate, showing that the VGluT2 participated in the release of glutamate from dopaminergic cells (Fortin et al., 2012; Hnasko et al., 2012). Surprisingly, when similar optogenetic experiments were performed to evaluate whether the dopaminergic neurons also released glutamate in the dorsal striatum, this release was not observed (Stuber et al., 2010). This last finding provided evidence that not all post-synaptic targets of the dopaminergic neurons receive the co-release of dopamine-glutamate. Consequently, current research has focused on investigating whether dopaminergic axons co-release glutamate at their different targets (Kabanova et al., 2015; Ellwood et al., 2017; Mingote et al., 2017). Specific to our research, the first studies documenting

the possibility that the VTA axons may release glutamate on prefrontal cortex (PFC) neurons were done by extracellular electrical stimulation in the VTA while recording PFC neurons (Mercuri et al., 1985; Lavin et al., 2005). Particularly important for our research, Lavin et al. (2005) presented a series of experiments strongly arguing in favor of the VTA providing a glutamatergic signal on PFC neurons, but due to the nature of their extracellular stimulation (it can activate fibers of passage or the VTA glutamatergic neurons; Morales and Root, 2014), no definitive answer was provided as to whether VTA dopaminergic neurons could directly release glutamate on PFC neurons. Later on, new studies searching for the co-release of glutamate by dopaminergic axons in the PFC have shown two opposite results. One study showed that dopaminergic axons do not co-release glutamate in the PFC (Mingote et al., 2015), whereas other works strongly suggested not only co-release of glutamate in PFC (Kabanova et al., 2015; Ellwood et al., 2017), but that these axons specifically innervate GABAergic fast-spiking interneurons (Kabanova et al., 2015) suggesting a specific control of the activity in PFC through the VTA→PFC(interneurons)→PFC(projection-neurons) circuit. However, the wide distribution of the dopaminergic cells from the VTA projecting to the PFC (Morales and Root, 2014) suggested an heterogeneous innervation onto the different PFC cell types. Therefore, with the aim to update these views, we performed whole cell recordings from neurons in the PFC while photo-activating the incoming VTA axons to ask if their release of glutamate was exclusive to control the cortical interneurons or they could also directly release glutamate onto the glutamatergic PFC neurons.

MATERIALS AND METHODS

Animals

All procedures were approved by the Institutional Committee for the Care and Use of Laboratory Animals of the Cell Physiology Institute, National Autonomous University of México (CICUAL N° FTA91-16) and the National Norm for the use of Animals (NOM-062-ZOO-1999). Transgenic animals used in the experiments resulted from the backcrossing of TH-Cre mice (Tyrosine Hydroxylase, FI12 line, kindly donated by Professor Dr. Rui M. Costa, from the Champalimaud Center for the Unknown) into Black C57BL/6J for at least six generations. Male and female TH-Cre mice of 2–3 months of age were obtained from our breeding colony in our institutional animal facility (the TH-Cre line was conserved in heterozygosity) and were housed under a 12 h light/dark cycle (lights on at 6:00 am) with *ad libitum* access to food and water before experiments.

Stereotaxic Virus Injections

To perform surgeries, animals were anesthetized using a mix of oxygen (1 liter/min) and 1% isoflurane (1%–2% for interventional procedures). After anesthesia and aligning of the animal's head on the stereotactic apparatus, using an arm with 10 μm resolution (model 961, Kopf instruments), each animal was unilaterally injected using a glass pipette (>25 μm and <50 μm) with 500 nL of viral stock solution (AAV2.1-Ef1a-DIO-hChR2-(H134R)-eYFP titer 1×10^{12}; Penn vector core, UPENN), using a nanoject-II (Drummond Scientific) programed to deliver 4.6 nanoliters by pressure each 5 s (23.6 nL/s rate) into the VTA. The coordinates for the injection with respect to Bregma were: anteroposterior −3.0 mm, mediolateral ±0.4 mm and dorsoventral −4.4 mm using as reference the Mouse Atlas (Paxinos and Franklin, 2004). The withdrawal of the pipette was done after 15–20 min to allow diffusion of the virus, the skin of the animals was subsequently sutured, and full recovery of each animal was monitored.

Ex vivo Brain Slices and Data Acquisition

After allowing 10–14 days for ChR2 expression, the animals were deeply anesthetized with ketamine (120 mg/kg, i.p.; Anesket) and xylazine (30 mg/kg, i.p.; Bayer) and perfused transcardially with an ice-cold perfusion solution containing (in mM): 60 NaCl, 100 sucrose, 20 D-glucose 2.5 KCl, 5 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 1 $CaCl_2$ (pH 7.3), saturated with 95/5% O_2/CO_2.

Coronal or angled slices (250–300 microns) were obtained at the PFC level (angled slices had a 30–40 degree-angle from the coronal axis as illustrated in **Figure 1A**) using a vibratome (3000 Ted pella), slices were then transferred to a storage chamber with oxygenated artificial cerebrospinal fluid (aCSF) containing (in mM): 125 NaCl, 3 KCl, 1.3 $MgCl_2$, 2.6 $CaCl_2$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 10 glucose, 3 sodium pyruvate (pH 7.3, 310 mOsmol/L) and allowed for 1 h recovery at room temperature. Single slices were transferred to a submerged recording chamber and superfused continuously with oxygenated aCSF (3–5 mL/min). Whole-cell recordings were performed on PFC neurons at room temperature, with micropipettes made from borosilicate glass (Harvard apparatus 30-0057) and fire polished for DC resistances of 6–10 MΩ. Internal solution was (in mM): 10 NaCl, 10 HEPES, 10 EGTA/KOH, 120 $KMeSO_3$, 2 $MgCl_2$, 1 $CaCl_2$, 2.4 Na^+-ATP, 1.2 Na^+-GTP and 3.3 biocytin (pH 7.2, 290 mOsmol/L). Neurons were visualized using an infrared filter with an upright microscope (Scientifica electrophysiology) and a digital camera (Evolution VF FAST mono 12-bit 32-0103B124). Whole cell recordings were acquired through a PC-501A amplifier (Warner Instrument Corp.) and data was digitized through a NIDAQ (CB 68LP, National Instruments) and Im-Patch (open access software designed in LabView).

Optogenetic Activation of VTA ChR2:TH-Cre Axons Into the PFC

Once intracellular access was obtained (holding potential −70 mV in voltage clamp or resting membrane potential in current clamp), bath application of 4-aminopiridine (40 μM, A0152 Sigma-Aldrich) was used to improve activation of ChR2 expressing axons (Petreanu et al., 2009), synaptic responsivity (Flores-Hernández et al., 1994), and the detection of the postsynatic responses (Kabanova et al., 2015). For optical stimulation of VTA:TH-Cre ChR2-eYFP axons in the PFC,

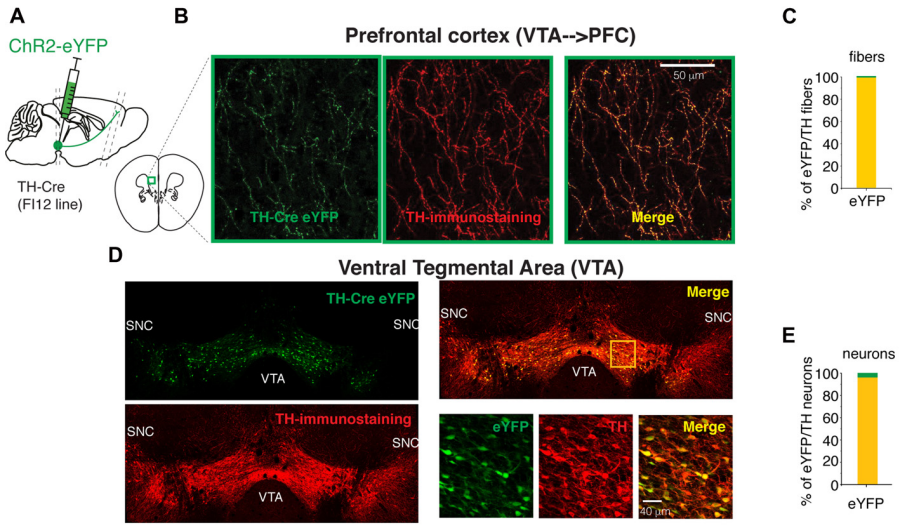

FIGURE 1 | The FI12:TH-Cre mouse line is specific to targeting the midbrain dopaminergic neurons. **(A)** Diagram of a sagittal mouse slice depicting the site of viral injection to express either eYFP or Channelrhodopsin-2 (ChR2)-eYFP. **(B)** Photomicrographs of maximum intensity projection from a Z-stack of TH-Cre eYFP expressing fibers in the prefrontal cortex (PFC; prelimbic, PrL cortex). These projections arise from the cell bodies labeled in the ventral tegmental area (VTA), the z-stack is from 10 photomicrographs taken every 2 microns. **(C)** Specificity to express eYFP in the TH-expressing PFC fibers. **(D)** Photomicrographs of the injected site in the VTA of a mouse FI12:TH-Cre with AAV2.1 DIO-eYFP. The three bottom right panels show the magnification of the yellow square in the upper right panel. **(E)** Specificity to express eYFP in VTA TH Cre/TH+ neurons. For this figure we quantified all cells per slice from the VTA (see "Materials and Methods" section) from three animals.

20 ms pulses of blue light (otherwise specified; 470 nm; 3 mW; CoolLED pE-100) were delivered through the same objective used to visualize the cells (40×). Once a postsynaptic response was detected, traces were recorded every 10 s. The experimental protocol continued by first varying the holding potential to obtain the reversal potential of the postsynaptic response. Then, glutamatergic (CNQX; 10 µM, C239 Sigma-Aldrich) or the GABA-A (SR-95531; 10 µM S106 Sigma-Aldrich) antagonists were added to the perfusate. At least 30 traces were acquired in the control and the antagonist conditions.

VTA and PFC Immunostaining to Identify Cell Bodies and Fibers

The brains from animals injected to express eYFP in the TH-Cre VTA neurons were obtained by first deeply anesthetizing and transcardially perfusing the animals with PBS 0.1 M and 4% paraformaldehyde (PFA). After overnight post-fixation in 4% PFA, brains were washed in PBS five times, and coronal or angled sections were obtained from the VTA or PFC in PBS 1% (50 µm slices) using a vibratome (3000 Ted pella). For immunostaining, the tissue sections were permeabilized with 0.3% Triton X-100 in PBS for 10 min at room temperature. After a blocking step (incubation for 40 min, at room temperature with 10% FBS, 0.3% Triton X-100:PBS), and depending on the experiment, sections were incubated with a primary antibody: 12 h at room temperature when using the polyclonal antibody against TH, 1:500 dilution (AB152, Merck Millipore) or 18–24 h when using the monoclonal antibody against CaMKII, 1:1,000 dilution (EP 1819Y, Abcam). Next, after five washes with PBS, a secondary antibody conjugated with the Cy3 or alexa 594 in the case of the TH primary antibody (711-165-152, Jackson ImmunoResearch) or a secondary antibody coupled with Alexa 647 in the case of CaMKII (711-605-152 Jackson ImmunoResearch) were incubated for 2 h in a 1:1,000 dilution. When necessary, DNA was counterstained with DAPI or Hoechst. Slices containing the PFC and the VTA were mounted and images were acquired with an LSM710 laser-scanning confocal microscope (Carl Zeiss). 20× magnification Z-stacks of two channels, one to detect eYFP and one for TH-Cy3 or TH-alexa 594 (excitation lasers were 488 and 543 for eYFP and Cy3 or alexa 594, emission 507–560 nm and 585–648 nm, respectively; 424 × 424 × 20 microns, 2 microns interslice). One slice every three in the VTA area (covering from −2.8 mm to −4.0 mm anteroposterior from Bregma) was selected for the quantification of eYFP+/TH+ cells. Quantification of eYFP-TH+ fibers in the PFC was done through Z-stacks taken from a randomly generated grid inside the limits of the recording areas (prelimbic, PrL and infralimbic, IL PFC; slice anteroposterior to Bregma 1.54 mm). The Z-stacks were imported to ImageJ, then a maximum projection image was obtained and fibers were quantified if they crossed a randomly positioned grid of 20 microns through more than two horizontal lines (Grider et al., 2006).

Recorded cells were processed for anatomy through a reaction to detect Biocytin (1:100, Streptavidin-Cy3, 434315 Thermo Fisher Scientific) and further processed to detect CaMKII. Once samples were mounted, a Z-stack of the area of interest was acquired, images were imported to ImageJ and estimations were performed.

Statistical Analysis

All data is presented as mean ± SEM, unless otherwise specified in the text. The significance level used was $p < 0.05$. For non-paired comparisons Mann-Whitney U test was performed, for percentage distributions comparison Chi square test were used and for paired samples Wilcoxon *t*-test was used. All statistic analyses were performed using Graph Pad and MATLAB.

Data Availability

All data is available upon contact with the corresponding author.

RESULTS

To elucidate whether the dopaminergic neurons from the VTA release glutamate in the PFC, the specific activation of their axons in PFC was required. To achieve this specific activation of the VTA dopaminergic axons, the mouse line FI12:TH-Cre, that expresses the enzyme Cre recombinase under the promoter for tyrosine hydroxylase was used (Gong et al., 2007; da Silva et al., 2018). **Figure 1** shows photomicrographs of eYFP-expressing cell bodies in the VTA (**Figure 1D**) and their axonal projections to the PFC (**Figures 1A,B**).

The quantification of eYFP+/TH+ fibers and cell bodies in randomly selected Z-stacks of the PFC (two quadrants of 150 microns from a randomly positioned grid on PFC were used to estimate the eYFP-fibers) and all cells transfected in the VTA ($n = 3$ animals) showed the specificity of the FI12:TH-Cre for targeting the cell bodies of VTA dopaminergic neurons (defined as a TH+ cell) was 96.32% (**Figure 1E**). Furthermore, the specificity of dopaminergic fibers in the PFC originating from VTA neurons was 99% (**Figure 1C**). This specificity allowed us to use the FI12:TH-Cre mouse line to express proteins of interest in VTA neurons and their fibers reaching the PFC.

Heterogeneous Postsynaptic Currents Are Evoked in Prefrontal Cortex by the Activation of the VTA-ChR2:TH-Cre Axons

In order to answer whether the selective activation of the VTA TH-Cre axons release glutamate in the PFC, we expressed the light sensitive protein ChR2 (Boyden et al., 2005) into the TH-Cre neurons of the VTA, and 10–14 days later PFC brain slices were obtained. Using these brain slices, we asked whether performing whole cell recording from PFC neurons and light activating the VTA TH-Cre axons expressing ChR2-eYFP (VTA-ChR2:TH-Cre axons) could evoke postsynaptic currents (PSCs), by light activating the VTA-ChR2:TH-Cre axons in the PFC, as previously reported (Kabanova et al., 2015; Ellwood et al., 2017). Following these procedures and in an attempt to improve the activation of ChR2 expressing axons (Petreanu et al., 2009), the synaptic responsivity (Flores-Hernández et al., 1994) and the detection of the postsynatic responses (Kabanova et al., 2015) we added 4-aminopyridine (4-AP) to the extracellular recording solution. In agreement with the literature, 4-AP facilitated the amplitude of PSC recorded in PFC neurons in response to light activation of the VTA-ChR2:TH-Cre axons (the normalized amplitude increased in average to 1.6 times, seven cells, from seven animals; $p < 0.05$ Wilcoxon test; **Figure 2A**, top panel; amplitude in ASCF 19 ± 4 pA vs. 29 ± 8 in 4-AP $p < 0.05$ Wilcoxon test; rise and decay time of 2.7 ± 0.3 and 13 ± 1.9 ms respectively). Similarly, the delivery of 20 vs. 10 or 1 ms pulses of light facilitated the amplitude to 1.3 times (six cells from six different animals, $p < 0.05$ Wilcoxon test, **Figure 2A**, bottom panel). Four of these cells had a single component

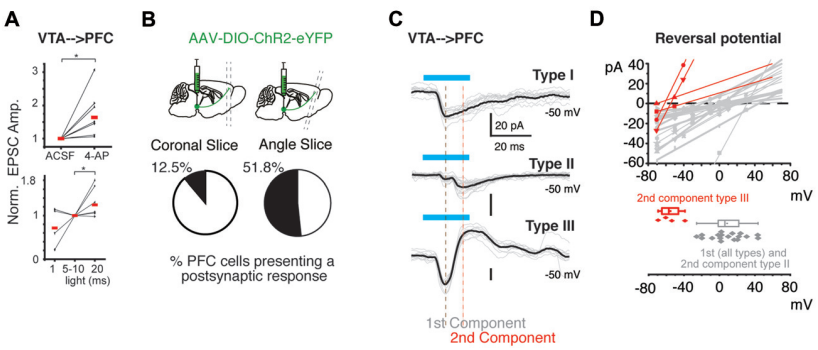

FIGURE 2 | Heterogeneity of postsynaptic currents (PSCs) evoked in PFC by the activation of VTA-ChR2 axons. **(A)** Upper panel, normalized amplitude of PSCs recorded before and in the presence of 4-aminopyridine (4-AP; 40 μM), lower panel, normalized amplitude of PSCs in response to different length of light pulses. **(B)** Representative diagrams of sagittal brain slices depicting the angled cut to obtain PFC brain slices. Bottom pie charts: probability to evoke postsynaptic responses by the photo-activation of VTA:TH-Cre-ChR2-eYFP axons in PFC. **(C)** Two categories of postsynaptic responses were detected: Category A: presents only one component (Type 1: only inward current). Category B: presents two components (Type II: present two inward currents; Type III presents two components one inward current followed by an outward current). Blue lines depict the light stimulus to activate ChR2. The black and red vertical lines depict the points where the first and second component amplitude was measured. For all types of connections, a smaller light pulse (1–10 ms) did not change the number of postsynaptic components but the amplitude was smaller (see "Heterogeneous Postsynaptic Currents Are Evoked in Prefrontal Cortex by the Activation of the VTA-ChR2:TH-Cre Axons" section). **(D)** Upper panel, lines are linear fits of the experimental data (points) per cell varying the holding potential from −70 mV to +20 mV. Bottom panel: distribution of the reversal potential calculated from the data above. *$p < 0.05$ Wilcoxon test.

using 1 or 10 ms pulses of light, and no additional components appeared using 20 ms pulses. Strikingly, it was also observed that the probability to detect postsynaptic responses was dependent on the type of brain slices used. Using brain slices with an angle (see "Materials and Methods" section) yielded a higher probability to detect postsynaptic responses (12.5% PSC detected in coronal slices; 3 out of 24 recorded neurons from 12 slices from 9 animals; vs. 51.8% in angled slices; 41 out of 79 recorded neurons from 35 slices from 29 animals; $p < 0.05$; χ^2 test; **Figure 2B**).

These PSCs were heterogeneous and grouped into two categories: Type I, which have a single component (20 out of 26 cells; 20 slices, 12 animals) and a second category: Type II and III, which have two components (Type II showed a second component that was an inward current at −50 mV; 4 out of 26 cells; four slices from four animals) and Type III showed a second component that was an outward current at −50 mV (4 out of 24 cells; four slices from four animals; **Figure 2C**). Most of these recordings were performed in angled slices (see **Table 1**). By performing a protocol to infer the reversal potential of these PSCs (varying the holding potential from −70 mV to +20 mV), and measuring the peak amplitude for the first and the second postsynaptic components, it was observed that the first postsynaptic component from all recordings has a higher reversal potential than the second component for Type III (mean of the first component 9 ± 4.7 mV vs. −55 ± 6.4 mV for the second component (Type III); $p < 0.05$; Mann Whitney U test; **Figure 2D**; reversal potential for Type II was 0 mV). These measurements of the first component and the second component of Type III resembles the theoretical reversal potential for PSCs mediated by glutamate and GABA, respectively.

The Postsynaptic Responses Evoked in the Prefrontal Cortex by the Photo-Activation of VTA-ChR2:TH-Cre Axons Are Mediated by Glutamate

To evaluate if the postsynaptic responses recorded on PFC neurons by the photo-activation of the VTA-ChR2:TH-Cre axons was mediated by glutamate, in nine of the postsynaptic responses detected, the AMPA glutamate receptor antagonist (CNQX 10 μM; nine slices from nine animals) was added to the bath perfusion. CNQX decreased the mean amplitude of the postsynaptic response to 9% of its original amplitude (control amplitude 52 ± 19 pA vs. 5 ± 0.7 pA in the presence of CNQX; $p < 0.05$; Mann Whitney U test; partial wash 20 ± 7 pA, **Figure 3A** and fourth column in **Figure 3B**).

TABLE 1 | Postsynaptic currents (PSCs) evoked by the activation of VTA-ChR2:TH-Cre axons onto prefrontal cortex (PFC) neurons.

	Type	Postsynaptic current (s; −50 mV)	*n* total	*n* per slice type	
				Coronal	Angled
Category one	I	Inward↓	*n* = 20	*n* = 2	*n* = 18
Category two	II	Inward-inward↓↓	*n* = 4	*n* = 1	*n* = 3
	III	Inward-outward↓↑	*n* = 4		*n* = 4

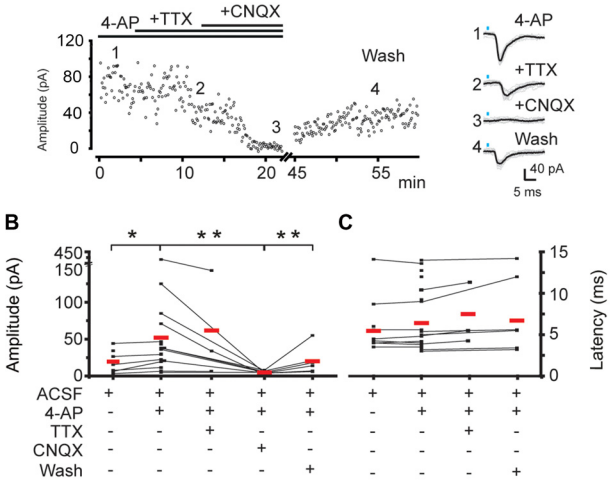

FIGURE 3 | The postsynaptic responses evoked in PFC by the photo-activation of VTA-ChR2:TH-Cre axons are mediated by glutamate. **(A)** Left panel, time course for the peak amplitude of individual traces evoked by the photo-activation of VTA-ChR2:TH-Cre axons in PFC. Right panel, average of at least 20 traces (black traces) corresponding to the numbers depicting the time course. **(B)** Individual mean amplitude of every experiment in the different presented conditions (black points). Lines represent the experiments in which the same cell undergoes different conditions. Red lines are the mean of each condition. **(C)** Latency of the responses for the amplitudes measured in **(B)**. *Wilcoxon paired test $p < 0.05$; **Wilcoxon paired test and Mann Whitney U test $p < 0.05$.

This suggests that the main neurotransmitter responsible for these postsynaptic signals is glutamate. Note that the latency for the mean postsynaptic response was not different before vs. during the partial wash of CNQX (control latency 7 ± 0.9 ms, latency after CNQX wash 7 ± 1.0 ms, $p > 0.05$; Mann Whitney U test, **Figure 3C**). Additionally, in three cells (out of 12, six slices, six animals) we achieved the recordings of postsynaptic responses in the presence of 4-AP+TTX (1 μM) arguing in favor of the VTA→PFC connection as monosynaptic (**Figure 3A** and third column in **Figure 3B**). Interestingly in these three cases the latency to detect the postsynaptic response was increased (see the right panel in **Figure 3A**), a feature of the EPSC evoked by light activation of ChR2 axons (Holloway et al., 2013), Two of these three cells were revealed as pyramidal neurons (further documented in "Photoactivation of VTA ChR2:TH-Cre Axons in the PFC Elicits Postsynaptic Currents Directly on Projection Neurons and Putative Interneurons" section).

In 30% of the Cases Under 4-AP and the Optogenetic Activation of the VTA Axons Onto PFC Neurons, the Blockade of the GABA-A Receptors Enhanced the Postsynaptic Responses

Given that the release of glutamate from a subpopulation of the VTA dopaminergic neurons in the PFC has been shown to exclusively impinge on PFC interneurons (Kabanova

et al., 2015), and IPSCs mediated by VTA-induced feedforward activation of local PFC interneurons has been suggested (Lavin et al., 2005), we asked whether the blockage of GABA-A receptors could affect the postsynaptic responses detected. For this purpose, we bath applied the specific GABA-A antagonist, SR-95531, and evaluated the changes in the postsynaptic signals detected (**Figure 4**). Consistent with the idea that the first component of the postsynaptic response in PFC is mediated mainly by glutamate, the application of a GABA-A antagonist did not modulate this first component (control area under the curve = 309 ± 61 pA/ms vs. 361 ± 122 pA/ms; 13 cells from 13 slices from 10 animals; $p > 0.05$; Mann Whitney U test; **Figure 4A**). However, in agreement with the idea that some of the dopaminergic axons of the VTA directly innervate GABAergic interneurons in the PFC, (Lavin et al., 2005; Kabanova et al., 2015), in 30% of the cases (4 out of 13 cells, from 10 animals) where the GABA-A receptors were blocked, the second glutamatergic postsynaptic component increased dramatically. This effect can be seen in the upper three panels of **Figure 4B** (control, +SR-95531, +SR-95531+CNQX) of one cell recorded in voltage clamp (black traces) and in current clamp (orange traces). The panel at the bottom of the same figure shows that in all cases where the blockade of the GABA-A receptors was followed by the subsequent bath application of CNQX, in the presence of SR-95531 (independently of been connections Type II or III), it abolished completely the postsynaptic responses (area under the curve: control = 91 ± 142 pA/ms; SR-95531 = 10,146 ± 5,604 pA/ms; $p < 0.05$; Mann Whitney U; SR-95531 + CNQX 10 ± 10 pA/ms; six out of six cells tested from six animals).

In summary, the blockade of the glutamatergic transmission practically abolished the postsynaptic responses detected, and, in a subset of cells (30%), the blockade of the GABA-A receptors enhanced the PSCs that were eliminated when blocking the glutamatergic transmission (**Figure 4C**).

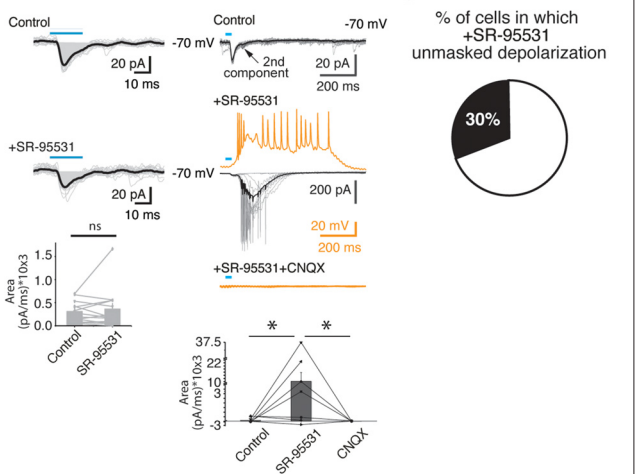

FIGURE 4 | The blockade of the GABA-A receptors enhanced the glutamatergic PSCs evoked by the activation of VTA-ChR2 axons in PFC. **(A)** Upper two panels, representative example of a Type I postsynaptic response in control condition, and in the presence of the antagonist of GABA-A receptors (SR-95531; middle panel). The lower panel shows the area under the curve for a group of cells (13 cells, from 13 slices from 13 animals). **(B)** Representative recordings from a Type III postsynaptic response in voltage (black/gray traces) and current clamp (orange traces) showing that the bath application of the GABA-A antagonist receptors enhanced an inward current (voltage clamp) or a depolarization (current clamp) that later on was blocked by the co-application of the AMPA receptors antagonist (CNQX). Note that when in voltage clamp (control condition) the holding potential is −70 mV, which shows both postsynaptic components as inward currents. The bottom panel shows the summary (area under the curve) for the Type II-III postsynaptic responses that undergo the blockade of the GABA-A and AMPA receptors. **(C)** The proportion of PFC neurons that presented the depolarization after SR-95531 application. *$p < 0.05$ Mann Whitney U test.

Photoactivation of VTA ChR2:TH-Cre Axons in the PFC Elicits Postsynaptic Currents Directly on Projection Neurons and Putative Interneurons

To reveal the identity of PFC neurons that showed a glutamatergic EPSC from VTA ChR2:TH-Cre, recorded neurons were filled with biocytin and subsequently processed for morphological and immunohistochemical analysis (e.g., **Figure 5A**). Sholl analysis was performed on 17 recovered neurons. However, this analysis failed to reveal the identity of the cells (no clear distinction between pyramidal vs. interneurons could be reached; **Figure 5B**), likely due to an incomplete filling of the cells. Therefore, slices were resectioned and immunostained for calcium calmodulin Type II protein (CaMKII; a molecular marker used to differentiate glutamatergic neurons from interneurons in cortex (Pinto and Dan, 2015; **Figure 5D**). Thus, every cell showing positive label for CaMKII+ was designated as a pyramidal glutamatergic neuron and the CaMKII− as a putative interneuron. Following this procedure, the position and proportion of pyramidal neurons vs. putative interneurons was obtained as shown in **Figure 5E**; each dot corresponds to a recorded cell with a postsynaptic light-evoked response, superimposed on the average slice recorded (in red: CaMKII+: glutamatergic pyramidal neurons and in black CaMKII−: putative interneurons). This analysis showed that 12 out of the 19 identified neurons that received the direct glutamate release from the photo-activation of the VTA-ChR2:TH-Cre axons in the PFC were pyramidal neurons (63%). Three pyramidal and one putative interneuron received Type III connections, three pyramidal neurons presented connections Type II, and 19 Type I. Importantly from the five cells where the GABA-A antagonist enhanced the PSCs (subsequently blocked by CNQX; **Figure 4**), three were pyramidal and two putative interneurons. A comparison between neurons recorded by layers or recorded in PrL vs. IL cortex showed no difference in the proportion of CaMKII+ vs. CaMKII− recorded neurons ($p > 0.05$; χ^2 test; **Figures 5F,G**).

In summary, based on our classification of the neurons that received direct release of glutamate in response to photo-activation of the VTA-ChR2:TH-Cre axons in the PFC, a similar proportion of pyramidal (CaMKII+) and putative interneurons (CaMKII−), received glutamate release.

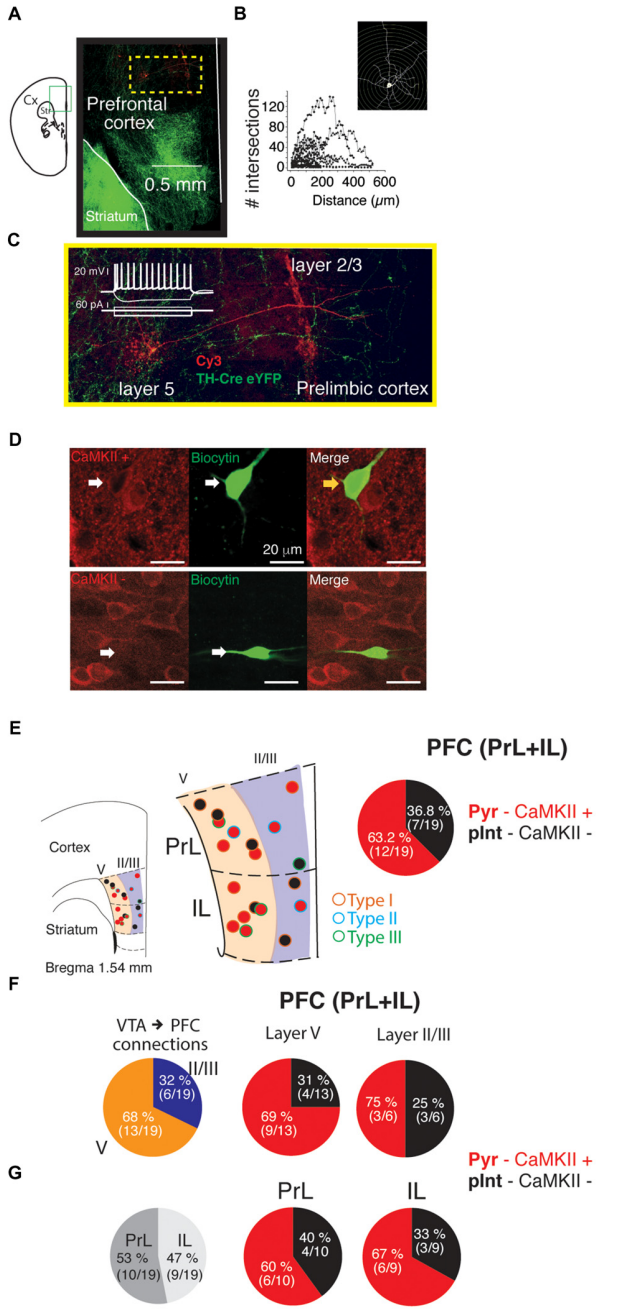

FIGURE 5 | Proportion of calcium calmodulin type II (CaMKII)+ and CaMKII− PFC neurons that received the direct release of glutamate by the photo-activation of VTA-ChR2:TH-Cre axons. **(A,C)** Photomicrograph of a PFC neuron filled with biocytin labeled with Cy3. **(B)** Sholl analysis for 17 neurons recorded and processed as in **(A)**. **(C)** Magnification of the yellow square from **(A)** to better depict the neuron dendrites. Inset is the voltage responses (top) to square pulses of current (bottom). **(D)** Example of a CaMKII+ or CaMKII− neurons. **(E)** Left, diagram representing a section of a coronal brain slice to depict where the cells were recorded (red circles: CaMKII+ pyramidal neurons and black circles: CaMKII− putative interneurons). The panel in the middle represents a magnification of prelimbic (PrL) and infralimbic (IL) cortex representing by colors the layers where the cells were recorded. Right pie chart, proportion of pyramidal

(*Continued*)

FIGURE 5 | Continued
neurons vs. putative interneurons grouped as PFC neurons recorded. **(F)** Proportion of cells recorded by layer (left), and pyramidal neurons vs. putative interneurons recorded by layer (middle and right). **(G)** Proportion of pyramidal and putative-interneurons recorded in PrL or IL cortex.

DISCUSSION

Overall, the data presented here support the idea that the VTA dopaminergic neurons release glutamate on the PFC neurons. Notably, while our results support previous reports of glutamate release by VTA dopaminergic axons in the PFC (Mercuri et al., 1985; Lavin et al., 2005; Kabanova et al., 2015; Ellwood et al., 2017), our study specifically identified that this glutamate release occurs not only onto interneurons but also onto pyramidal neurons.

Why Have Some Groups Documented the Release of Glutamate From VTA Axons in the PFC and Others Have Not?

The first attempts to document the capability of VTA axons to release glutamate on PFC neurons were by electrical (or iontophoretic) activation of the VTA while electrophysiologically recording in the PFC *in vivo*. These studies revealed postsynaptic responses that were blocked by glutamatergic antagonists and in some cases enhanced by GABA-A antagonist (Mercuri et al., 1985; Lavin et al., 2005). However, due to the nature of their VTA activation in those studies (as extracellular stimulation may activate fibers of passage or the VTA glutamatergic neurons that project to PFC; Morales and Root, 2014), no conclusive answer was provided to whether the VTA dopaminergic neurons directly released glutamate on PFC neurons. Latter with the use of transgenic mice, the answer to this question proposed two different views. In the first view, two groups documented glutamate co-release from VTA dopaminergic axons in the PFC using either a transgenic line that targets a specific subpopulation of dopaminergic neurons in the medial VTA (Kabanova et al., 2015) or a TH-Cre line (Ellwood et al., 2017), as in this study. In a second view, a different group using a DAT-IRES-Cre mouse line reported that such glutamate co-release did not exist (Mingote et al., 2015). This discrepancy raises the question of what may be the differences between these transgenic lines? The main difference could be that the TH-Cre and the DAT-Cre mouse lines target overlapping but not identical populations of VTA neurons (Björklund and Dunnett, 2007; Lammel et al., 2015; Stuber et al., 2015). These lines likely target different populations or compartments of the VTA (e.g., the medial vs. the lateral VTA), as these different compartments have been shown to differentially project to PFC (Morales and Root, 2014; Kabanova et al., 2015). Of crucial consideration is that when using transgenic lines to target specific populations of neurons of the midbrain, the specificity of each line may vary. This is especially true for the TH-Cre lines (Lammel et al., 2015), nonetheless it must be noted that the particular mouse line used in this study (FI12) has been documented for its specificity to target midbrain dopaminergic neurons

(Gong et al., 2007; da Silva et al., 2018), and we further validated it in this study. By quantifying the cell bodies in the VTA and PFC axonal fibers originating in the VTA, we estimated a 96% and a 99% of specificity, respectively (**Figure 1**). This high level of specificity allowed us to support the idea that the release of glutamate in PFC evidenced by this study comes from a specific subset of VTA dopaminergic neurons in the FI12:TH-Cre line.

Intriguingly, a second possibility to explain why some groups have not detected the release of glutamate from the VTA dopaminergic neurons in the PFC may come from the different recording conditions in each study. Specifically we improved the reliability to detect the release of glutamate from VTA dopaminergic fibers into the PFC by using 4-AP in the extracellular solution (Petreanu et al., 2009; Kabanova et al., 2015), 20 ms pulses of light and an angled slice (**Figures 2A,B**). Presumably, and according to our data, this protocol improves the activation of the ChR2 expressing axons (Petreanu et al., 2009) the detection of the postsynatic responses (Kabanova et al., 2015) and better preserves incoming VTA axons in the angled slices, as the cell bodies from the VTA do not remain in the brain slices.

Different Types of Postsynaptic Responses Due to the Activation of the VTA Dopaminergic Axons in PFC

Knock out animals with the deletion of the VGluT2 specifically from dopaminergic neurons, which removes their ability to co-release glutamate (Hnasko et al., 2012), show deficits in their motor control, their response to amphetamine or cocaine, and behavioral despair (Birgner et al., 2010; Alsio et al., 2011; Hnasko et al., 2012). Optogenetic manipulations of the dopaminergic VTA→PFC fibers (that release dopamine and glutamate) caused mice to maintain or deviate from previously learned cue–reward associations (Ellwood et al., 2017). The loss of mesocortical dopaminergic neurons that release glutamate in the PFC increases the perseverative behaviors and present alterations in impulsivity and attention (Kabanova et al., 2015). All these studies highlight the control that the VTA→PFC axons exert on PFC functions. However, to fully understand this control, we must first identify the full connectivity of this circuit. In 2015 Kabanova and colleagues (Kabanova et al., 2015) presented the first study of specific functional connectivity from VTA dopaminergic neurons that release glutamate on PFC neurons, pointing out a subpopulation of dopaminergic cells in the medial VTA that release glutamate exclusively on PFC interneurons. This finding raised the possibility that the VTA could exert control on the PFC projection neurons through GABAergic interneurons. Nevertheless, the heterogeneous distribution of the VTA fibers within the PFC (**Figure 1B**), data from the first author of this study during his bachelor thesis (Pérez-Lopéz, 2016), identifying that putative PFC pyramidal neurons receive glutamate release from VTA axons, and the possibility that a bigger population than the one described by Morales and Root (2014) and Kabanova et al. (2015) could innervate the PFC, kept the possibility of a more heterogeneous innervation from the dopaminergic VTA neurons in the PFC. Therefore, when investigating this possibility, we identified that most of the times that we were able to evoke postsynaptic responses in PFC, the neurons recorded were electrophysiologically identified as pyramidal-like neurons (see inset in **Figure 5C** white color). Nonetheless, since neither the sorting of the basic electrophysiology parameters in current clamp (input resistance, resting membrane potential and shape of action potentials; data not shown) nor the anatomical reconstruction (performing a Sholl analysis: **Figure 5B**), yielded a clear identification of the cells as pyramidal neurons vs. interneurons, an analysis of the recorded cells was performed to label for a the molecular marker of pyramidal neurons, CaMKII (Pinto and Dan, 2015; **Figure 5D**). The labeling of CaMKII showed that more than 60% of the recorded neurons were pyramidal neurons (12 out of 19). Additionally in a separated experiment the blockage of the GABA-A receptors, presented a strong depolarization linked to the optogenetic stimulation of VTA-axons in PFC (30% of the cases; **Figure 4C**), suggesting that as previously reported, some of the VTA dopaminergic axons release glutamate onto PFC GABAergic interneurons (Lavin et al., 2005; Kabanova et al., 2015; **Figure 4B**). This data highlights that around 70% of the VTA-TH axons release glutamate directly onto pyramidal PFC neurons. Notably, since our strategy to target VTA neurons did not differentiate between dopaminergic subpopulations (Björklund and Dunnett, 2007), an open question is whether the VTA-TH axons that innervate pyramidal PFC neurons come from a specific subpopulation in the VTA that directly drives the activation of PFC neurons (parallel to the one innervating the PFC interneurons).

When Could the Co-release of Dopamine and Glutamate by VTA Axons in PFC Be Relevant?

Despite the fact that in our study we focused only on the capability of VTA axons in PFC to release glutamate, the ability of these same axons to release dopamine in the PFC has been previously documented (Garris et al., 1993; Hedou et al., 2001; Phillips et al., 2004; Lavin et al., 2005; Ellwood et al., 2017).

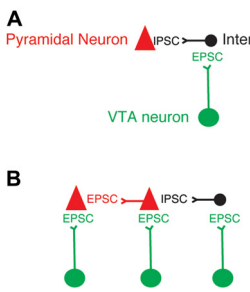

FIGURE 6 | Updated model of VTA axons that release glutamate in PFC neurons. **(A)** Model of the exclusive innervation from VTA dopaminergic axons that release glutamate onto the GABAergic PFC interneurons. **(B)** Updated model based on the conclusion of this study.

Evidence from recording the local field potential in the PFC while stimulating the VTA showed a signal with three components, two fast components that correspond to the fast depolarization evoked by the release of glutamate triggered by VTA activation, and a third component of opposite amplitude that corresponds to an inhibitory postsynaptic response evoked by this same activation (Lavin et al., 2005). Importantly, at the level of EEG recordings, a switch to frequencies linked to the arousal state has been documented during electrically or optogenetically activation of the VTA (Solt et al., 2014; Taylor et al., 2016), this switch may come from the ability of VTA cells to co-release dopamine and glutamate on their different targets. Therefore, to answer the question of when it could be relevant that the VTA axons co-release dopamine and glutamate in the PFC two general ideas could be proposed. The first hypothesis proposes that the rapid release of glutamate from these axons may convey the fast salience of a stimulus, while the slower actions of dopamine may control the activity of PFC neurons on a slower scale (Lavin et al., 2005; Buchta et al., 2017). A second proposal is that the patterns of activation of VTA dopaminergic-glutamatergic axons in the PFC control whether mouse maintains or deviates from previously learned cue reward associations (Ellwood et al., 2017).

GENERAL CONCLUSION

This study showed that the axons from the VTA TH+ neurons that release glutamate on PFC directly innervate PFC pyramidal neurons. This finding expands the range of possibilities that VTA dopaminergic axons may have to modulate the spiking activity and functions of PFC neurons (**Figure 6**). Specific experiments evaluating the contribution of the release of glutamate from VTA axons onto pyramidal neurons vs. interneurons in PFC are necessary to fully visualize the functions of the co-release of glutamate from dopaminergic axons in the PFC.

AUTHOR CONTRIBUTIONS

JLP-L and FT designed and wrote the study. RC-L performed the 4-AP+TTX experiments. JOR-J provided the genotyping and technical support for the development of the study.

FUNDING

This work was supported by the Ciencia Básica Consejo Nacional de Ciencia y Tecnología (CONACyT) grant 220412, Fronteras de la Ciencia, CONACyT grant 2022 and the Dirección General de Asuntos del Personal Académico (DGAPA)-PAPIT-Universidad Nacional Autónoma de México (UNAM) grants IA200815, IN226517 to FT, master fellowship CONACyT 779917 to JLP-L and PhD fellowship CONACyT 332379 to RC-L.

ACKNOWLEDGMENTS

We thank Dr. Rui Costa for the TH-Cre mice, Dra. Elvira Galarraga and Biol. Dagoberto Tapia for electrophysiology *ex vivo* support, Dra. Gabriela J. Martins and MD. Anil Verma Rodríguez for help in editing the manuscript. JLP-L is a master student of Programa de Maestría en Ciencias Bioquímicas, Universidad Nacional Autónoma de México (UNAM) and received the fellowship 779917 from CONACyT. RC-L is a PhD student of Programa de Doctorado en Ciencias Biomédicas, UNAM and received the fellowship 332379 from CONACyT.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neural Circuits

REVIEW
published: 18 December 2018
doi: 10.3389/fncir.2018.00113

Glutamate Cotransmission in Cholinergic, GABAergic and Monoamine Systems: Contrasts and Commonalities

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Multiple discoveries made since the identification of vesicular glutamate transporters (VGLUTs) two decades ago revealed that many neuronal populations in the brain use glutamate in addition to their "primary" neurotransmitter. Such a mode of cotransmission has been detected in dopamine (DA), acetylcholine (ACh), serotonin (5-HT), norepinephrine (NE) and surprisingly even in GABA neurons. Interestingly, work performed by multiple groups during the past decade suggests that the use of glutamate as a cotransmitter takes different forms in these different populations of neurons. In the present review, we will provide an overview of glutamate cotransmission in these different classes of neurons, highlighting puzzling differences in: (1) the proportion of such neurons expressing a VGLUT in different brain regions and at different stages of development; (2) the sub-cellular localization of the VGLUT; (3) the localization of the VGLUT in relation to the neurons' other vesicular transporter; and (4) the functional role of glutamate cotransmission.

Keywords: cotransmission, glutamate, vesicular transporters, synapse, VGLUT

OPEN ACCESS

Edited by:
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Received: *29 August 2018*
Accepted: *03 December 2018*
Published: *18 December 2018*

Citation:
Trudeau L-E and El Mestikawy S (2018) Glutamate Cotransmission in Cholinergic, GABAergic and Monoamine Systems: Contrasts and Commonalities. Front. Neural Circuits 12:113. doi: 10.3389/fncir.2018.00113

INTRODUCTION ON COTRANSMISSION AND THE DISCOVERY OF VGLUTs

Stricto sensu, cotransmission implies that two neurotransmitters are released from the same neuron. The complementary term "corelease" is considered by many to imply simultaneous release of two transmitters from the same vesicles. For corelease to occur, two classic transmitters [glutamate, GABA, dopamine (DA), acetylcholine (ACh), serotonin (5-HT), norepinephrine (NE)] must be stored within the same synaptic vesicle (SV) in the readily releasable pool (RRP). In the absence of actual corelease from the same SVs (**Figure 1A**), cotransmission could involve the synchronous release of two different sets of vesicles containing different neurotransmitters (**Figure 1B**). In this review we will use the term cotransmission in a broad way to refer to neurons that can release more than one classic transmitter (glutamate, GABA, DA, ACh, 5-HT, NE). This release sometimes occurs from the same terminals, but it could also arise from different varicosities established by a given neuron (**Figure 1C**).

Glutamate is the major excitatory transmitter in the brain. To transmit an excitatory signal, neurons must have the capacity to accumulate glutamate inside SVs that will undergo regulated fusion with the synaptic plasma membrane (Takamori et al., 2000, 2001). Proton-dependent

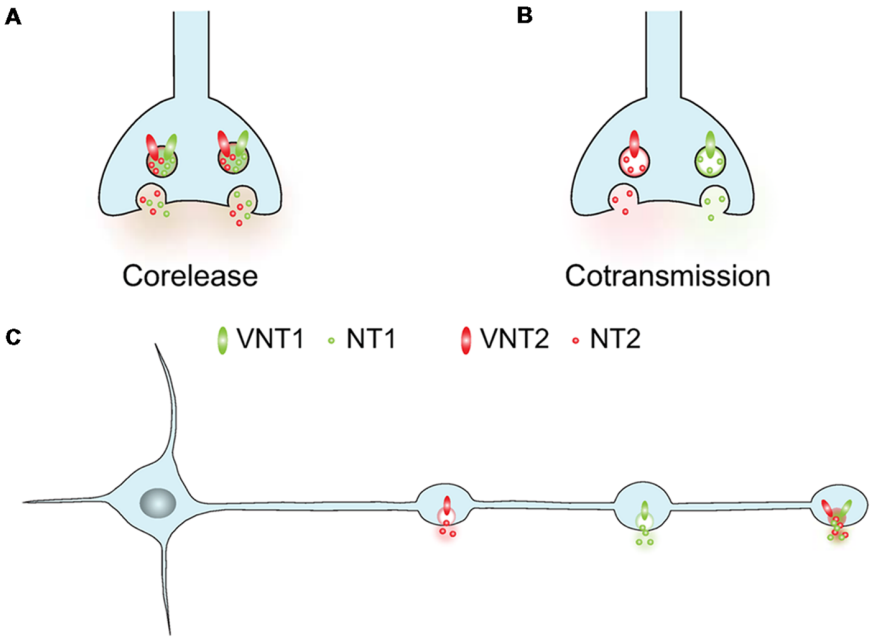

FIGURE 1 | The co-expression of two vesicular neurotransmitter transporters by a single neuron generates multiple signaling possibilities. **(A,B)** Two different vesicular neurotransmitter transporters (VNT1 or VNT2) can either be addressed to the same synaptic vesicles (SVs) or segregated in different SVs. In the first case the two transmitters (NT1 and NT2) will be coreleased simultaneously, in the second one they can be differentially released. **(C)** Within a neuron, two different VNTs can be targeted the same or to different varicosities. With these two options, a neuron can release two different NTs from various varicosities or corelease two NTs from a single varicosity.

solute carriers fulfill this critical accumulation of glutamate in SVs. These transporters belong to the SLC17 family and include VGLUT1 (SLC17A7), VGLUT2 (SLC17A6) and VGLUT3 (SLC17A8; for review, see El Mestikawy et al., 2011; Anne and Gasnier, 2014). VGLUT1-3 are secondary transporters and their activity is driven by a proton gradient established by the vacuolar-type H^+-ATPase (V-ATPase; Edwards, 2007; Omote et al., 2011). VGLUT1 was the first member of the family to be identified and was initially characterized as a putative brain-specific Na^+-dependent transporter of inorganic phosphate (Pi; originally named BNPI; Ni et al., 1994). The H^+-driven transport of glutamate inside SVs by VGLUT1 was demonstrated 6 years later (Bellocchio et al., 2000; Takamori et al., 2000). Interestingly, heterologous expression of "BNPI" in GABA primary neurons was found to be sufficient to confer a glutamatergic phenotype to these inhibitory neurons (Takamori et al., 2000), demonstrating that vesicular glutamate transporters (VGLUTs) are key to the acquisition of a glutamatergic phenotype by neurons. Taken together, these initial studies suggested that VGLUTs were able to transport two different substrates (glutamate and/or Pi) in different cellular locations (plasma membrane or SVs) and in opposite directions. This inversely oriented transport of glutamate and Pi remained controversial for more than two decades. However, a recent publication solved this mystery and established that VGLUTs are indeed dual carriers. Preobraschenski et al. (2014) recently demonstrated elegantly that VGLUT1 co-accumulates glutamate and Pi inside SVs in a proton-dependent manner when it is facing the cytoplasm. However, when facing the extracellular space, VGLUT1 mediates cytoplasmic Pi accumulation in a Na^+-dependent manner (Preobraschenski et al., 2014). In addition to glutamate and Pi, VGLUT1 potentially also transports chloride ions (Naito and Ueda, 1985; Maycox et al., 1988; Takamori, 2016; Preobraschenski et al., 2014). The influx of Pi in the cytoplasm of glutamatergic terminals could help to activate Pi-activated glutaminase and hence to replenish glutamate stores (Masson et al., 2006). However, the rationale for the transport of glutamate, Pi and chloride by VGLUT1 is not fully understood and remains to be formally established for VGLUT2 and VGLUT3.

Similarly to VGLUT1, VGLUT2 was first categorized as a Pi transporter and named DNPI (Aihara et al., 2000), shortly before its ability to accumulate glutamate in SVs was established by several teams (Bai et al., 2001; Fremeau et al., 2001; Herzog et al., 2001; Takamori et al., 2001; Varoqui et al., 2002). VGLUT1 and VGLUT2 are very similar in terms of structure and glutamate transport. However, they differ mainly by their distribution and by the probability of glutamate release they confer to neurons expressing them (Herzog et al., 2001; Fremeau et al., 2004; Herman et al., 2014).

In 2002, VGLUT3 was the last subtype of vesicular glutamate transporter to be identified (Fremeau et al., 2002; Gras et al., 2002; Schäfer et al., 2002; Takamori et al., 2002). This delayed discovery probably reflects the low abundance of VGLUT3 compared to VGLUT1 or VGLUT2. Despite its functional and structural similarity with VGLUT1 and

VGLUT2, VGLUT3 displays some atypical anatomical features. For example, VGLUT3 is found in a small population of glutamatergic neurons present in raphe nuclei, striatum or cortex and in inner hair cells. In addition, VGLUT3 is expressed by subpopulations of neurons releasing 5-HT, GABA (CCK-positive basket cells) or ACh (for review see El Mestikawy et al., 2011). VGLUT3 is also transiently expressed by GABA neurons of the cerebellum and auditory system (Gillespie et al., 2005; Gras et al., 2005; Case and Gillespie, 2011; Case et al., 2014). As will be further discussed below, VGLUT1 and VGLUT2 were also reported in subpopulations of ACh, DA or GABA neurons, although this expression can be dependent of the developmental stages (Dal Bo et al., 2004; Trudeau, 2004; Mendez et al., 2008; Zander et al., 2010; Ren et al., 2011). Electrophysiological and optogenetic methods played a decisive role to establish that in numerous cases, glutamate was indeed released by 5-HT neurons (Johnson, 1994; Varga et al., 2009; Sengupta et al., 2017), DA neurons (Sulzer et al., 1998; Bourque and Trudeau, 2000; Hnasko et al., 2010; Tecuapetla et al., 2010; Chuhma et al., 2014; Trudeau et al., 2014), ACh (Huh et al., 2008; Higley et al., 2011; Nelson et al., 2014; Frahm et al., 2015) and GABA neurons (Shabel et al., 2014; Root et al., 2018).

In summary, VGLUT1-3 are observed in numerous neuronal populations that were initially not considered glutamatergic. An implication of these discoveries is that glutamate cotransmission must regulate numerous brain functions. This issue will be discussed in section "What Are the Functional Implications of Glutamate Cotransmission in These Neuronal Populations?". But to date, optogenetic experiments convincingly established that glutamate cotransmission regulates reward-related behaviors (Birgner et al., 2010; Hnasko et al., 2010; Witten et al., 2010; Adamantidis et al., 2011; Alsiö et al., 2011; Liu et al., 2014) and auditory or respiratory functions (Abbott et al., 2014; Burke et al., 2014). The implication of glutamate cotransmission in other brain functions such as memory, fear or stress is not clearly established (Balázsfi et al., 2018).

CHARACTERIZATION OF VGLUTs IN MONOAMINE, CHOLINERGIC AND GABA NEURONS REVEALS HIGHLY HETEROGENEOUS EXPRESSION IN DIFFERENT NEURONAL POPULATIONS

The identification of VGLUTs in monoamine, cholinergic and GABA neurons raised a lot of interest in the potential roles of glutamate released as an additional neurotransmitter by these neurons. Exploration of the pattern of expression of these transporters in such neurons, mostly in the adult brain, revealed surprises. One of these is that in these different neuronal populations, there are wide variations in the proportion of neurons containing detectable levels of a VGLUT in the adult rodent brain. As an example, in the DA system, while subsets of ventral tegmental area (VTA) DA neurons contain VGLUT2 and release glutamate in the ventral striatum, adult *substantia nigra compacta* (SNc) DA neurons typically contain only very low levels or no vglut2 mRNA and thus do not establish many glutamatergic synapses in the dorsal striatum, as revealed by optogenetic stimulation and patch-clamp electrophysiology (Stuber et al., 2010). The examination of the distribution of vglut2 mRNA in the DA system revealed the existence of higher proportions of DA neurons containing vglut2 mRNA in the rostro-medial regions of the VTA (Yamaguchi et al., 2007; Li et al., 2013). The exact proportion of DA neurons containing vglut2 mRNA was found to be very low in some studies using *in situ* hybridization; approximately 0.1% of TH-positive neurons in the VTA were found to contain vglut2 mRNA in one of these studies performed in rats (Yamaguchi et al., 2007). In a separate study of the SNc, again in rats, these same authors reported similar numbers, with slightly less than 0.1% of SNc TH-positive neurons containing vglut2 mRNA (Yamaguchi et al., 2013). But such low proportions are likely to result from technical limitations as other studies reported much higher proportions in the VTA (30%–50%), both using *in situ* hybridization (Li et al., 2013) and single-cell RT-PCR (Mendez et al., 2008; Fortin et al., 2012; Li et al., 2013). Although much less studied at early developmental stages, the glutamatergic phenotype of DA neurons appears to be the rule rather than the exception early in development; estimates suggest that over 80% of DA neurons in the VTA and SNc expressed the vglut2 gene at some point of their embryonic development (Dal Bo et al., 2008; Steinkellner et al., 2018). Similarly to DA neurons, only a subset of NE/epinephrine neurons appears to display a glutamatergic phenotype and express vglut2. But in this system there is a striking distinction between different subgroups, with the majority (>80%) of C1, C2 and C3 adrenergic neurons and A2 NE neurons expressing vglut2 (Stornetta et al., 2002a,b; DePuy et al., 2013), partial (16%) expression in the A1 group and essentially no expression in the locus coeruleus (LC; Stornetta et al., 2002a). In keeping with these observations, epinephrine and NE neurons establish VGLUT2-positive synapses in target areas including for example the spinal cord and the dorsal motor nucleus of the vagus (Nakamura et al., 2004; DePuy et al., 2013). 5-HT neurons of the raphe nuclei are comparatively less heterogeneous in their expression of VGLUT3. Although initial studies proposed that most 5-HT neurons of the dorsal and medial raphe expressed *vglut3* mRNA (Gras et al., 2002; Herzog et al., 2004), subsequent investigations concluded that in fact only approximately 50% do so (Hioki et al., 2010; Voisin et al., 2016; Sos et al., 2017).

Cholinergic neurons in the brain are also dichotomous in their expression of VGLUT3. On one hand, most if not all cholinergic interneurons of the striatum express VGLUT3 (Gras et al., 2002; Herzog et al., 2004). On the other hand, within the basal forebrain, the expression of VGLUT3 is highly heterogeneous in cholinergic neurons; in this area approximately 30% of cholinergic neurons in the ventral pallidum of rats contain vglut3 mRNA, while virtually no expression is detected in the medial septum and vertical limb of the diagonal band of Broca (Nickerson Poulin et al., 2006). However, using a genetic fate-mapping approach, it was reported that approximately 50% of cholinergic neurons of the horizontal limb of the diagonal

band of Broca in mice have a history of vglut3 expression (Case et al., 2017).

The expression of VGLUTs in GABA neurons also illustrates broad heterogeneity. VGLUT3 is present in GABAergic basket cells of the hippocampus and cortex (Fremeau et al., 2002; Gras et al., 2002; Hioki et al., 2004; Somogyi et al., 2004; Fasano et al., 2017). In the CA1 and CA3 regions, it is only present in approximately 10%–25% of CCK-positive interneurons, with no expression in VIP-positive interneurons (Somogyi et al., 2004; Fasano et al., 2017; Del Pino et al., 2017). Similarly, in the basal amygdala, vglut3 mRNA is found in approximately 25% of CB1/CCK-positive GABAergic neurons (Omiya et al., 2015). In the bed nucleus of the stria terminals, only less than 10% of GABA neurons appear to express vglut3 (Kudo et al., 2012). In a recent study, neurons containing both VGLUT2 and the GABAergic markers glutamic acid decarboxylase (GAD) and vesicular GABA transporter VGAT were detected in the VTA, entopeduncular nucleus (EPN) and supramammillary (SUM) nucleus (Root et al., 2018). In the VTA, a little over 20% of neurons were found to have this phenotype, while in the entopeduncular and lateral supramammillary nuclei, this was approximately 50% and 35%, respectively.

Together, these findings clearly show that VGLUT expression is highly heterogeneous in multiple classes of neurons, suggesting the possibility that cotransmission may allow sub-circuits to be defined within existing projections.

STUDIES OF THE SUBCELLULAR LOCALIZATION OF VGLUTs REVEAL COMPLEX MODES OF COTRANSMISSION

The complexity of glutamate cotransmission is further highlighted in studies that examined the subcellular localization of VGLUTs in neurons using glutamate as a cotransmitter. Indeed, whether VGLUTs in neurons using other neurotransmitters are localized to the same terminals releasing the neuron's other classical transmitter, or even on the same vesicles, places some clear constraints on the mode of cotransmission. In DA neurons, initial studies evaluating the localization of glutamate immunoreactivity in single neuron cultures highlighted the existence of terminals positive for glutamate but negative for the DA biosynthetic enzyme tyrosine hydroxylase (TH; Sulzer et al., 1998). This suggested that release sites for glutamate and DA might be at least partially segregated along axonal segments (**Figure 1**). Subsequently, following the immuno-purification of SVs from rat ventral striatum, evidence was provided suggesting that at least a subset of vesicles containing VMAT2 co-immunoprecipitated with vesicles containing VGLUT2 (Hnasko et al., 2010). This suggested that, at least in juvenile rats, a subset of vesicles may contain both vesicular transporters and hence transmitters. However, subsequent anatomical investigations did not support an extensive colocalization of glutamatergic and dopaminergic markers in axon terminals established by DA neurons in both adult rats and mice. First, immunolocalization of VMAT2 and VGLUT2 at the ultrastructural level in rat nucleus accumbens revealed that although the two proteins could be detected in closely located terminals along a given axon, they are essentially never colocalized (Zhang et al., 2015). A similar conclusion was reached in mouse nucleus accumbens for the localization of VGLUT2 and dopaminergic markers like TH and DAT in two independent studies (Zhang et al., 2015; Fortin et al., 2018). Together these results suggest that if VGLUT2 and VMAT2 can possibly be found on the same vesicles in a subset of vesicles or at early developmental periods, DA and glutamate release sites established along dopaminergic axons appear to be mostly segregated.

A similar segregation of 5-HT terminal markers and VGLUT3 in the axonal projections of raphe neurons in the mouse has also been reported both *in vitro*, in single neuron cultures (Fremeau et al., 2002) and *in vivo* (Schäfer et al., 2002; Nakamura et al., 2004; Voisin et al., 2016). Only a subset of VGLUT3-positive terminals established by 5-HT neurons in regions including the striatum, the lateral septum, the hippocampus and the spinal cord are immunopositive for the 5-HT reuptake site SERT or 5-HT itself. Co-immunostaining for VMAT2 and VGLUT3 also provided evidence for heterogeneous sets of terminals in the cortex and hippocampus, with only a subset expressing both markers (Schäfer et al., 2002; Amilhon et al., 2010). Surprisingly, in contrast to these results, one study performed in the rat suggested that most SERT-positive terminals established by individually-labeled dorsal raphe neurons are also VGLUT3-positive (Gagnon and Parent, 2014). The segregation of VGLUTs in the terminals of DA and 5-HT neurons may possibly find its origin in the differential trafficking of VMAT2 and VGLUTs, as suggested in an *in vitro* study studying the trafficking of VMAT2 and VGLUT1 in hippocampal and dopaminergic neurons (Onoa et al., 2010).

The situation appears to be drastically different in adrenergic neurons as, for example, most terminals of adrenergic C1 neurons in the dorsal motor nucleus of the vagus, visualized after conditional expression of ChR2, appear to be TH-immunoreactive and to also be VGLUT2-immunopositive (DePuy et al., 2013). Intriguingly, adrenergic projections in the paraventricular nucleus of the hypothalamus of both rats and mice appear to be more heterogeneous in their expression of VGLUT2, with only approximately 20% of terminals labeled with the adrenaline biosynthetic enzyme phenylethanolamine N-methyltransferase (PNMT) also positive for VGLUT2 (Johnson et al., 2018). Similarly, only a subset of DA beta-hydroxylase-positive terminals in the intermediolateral cell column of the spinal cord were found to be VGLUT2-postive (Nakamura et al., 2004).

In cholinergic interneurons, VGLUT3 is abundantly expressed in axonal-like varicosities containing the vesicular ACh transporter (VAChT) or choline acetyltransferase (ChAT; Gras et al., 2002, 2008; Sakae et al., 2015; Kljakic et al., 2017). Immuno-isolation of cholinergic vesicles from the rat striatum further suggested that VAChT and VGLUT3 may be together on subsets of SVs (**Figure 1**), a configuration allowing

for a synergistic interaction between the two transmitters, leading to enhanced vesicular packaging of ACh or glutamate, a mechanism named vesicular synergy (Gras et al., 2008; Frahm et al., 2015). But an evaluation of cholinergic terminals emanating from basal forebrain cholinergic neurons revealed that while VGLUT3 and ChAT are highly co-localized in terminals in regions such as the basolateral amygdaloid nucleus, they are typically found on separate populations of terminals in other regions including the olfactory bulb, the reticular thalamic nucleus and the entorhinal cortex (Nickerson Poulin et al., 2006). VGLUT3 is also found in subsets of GABAergic terminals. For example, VGLUT3 is transiently found with the vesicular GABA/glycine transporter (VIAAT or VGAT) in the axon terminals of neurons of the medial nucleus of the trapezoid body in the lateral superior olive (Gillespie et al., 2005). In these projections, it appears that only a subset of terminals labeled with VIAAT are also immuno-positive for VGLUT3 (Gillespie et al., 2005). A subset of GABAergic terminals co-labeled with VGLUT3 has also been reported in the intermediolateral cell column of the spinal cord (Stornetta et al., 2005). In the terminals of hippocampal GABA neurons, ultrastructural and biochemical evidence for the presence of VGLUT3 on vesicles containing VIAAT has been provided (Stensrud et al., 2013, 2015). Indeed, VGLUT3 seems to be expressed by VIAAT-positive SVs and furthermore these SVs are able to accumulate [^{3}H]glutamate (Fasano et al., 2017). Arguing in favor of the fact that such colocalization could lead to corelease of glutamate and GABA from the same vesicles (**Figure 1**), overexpression of VGLUT3 in GABA neurons *in vitro* has been shown to allow for co-occurring GABA- and glutamate-mediated synaptic currents (Zimmermann et al., 2015).

In contrast to these previous results highlighting release of GABA and glutamate from the same terminals, recent work characterizing the co-expression of VGLUT2, VIAAT and glutamic acid decarboxylase (GAD) by neurons of the VTA, EPN and SUM nuclei (Root et al., 2018), provided evidence for segregation of release sites for the two neurotransmitters. These authors reported that GABA/glutamate neurons from the VTA or EPN send projections to the lateral habenula (LHb) where they form asymmetric (excitatory) and symmetric (inhibitory) synapses. However, these "mixed" axons segregate VGLUT2 and VIAAT onto separate SVs and therefore appear to release GABA or glutamate from distinct release sites, further highlighting the complex pattern adopted by various co-transmitting neurons (**Figure 1**).

The presence of VGLUT3 in the somatodendritic compartment of 5-HT, cholinergic and cortical neurons is also particularly intriguing and unique in comparison to VGLUT1 and VGLUT2, that are exclusively present in axon terminals (Fremeau et al., 2002; Gras et al., 2002; Harkany et al., 2004; Herzog et al., 2004; Somogyi et al., 2004; Calizo et al., 2011). It has been considered that VGLUT3-containing vesicles in the dendrites of neurons may be involved in the dendritic release of glutamate and retrograde signaling. This possibility has recently received direct support from experiments showing that stimulation of the dendrites of glycinergic amacrine cells of the retina leads to VGLUT3-dependent glutamate-mediated activation of ganglion cells (Haverkamp and Wässle, 2004; Johnson et al., 2004; Marshak et al., 2015; Tien et al., 2016; Chen et al., 2017).

WHAT ARE THE FUNCTIONAL IMPLICATIONS OF GLUTAMATE COTRANSMISSION IN THESE NEURONAL POPULATIONS?

Despite the widespread existence of glutamate cotransmission in the brain and much recent progress, we still know very little about its molecular and cellular modalities or its functional implications. A question that arises is whether two neurotransmitters within the same synapse influence each other's signaling? The existence of vesicular synergy clearly illustrates how glutamate, perhaps through its negative charge, can influence the quantum size of ACh-, DA-, 5-HT- and GABA-containing SVs and conversely, how ACh can increase the accumulation of glutamate in SVs (Gras et al., 2008; Amilhon et al., 2010; Hnasko et al., 2010; Zander et al., 2010; Frahm et al., 2015; Voisin et al., 2016). Interestingly, in heterologous cells (HEK293T) cotransfected with VGLUT2 and VMAT2, glutamate (through the presence of VGLUT2) produces a robust and stable acidification of SVs (Hnasko et al., 2010). The activity of VMAT2 and VAChT is strongly dependent on the existence of the intralumenal ΔpH. Therefore, the basic mechanism underlying vesicular synergy could be the existence of this increased influx of protons due to VGLUT2 or VGLUT3 activity. The existence of such a mechanism has received partial support from functional experiments carried out in drosophila DA neurons. Aguilar et al. (2017) reported that neuronal depolarization induces a VGLUT-dependent hyper acidification of SVs that causes a small increase in the vesicular uptake of FFN206, a fluorescent VMAT substrate. These data provide some support for the hypothesis that the vesicular quantum of neurotransmitter is modulated at least in part by neuronal activity and that vesicular synergy could help to dynamically increase DA content in SVs to meet the varying demands of neuronal activity. However, for such vesicular synergy to occur, both vesicular transporters need to be present on at least a subset of the same SVs. However, the evidence in favor of the co-expression of VGLUT2 and VMAT2 on the same vesicles is not strong. While immunopurification of SVs from juvenile rat striatum suggested co-purification of vesicles containing both vesicular transporters (Hnasko et al., 2010), others provided evidence for differential localization of both transporters to different axon terminals in the mouse (Zhang et al., 2015; Fortin et al., 2018). In the drosophila brain, it has been estimated that less than 5% of dopaminergic terminals contain the drosophila VGLUT (Aguilar et al., 2017).

The presence of VAChT and VGLUT3 on the same vesicles received support from functional experiments (effect of glutamate on ACh vesicular accumulation i.e., vesicular synergy) and by vesicle immuno-purification experiments (Gras et al.,

2008). However, these are bulk methods that could lead to "false-positive" vesicular colocalization. For this and other suspected cases of vesicular synergy, higher resolution imaging techniques such as Stimulated Emission Depletion (or STED) microscopy (Hell and Wichmann, 1994) or immuno-electron microscopy are needed to provide stronger support for co-localization at the level of single vesicles. STED has recently been used to investigate the distribution of VGLUT3 variants in cholinergic varicosities (Ramet et al., 2017) and could help to examine the presence of VGLUTs and other vesicular transporters on the same SVs.

Recently, in SV immunopurification experiments, VGLUT2 and VGAT were also found to be present in different sets of vesicles in the rat LHb, an observation confirmed by immuno-gold electron microscopy (Root et al., 2018).

Broadly speaking, glutamate corelease may act to amplify postsynaptic activation of target cells, either through activation of ionotropic or metabotropic glutamate receptors. In line with this, genetic deletion of VGLUT3 from striatal cholinergic interneurons leads to impaired activation of striatal GABAergic fast-spiking interneurons (Nelson et al., 2014). But glutamate released through cotransmission can also activate metabotropic glutamate receptors. For example, in the hippocampus, Fasano et al. (2017) recently showed that VGLUT3-mediated glutamate release by basket cells tones-down local GABA transmission by stimulating metabotropic glutamate receptors. This inhibition of local GABA tone by VGLUT3-dependent glutamate alters hippocampal network properties (plasticity and oscillations). The presence of VGLUT3 in basket cells could thus have important consequences for spatial learning or mood regulation.

However, we still do not know how and when glutamate and other transmitters are coreleased from VGLUT3-positive terminals. For example, are the two transmitters released simultaneously or differentially? This question has not been tackled yet in the striatum or in the hippocampus. However, interesting data have started to accumulate in other brain areas such as the interpeduncular nucleus (IPN) or the amygdala. In these areas, such "bilingual" neurons release either glutamate or their cognate transmitter (ACh or 5-HT) depending on their firing pattern. The IPN receives a dense cholinergic innervation from the medial Hb; these terminals massively co-express VGLUT1 (Ren et al., 2011). In the IPN, brief optogenetic stimulation of medial Hb terminals produces fast glutamatergic EPSCs, whereas tetanic stimulation is necessary to evoke slower nicotinic responses (Ren et al., 2011). Therefore, quite surprisingly, these so-called "cholinergic" neurons release more easily glutamate than ACh. Interestingly, in these fibers, ACh was shown to synergistically increase glutamate accumulation and release (Frahm et al., 2015), in line with the previous demonstration of vesicular synergy between glutamate and ACh by Gras et al. (2005) in the striatum. Similarly, in the basal amygdala, low frequency ($\leq$1 Hz) optogenetic stimulation of 5-HT fibers evokes glutamate release whereas higher frequencies (10–20 Hz) are required to release 5-HT (Sengupta et al., 2017). These two thought-provoking publications therefore suggest that the preferentially-released transmitter by these cholinergic and serotoninergic fibers is glutamate. Overall, they show that glutamate and its associated transmitters (here ACh or 5-HT) can be used in different firing conditions by the same neurons. These important and challenging findings indirectly imply that the two transmitters are stored in different SVs (as depicted in **Figure 1B**) and seriously question the initially proposed mechanistics of vesicular synergy. In the initial model of vesicular synergy, glutamate was acting as a counter ion allowing the accumulation of additional protons and hence more ACh (see El Mestikawy et al., 2011). This model implies that VGLUT3 and VAChT were present on the same SVs and is clearly in conflict with the differential release of glutamate (at low firing frequency) and ACh or 5-HT (at higher frequency). Therefore, further investigations will be necessary to fully understand vesicular synergy and the differential release of glutamate and ACh or 5-HT from the same neurons.

What are some of the broader functional implications of co-transmission? In other words, are both transmitters used to fulfill similar or different functions? Optogenetic stimulation does not readily allow to answer this question as it typically triggers release of both transmitters, albeit to possibly different extents according to firing frequency, as discussed previously. In contrast, this problem was investigated in cholinergic interneurons with the use of genetic deletion in mice of either VAChT or VGLUT3. As mentioned previously, cholinergic interneurons from the ventral striatum (or nucleus accumbens, NAc) co-express VAChT and VGLUT3 and consequently signal with both ACh and glutamate (Gras et al., 2008; Guzman et al., 2011; Sakae et al., 2015). The involvement of the NAc in reward-guided behavior and vulnerability to substance use disorders has been abundantly documented since the mid-80s and the seminal experiments of Di Chiara and Imperato (1985) and Imperato and Di Chiara (1986). In addition, immunotoxin-mediated ablation of cholinergic interneurons in the NAc clearly established the involvement of cholinergic interneurons in the sensitivity to the rewarding properties of cocaine (Kaneko et al., 2000; Hikida et al., 2001). However, selective deletion of VAChT from these interneurons and hence silencing of ACh signaling had little effect on the psychostimulant or rewarding properties of cocaine (Guzman et al., 2011). In contrast, the ablation of VGLUT3 recapitulates phenotypes reported with ablation of cholinergic interneurons; VGLUT3-knockout (KO) mice were more sensitive to the stimulant and rewarding effects of cocaine (Sakae et al., 2015). Therefore, as described above in the IPN, ACh seemed to play only a modest role and glutamate appeared to be the major transmitter in the regulation of reward-guided behaviors by cholinergic interneurons.

However, ACh and glutamate released by cholinergic interneurons have opposite effects on DA efflux in the NAc. Activation of presynaptic cholinergic receptors (nAChR) located on DAergic fibers powerfully stimulates DA release in the striatum (for review see Exley and Cragg, 2008). Therefore, ACh stimulates DA release in the NAc. In contrast, glutamate released by cholinergic interneurons binds to a metabotropic glutamate receptor (mGLUR) and inhibits DA release (Figure 2 in Sakae et al., 2015). Together with the numerous reports demonstrating

that ACh is a key frequency-dependent regulator of DA release in the striatum (Zhou et al., 2001; Rice and Cragg, 2004; Zhang and Sulzer, 2004; Threlfell and Cragg, 2011; Exley et al., 2012; Jennings et al., 2015), these observations suggest that much remains to be learned about the functional roles of ACh release by striatal interneurons.

The conditional ablation of *vglut2* from DA neurons provides further evidence for a key role of glutamate corelease in the regulation of psychostimulant-induced behaviors (Birgner et al., 2010; Hnasko et al., 2010; Alsiö et al., 2011). Selective ablation of *vglut2* from DA neurons blunted locomotor response to cocaine and amphetamine (Birgner et al., 2010; Hnasko et al., 2010). In contrast, and surprisingly, self-administration experiments showed that rewarding properties of cocaine (and sucrose) were enhanced in these mutants mice (Birgner et al., 2010). While initial studies were performed by Cre-driven gene deletion dependent on the DAT, and thus caused deletion of the *vglut2* gene during the embryonic period, a recent study used a tamoxifen-inducible strategy to KO *vglut2* from DA neurons in adult mice. This work reported unaltered locomotor sensitization to amphetamine and cocaine in the absence of VGLUT2 (Papathanou et al., 2018). A possible interpretation of the discrepancy between the effects of embryonic vs. adult KO of *vglut2* on the response of mice to psychostimulants is that embryonic gene deletion leads to perturbed development of the DA system. More work will be needed to examine this question directly. However, some indirect support for this possibility has been obtained. Although constitutive KO of VGLUT2 is lethal immediately after birth due to absence of breathing (Wallén-Mackenzie et al., 2006), conditional KO of this VGLUT in DA neurons during embryonic development using a DAT-Cre driver reduced DA release in the ventral striatum and reduced the density of TH-positive terminals in this brain region (Hnasko et al., 2010; Fortin et al., 2012). Interestingly, as mentioned previously, VGLUT2 appears to be expressed broadly during the embryonic period in mesencephalic DA neurons (Dal Bo et al., 2008; Steinkellner et al., 2018). Glutamate release or other functions of VGLUT2 in these neurons during the embryonic period could therefore play key roles in the early establishment of dopaminergic pathways. Constitutive KO of VGLUT3 in mice has only limited effects on adult brain networks. For example, Voisin et al. (2016) thoroughly examined the effect of deleting VGLUT3 on the maturation of the 5-HT system. Absence of VGLUT3 did not modify the number of 5-HT neurons, nor the global density of their axons or dendrites in the adult brain. However, when examined *in vitro*, VGLUT3-KO 5-HT neurons showed reduced survival, suggesting a potential role in their basal vulnerability.

Clearly much remains to be done to better understand the functional implications of glutamate cotransmission. Further, more extensive studies using conditional and inducible genetic deletion of VGLUTs at different developmental stages in select neuronal populations, coupled with functional analyses will likely provide further insights in the coming years.

In the present review, we provided an overview of glutamate cotransmission in different classes of neurons. What emerges from this global comparison is the particularly heterogeneous nature of glutamate cotransmission in different brain nuclei. There are wide variations in the proportion of neurons expressing a VGLUT in different brain regions and at different stages of development. The sub-cellular localization of VGLUTs in neurons using multiple classical neurotransmitters is also clearly heterogeneous, with frequent segregation of the VGLUTs in relation to the neurons' other vesicular transporter. Finally, studies of the functional roles of glutamate cotransmission clearly need to be strengthened by additional studies evaluating these roles at different developmental stages and in disease conditions.

AUTHOR CONTRIBUTIONS

L-ET and SEM jointly wrote the manuscript.

FUNDING

Work in the Trudeau laboratory was supported by grants from the Canadian Institutes of Health Research and the Brain Canada (Multi-Investigator Research Initiative) and Krembil Foundations. Work in the El Mestikawy laboratory was supported by grants from the Brain Canada Foundation (Multi-Investigator Research Initiative), the Fond de Recherche Santé Québec (FRQS 30582), the Natural Sciences and Engineering Research Council (RGPIN/386431-2012 and RGPIN/04682-2017), the ERANET-Neuron Joint Transnational Call for European Research Projects on Mental Disorders and research projects on synaptic dysfunction in disorders of the central nervous system JTC 2013 and 2017, the Djavad Mowafaghian Foundation the Agence Nationale de la Recherche (ANR, ANR-13-SAMA-0005-01), the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale and Université Pierre et Marie Curie.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neural Circuits

ORIGINAL RESEARCH
published: 29 August 2018
doi: 10.3389/fncir.2018.00064

Targeting VGLUT2 in Mature Dopamine Neurons Decreases Mesoaccumbal Glutamatergic Transmission and Identifies a Role for Glutamate Co-release in Synaptic Plasticity by Increasing Baseline AMPA/NMDA Ratio

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Edited by:
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Received: 10 April 2018
Accepted: 31 July 2018
Published: 29 August 2018

Citation:
Papathanou M, Creed M, Dorst MC, Bimpisidis Z, Dumas S, Pettersson H, Bellone C, Silberberg G, Lüscher C and Wallén-Mackenzie Å (2018) Targeting VGLUT2 in Mature Dopamine Neurons Decreases Mesoaccumbal Glutamatergic Transmission and Identifies a Role for Glutamate Co-release in Synaptic Plasticity by Increasing Baseline AMPA/NMDA Ratio. Front. Neural Circuits 12:64. doi: 10.3389/fncir.2018.00064

Expression of the *Vglut2/Slc17a6* gene encoding the Vesicular glutamate transporter 2 (VGLUT2) in midbrain dopamine (DA) neurons enables these neurons to co-release glutamate in the nucleus accumbens (NAc), a feature of putative importance to drug addiction. For example, it has been shown that conditional deletion of *Vglut2* gene expression within developing DA neurons in mice causes altered locomotor sensitization to addictive drugs, such as amphetamine and cocaine, in adulthood. Alterations in DA neurotransmission in the mesoaccumbal pathway has been proposed to contribute to these behavioral alterations but the underlying molecular mechanism remains largely elusive. Repeated exposure to cocaine is known to cause lasting adaptations of excitatory synaptic transmission onto medium spiny neurons (MSNs) in the NAc, but the putative contribution of VGLUT2-mediated glutamate co-release from the mesoaccumbal projection has never been investigated. In this study, we implemented a tamoxifen-inducible Cre-LoxP strategy to selectively probe VGLUT2 in mature DA neurons of adult mice. Optogenetics-coupled patch clamp analysis in the NAc demonstrated a significant reduction of glutamatergic neurotransmission, whilst behavioral analysis revealed a normal locomotor sensitization to amphetamine and cocaine. When investigating if the reduced level of glutamate co-release from DA neurons caused a detectable post-synaptic effect on MSNs, patch clamp analysis identified an enhanced baseline AMPA/NMDA ratio in DA receptor subtype 1 (DRD1)-expressing accumbal MSNs which occluded the effect of cocaine on synaptic transmission. We conclude that VGLUT2 in mature DA neurons actively contributes to glutamatergic neurotransmission in the NAc, a finding which for the first time

highlights VGLUT2-mediated glutamate co-release in the complex mechanisms of synaptic plasticity in drug addiction.

Keywords: cocaine, amphetamine, addiction, substance use disorder, ventral tegmental area (VTA), striatum, medium spiny neurons

INTRODUCTION

Drug addiction is a multifaceted neuropsychiatric disease characterized by a neurobiological interplay between the reinforcing effect of supraphysiological dopamine (DA) levels upon initial drug intake and the lasting alterations in glutamatergic synaptic strength upon repeated drug consumption (Volkow and Morales, 2015; Lüscher, 2016). Converging on medium spiny neurons (MSNs) in the nucleus accumbens (NAc) of the ventral striatum, the main DA input is derived from DA neurons located in the ventral tegmental area (VTA) of the midbrain while substantial glutamatergic innervation originates in numerous cortical and limbic sources including the prefrontal cortex, amygdala and hippocampus (Yager et al., 2015). In addition to forebrain-derived glutamatergic transmission within the NAc, a group of VTA DA neurons possess the capacity for glutamate release within this area. Based on their unique capacity to co-release glutamate and DA, these VTA neurons, often referred to as "dual-signaling," "bi-lingual," "combinatorial" or "co-releasing" neurons (El Mestikawy et al., 2011; Trudeau et al., 2014; Pupe and Wallén-Mackenzie, 2015; Morales and Margolis, 2017), might be of particular interest to drug addiction, but their role in VTA circuitry and behavior remains to be fully clarified.

Historically, pioneering electrophysiological experiments in cell culture and brain slices led to the first evidence that VTA DA neurons can release glutamate, which was detected as excitatory postsynaptic currents (EPSCs) (Sulzer et al., 1998; Bourque and Trudeau, 2000; Joyce and Rayport, 2000; Chuhma et al., 2004, 2009; Dal Bo et al., 2004; Lavin et al., 2005). Expression of the *Vglut2 (*aka *Slc17a6)* gene encoding the Vesicular glutamate transporter 2 (VGLUT2) in cultured VTA DA neurons subsequently provided the means for vesicular co-release of glutamate (Dal Bo et al., 2004). Histological gene expression analyses of *Vglut2* and *Tyrosine hydroxylase* (*Th*), encoding the rate-limiting enzyme of DA synthesis (TH), in adult rodents confirmed the presence of cells having both Vglut2 mRNA and TH immunoreactivity within a subset of cells in the medial VTA, intermingled with cells showing either *Th* or *Vglut2* gene expression only (Kawano et al., 2006; Yamaguchi et al., 2007, 2011, 2015; Nair-Roberts et al., 2008). In addition to mice and rats, the VGLUT2/TH combinatorial cell type has also been identified within the VTA of non-human primates and humans (Root et al., 2016). Vglut2 mRNA has been visualized by *in situ* hybridization throughout several developing brain regions at embryonal day (E) 12.5 in the mouse, and co-localizes already at this stage with TH immunoreactivity within midbrain DA neurons (Birgner et al., 2010; Nordenankar et al., 2014). RT-PCR experiments have shown that 25% of Th-positive DA neurons express the *Vglut2* gene at birth, while only 14% keeps this expression after 6 weeks (Mendez et al., 2008). In rats, even fewer *Vglut2*-expressing DA neurons have been reported at adulthood (Yamaguchi et al., 2007). Immunohistological co-labeling analysis of terminals in the NAc further proposed age as factor of importance for the ability for glutamate-DA co-release, as the extent of co-localization between VGLUT2 and TH proteins regressed with age and was not visible in adult rats (Bérubé-Carrière et al., 2009; Moss et al., 2011). Optogenetic stimulations carried out in DA transporter (DAT)-Cre transgenic mice expressing Channelrhodopsin-2 (ChR2) in the VTA, however, demonstrated the presence of DAT-Cre-dependent EPSCs in accumbal MSNs of adult mice, thus verifying mesoccumbal glutamate release from DA neurons located within the VTA (Stuber et al., 2010; Tecuapetla et al., 2010). Responses were blocked by the selective AMPA antagonist DNQX, thus verifying their glutamatergic nature. It was also established that the recorded glutamate was released directly from DA neurons, that it occurred independent of DA activity and that glutamate release was accompanied by DA release (Stuber et al., 2010; Tecuapetla et al., 2010). Recently, optogenetic stimulations were shown to cause glutamate and DA release from distinct sites and vesicles originating within the same mesoaccumbal axons, thereby supporting dual release (co-release) from one axon but from distinct axonal substructures (Zhang et al., 2015).

The lack of selective approaches in experimental animals has made it challenging to address the behavioral role of glutamate-DA co-release (Pupe and Wallén-Mackenzie, 2015; Morales and Margolis, 2017). However, implementing DAT-Cre-driven targeting of the *Vglut2* gene, generating $Vglut2^{lx/lx;DAT\text{-}Cre}$ conditional knockout (KO) mice, several studies have implicated glutamate-DA co-release in reward processing of relevance to addiction (Birgner et al., 2010; Hnasko et al., 2010; Alsiö et al., 2011; Fortin et al., 2012). For example, $Vglut2^{lx/lx;DAT\text{-}Cre}$ KO mice showed altered psychomotor activity in response to both acute and repeated administration of drugs of abuse, including amphetamine and cocaine (Birgner et al., 2010; Hnasko et al., 2010; Fortin et al., 2012). Glutamatergic EPSCs in accumbal MSNs were shown to be completely abolished in such KO mice (Hnasko et al., 2010; Stuber et al., 2010; Wang et al., 2017), thus verifying the importance of VGLUT2 for mesoaccumbal glutamate-DA co-release. Further, when $Vglut2^{lx/lx;DAT\text{-}Cre}$ KO mice were tested in the operant self-administration paradigm, striking differences were observed when compared to control mice (Alsiö et al., 2011). $Vglut2^{lx/lx;DAT\text{-}Cre}$ KO mice consumed more sugar than controls and had increased consumption of cocaine at a low dose. The results suggested

that loss of VGLUT2 heightens the sensitivity to palatable food (sugar) and to addictive drugs (cocaine) (Alsiö et al., 2011).

Importantly, all gene targeting studies of mesoaccumbal glutamate-DA co-release performed so far suffer from the uncertainty that any observed phenotypes might depend on developmental circuitry adaptations, as the endogenous promoters of both *Vglut2* (Birgner et al., 2010; Nordenankar et al., 2014) and *Dat* (Ang, 2006) genes have embryonal onset. The proposed age-dependent decrease in *Vglut2* expression within the VTA toward adulthood alongside the developmental component of DAT-Cre-driven *Vglut2* gene targeting has made it impossible to dissociate the putative role of VGLUT2 in mature DA neurons from developmental compensatory adaptations. Further, on a molecular and circuitry level, while ablation of VGLUT2 has been shown to affect DA release in the NAc (Hnasko et al., 2010; Alsiö et al., 2011), the physiological role of the co-released glutamate in mesoaccumbal neurotransmission has remained unexplored.

To unambiguously pinpoint the role of *Vglut2* gene expression in mature DAT-Cre neurons, this study implemented a tamoxifen-inducible DAT-Cre transgene (DAT-CreERT2; Engblom et al., 2008) to control temporal aspects of recombination. We found that ablation of *Vglut2* gene expression in mature DA neurons significantly decreased excitatory post-synaptic currents (EPSCs) in the NAc. Behaviorally, and in stark contrast to developmental VGLUT2 targeting, DAT-CreERT2-induced VGLUT2 targeting in adulthood did not disturb psychostimulant-induced locomotion. By addressing synaptic plasticity in the NAc, we found that DA receptor 1 (DRD1)-expressing MSNs showed normal rectification index (RI) but increased baseline AMPA/NMDA ratio, which cocaine did not increase further. This study thereby identifies a role for VGLUT2-mediated glutamate co-release from DAT-Cre-positive neurons in synaptic plasticity of putative relevance to drug addiction.

MATERIALS AND METHODS

Animal Housing

Animals were housed on a standard 12 h sleep/wake cycle (7:00 A.M. lights on, 7:00 P.M. lights off). Mice were provided with food and water *ad libitum* unless stated otherwise and housed according to Swedish legislation (Animal Welfare Act SFS 1998:56) and European Union legislation (Convention ETS 123 and Directive 2010/63/EU). All experiments were conducted with permission from the local Animal Ethical Committees (Uppsala University, UU/Karolinska Institutet, KI) and by the Institutional Animal Care and Use Committee (University of Geneva, UG), respectively. For glutamate recordings upon optogenetic stimulation, mice were transferred from UU to KI, Stockholm. For cocaine sensitization and electrophysiological recordings, mice were transferred from UU to UG.

Generation and Genotyping of Transgenic Mice

Genotyping of transgenic mice was performed by PCR analysis (**Supplementary Table S1**). The following Cre-drivers were implemented in the study: (i) DAT-Cre transgenic mouse line with embryonal onset of the transgene (Ekstrand et al., 2007), here abbreviated eDAT-Cre; and (ii) Tamoxifen-inducible DAT-CreERT2 mice (Engblom et al., 2008), here abbreviated txDAT-Cre. Mice of the eDAT-Cre and txDAT-Cre transgenic lines were bred with the Vglut2^{lx} conditional KO mouse line in which exons 4–6 are surrounded by LoxP sites to enable Cre-driven *Vglut2* gene targeting (Wallén-Mackenzie et al., 2006). DAT-Cre driven recombination of the floxed *Vglut2* gene directs *Vglut2* gene targeting to DA neurons either during development (eDAT-Cre-driver: *Vglut2*$^{eDAT\text{-}Cre}$ transgenic line) or in mature DA neurons upon tamoxifen treatment (txDAT-Cre-driver: *Vglut2*$^{txDAT\text{-}Cre}$ transgenic line), respectively. Offspring within these lines were used for behavioral and electrophysiological experiments. *Vglut2*$^{eDAT\text{-}Cre}$ and *Vglut2*$^{txDAT\text{-}Cre}$ transgenic lines were also bred to the floxed tdTom reporter line (B6;129S6-*Gt (ROSA) 26Sor*$^{tm14(CAG\text{-}tdTomato)Hze}$/J (Jackson Laboratory) and to the DRD1a-EGFP reporter line (Tg(Drd1-EGFP)X60Gsat/Mmmh; Gensat) for fluorescent visualization of Cre-driven activity and of neurons expressing the DA receptor subtype 1 (DRD1), respectively.

Tamoxifen Administration

All experimental mice containing the txDAT-Cre allele were treated with tamoxifen to induce recombination (Sigma, T-5648). Tamoxifen was dissolved in sunflower oil and ethanol (9:1) to a final concentration of 20 mg/ml. Animals (8–9 week old) were intraperitoneally injected with 2 mg of tamoxifen once daily for five consecutive days. Animals undergoing stereotaxic injections were treated with their last tamoxifen injection 10 days prior to surgery. All behavioral and electrophysiological experiments commenced no earlier than 1 week after last tamoxifen injection.

RNA Extraction and Nested PCR

Brain tissue from tamoxifen-treated *Vglut2*$^{lx/lx;txDAT\text{-}Cre\text{-}tg}$ and *Vglut2*$^{lx/lx;txDAT\text{-}Cre\text{-}wt}$ mice was collected 1 week after tamoxifen treatment, snap-frozen in −30°/−35°C isopentane and stored at −80°C until further use. The VTA was dissected out using the 2 mm brain punch and a brain matrice (Agnthos, Sweden) at −20°C. The RNA from the dissected VTA was extracted using the Qiagen RNeasy Plus Mini Kit according to manufacturer's guidelines. Ten nanogram of total RNA, Oligo $(dT)_{20}$ primer and the ThermoScriptRT kit (Invitrogen) was used for cDNA synthesis. PCR was performed using Phusion High-Fidelity DNA Polymerase kit (ThermoFisher Scientific) with a total volume of 20 µl. The first PCR reaction (PCR1) contained 1.5 µl of cDNA and the second PCR (PCR2) contained 1 µl of PCR1 (**Supplementary Table S2** for primer sequences and thermal conditions). Five microliter of the final product of PCR2 was subjected to agarose gel electrophoresis using 2.8% agarose.

Stereotaxic Injection of Optogenetic Virus

Vglut2$^{lx/lx;txDAT\text{-}Cre\text{-}tg}$, *Vglut2*$^{wt/lx;txDAT\text{-}Cre\text{-}tg}$ and *Vglut2*$^{wt/wt;txDAT\text{-}Cre\text{-}tg}$ littermate mice (>8 weeks; >20 g) were anesthetized with isoflurane and stereotaxically injected unilaterally into the VTA with 300 nl of AAV5-EF1a-DIO-ChR2(H134)-eYFP (titer 5.6×10^{12} vg/ml; UNC Gene Therapy, Chapel Hill, NC, USA) at a flow-rate of 100 nl per min at the following coordinates from Bregma: anterior-posterior −3.45, medial-lateral −0.2 and dorsal-ventral −4.4 according to Paxinos and Franklin (2012). Topical analgesic, Marcaine (1.5 mg/kg; AstraZeneca) was applied during surgery and Caprofen (5 mg/kg; Norocarp) was given subcutaneously pre- and post-surgery. All animals were allowed to recover for at least 2 weeks prior to electrophysiological recordings. Mice received tamoxifen treatment prior to virus injections as described above.

Histological Analysis

Immunohistochemistry

Deeply anesthetized mice were transcardially perfused with body-temperature phosphate-buffered saline (PBS) followed by ice-cold 4% formaldehyde. Brains were dissected and post-fixed overnight. The brains were then cryo-protected with 30% sucrose and cut using a cryostat at 60 μm slice thickness. Free-floating sections were processed for immunofluorescence according to standard protocols (Primary Antibodies: rabbit TH 1:1,000 #ab172, Millipore; chicken GFP 1:1,000 #ab13970, Abcam; Secondary antibodies: Donkey Anti-Rabbit Alexa Fluor 488 1:500; Donkey-Anti-Chicken Alexa Fluor 488 1:500; Donkey Anti-Rabbit Cy3 1:500). The signal of tdTom was detected by endogenous fluorescence without any additional use of an antibody. Images were captured using Mirax MIDI scanner or a Zeiss Confocal (LSM 700, 20× magnification) and analyzed using PanoramicViewer or Zen software. Manual quantification of TH-positive neurons in the VTA and SNc was performed on three sections along the anterio-posterior axis for each animal (*Vglut2*$^{lx/lx;txDAT\text{-}Cre\text{-}wt}$, $n = 3$; *Vglut2*$^{lx/lx;txDAT\text{-}Cre\text{-}tg}$, $n = 3$). Analysis was done by two-way ANOVA with Sidak *post hoc* test. Brain slices (250 μm) used for patch clamp recordings were fixed overnight in Lana's fixative, for detection of neurobiotin and GFP. The sections were subsequently washed overnight in 0.01 M phosphate buffered saline (PBS), followed by another overnight incubation with Cy5-conjugated streptavidin (1:1,000) in 0.1 M PB containing 0.3% Triton X-100. They were then washed and incubated in PBS with DAPI (1:1,000) for 30 min and washed again before mounting with Fluoromount Aqueous mounting medium (Sigma, USA) and analyzed. GFP was detected by endogenous fluorescence without any additional enhancement of signal.

In situ Hybridization

The brains of anesthetized mice were extracted and snap-frozen by rapidly immersing the tissue in ice-cold isopentane (−25°/−30°C for embryo E14.5, postnatal day 3; −30°/−35°C for adult). Colorimetric and double fluorescent *in situ* hybridization analyses were performed as previously described (Viereckel et al., 2016; Riboprobes corresponding to: slc17a6 NM_080853.3, sequence: 2315–3244; tdTomato, sequence: 84–653, slc6a3 NM_012694.2 sequence 1015–1938, th NM_012740.3, sequence 456–1453). All slides were scanned at 20× magnification on a NanoZoomer *2.0-HT*. The Ndp2.view software (Hamamatsu) was employed for viewing the images and manual counting was performed for quantification (Post-natal (P) day 3, $n = 3$; Adult, $n = 3$).

Behavioral Analysis

All behavioral experiments took place during the light-cycle (8:00 AM to 6:00 PM). All animals were adult (≥8 weeks) and habituated to handling for several days prior to behavioral assessment. Both male and female mice were used throughout the study.

Amphetamine-Induced Behavioral Sensitization

Locomotor behavior of *Vglut2*$^{lx/lx;eDAT\text{-}Cre\text{-}tg}$, *Vglut2*$^{lx/lx;eDAT\text{-}Cre\text{-}wt}$, *Vglut2*$^{lx/lx;txDAT\text{-}Cre\text{-}tg}$ and *Vglut2*$^{lx/lx;txDAT\text{-}Cre\text{-}wt}$ mice was recorded upon amphetamine injections (3 mg/kg i.p) using the Ethovision XT 13.0 software. *Vglut2*$^{lx/lx;txDAT\text{-}Cre\text{-}tg}$ and *Vglut2*$^{lx/lx;txDAT\text{-}Cre\text{-}wt}$ mice received tamoxifen treatment prior to the onset of the experiment. Amphetamine-induced locomotor activity was analyzed in open-field activity boxes (Makrolon® polycarbonate cages, 15 × 26 × 40 cm) containing 1.5 cm bedding and a transparent plexiglas lid. The mice were allowed to habituate to the boxes and surrounding environment for 30 min prior to drug administration. Locomotor activity was measured for 90 min consisting of (30 min baseline and 60 min drug-induced activity). The experimental setup consisted of saline treatment on day 1, followed by four consecutive days of amphetamine injections (days 2–5) and a subsequent acute amphetamine challenge 2 weeks later (day 19). Mice were then left to recover for 1 week and on day 26 received another saline injection followed by four subsequent amphetamine injections on days 27–30. After 1 week the mice received an acute amphetamine challenge (day 36), and a final amphetamine challenge 1 week later (day 44). Statistical analysis was performed with GraphPad Prism 7 using 2-way repeated measures ANOVA with Sidak *post hoc* test using genotype and time as variables (*Vglut2*$^{lx/lx;eDAT\text{-}Cre\text{-}tg}$, $n = 3$; *Vglut2*$^{lx/lx;eDAT\text{-}Cre\text{-}wt}$, $n = 3$; *Vglut2*$^{lx/lx;txDAT\text{-}Cre\text{-}tg}$, $n = 11$ and *Vglut2*$^{lx/lx;txDAT\text{-}Cre\text{-}wt}$, $n = 7$. Males $n = 15$, Females $n = 9$).

Electrophysiological Recordings

Patch Clamp Recordings Upon Optogenetic Stimulation

Post-synaptic currents upon optogenetic stimulation were analyzed by patch clamp recordings in brain slices from tamoxifen-treated *Vglut2*$^{lx/lx;txDAT\text{-}Cre\text{-}tg}$, *Vglut2*$^{wt/lx;txDAT\text{-}Cre\text{-}tg}$ and *Vglut2*$^{wt/wt;txDAT\text{-}Cre\text{-}tg}$ mice stereotaxically injected unilaterally into the VTA with AAV5-EF1a-DIO-ChR2(H134)-eYFP as described above. Mice were anesthetized using isoflurane and dissected brains immersed in ice cold cutting solution containing (in mM): KCl 2.5, $NaH_2PO_4 \cdot H_2O$ 1.25, $CaCl_2 \cdot 2H_2O$ 0.5, $MgCl_2 \cdot 6H_2O$ 7.5, Glucose 10, $NaHCO_3$ 25,

and Sucrose 205. Coronal sections 250 μm thick were cut on a VT1200S Vibratome (Leica, Japan) and recovered for 30 min in 35°C artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 125, KCl 2.5, $MgCl_2 \cdot 6H_2O$ 1, $NaH_2PO_4 \cdot H_2O$ 1.25, $CaCl_2 \cdot 2H_2O$ 2, Glucose 25, and $NaHCO_3$ 25. Slices were maintained at room temperature until recording at 35°C. Solutions were continuously oxygenated with carbogen (95% O_2, 5% CO_2) throughout the procedure. Borosilicate glass pipettes were pulled on a P1000 micropipette puller (Sutter Instruments, Novato, CA, USA) to a resistance of 4–6 MΩ and filled with intracellular solution containing (in mM): CsCl 10, $CsMeSO_3$ 110, HEPES 10, Na_2-Phosphocreatine 10, ATP-Mg 4, GTP-Na 0.3, TEA-Cl 10 and QX-314 Cl 1. For staining, 0.3% neurobiotin was added to the intracellular solution and detected as described above ("Immunohistochemistry" section). Neurons were identified using Infrared-Differential Interference Contrast (IR-DIC) imaging on a BX51WI (Olympus, Japan) upright microscope equipped with a 40× long-working-distance immersion objective. Neurons were selected by proximity to afferent fibers. Once a whole-cell patch was achieved, light evoked responses were recorded in $V_H = -70$ mV voltage-clamp on a MultiClamp 700B (Molecular Devices, Sunnyvale, CA, USA), digitized at 10 KHz on a ITC-18 (HEKA, Houston, TX, USA) and acquired with Igor Pro 6.3 (Wavemetrics, Tigard, OR, USA). Fibers were stimulated through an ocular-mounted blue LED producing 6.4 mW/mm^2 light under the objective, controlled through an SLA-1200-2 LED driver (Mightex, Pleasanton, CA, USA). When EPSCs could reliably be evoked, 5 μm NBQX was bath-applied and light-evoked EPSCs were again recorded. Peak amplitude was determined by subtracting the mean current in the 100 ms preceding light stimulation from the maximum deviation achieved in the 100 ms following light onset. Data were analyzed using unpaired Mann-Whitney test ($Vglut2^{wt/lx;txDAT\text{-}Cre\text{-}tg}$ and $Vglut2^{wt/wt;txDAT\text{-}Cre\text{-}tg}$ animals were pooled together as controls (txCtrl) as there were no significant difference between these groups; txCtrl, cells $n = 24$, 3 mice and $Vglut2^{lx/lx;txDAT\text{-}Cre\text{-}tg}$ (txKO), cells $n = 24$, 3 mice. Males $n = 4$; Females $n = 2$).

Patch Clamp Recording Upon Cocaine-Induced Behavioral Sensitization

Locomotor behavior of $Vglut2^{lx/lx;eDAT\text{-}Cre\text{-}tg}$, $Vglut2^{lx/lx;eDAT\text{-}Cre\text{-}wt}$, $Vglut2^{lx/lx;txDAT\text{-}Cre\text{-}tg}$ and $Vglut2^{lx/lx;txDAT\text{-}Cre\text{-}wt}$ mice carrying the DRD1-EGFP transgene (see "Generation and Genotyping of Transgenic Mice" section above) was recorded upon cocaine injections whereupon post-synaptic currents were recorded in patch clamp electrophysiology. $Vglut2^{lx/lx;txDAT\text{-}Cre\text{-}tg}$ and $Vglut2^{lx/lx;txDAT\text{-}Cre\text{-}wt}$ mice received tamoxifen treatment prior to the experimental start. Both male and female mice were used. Cocaine-induced locomotor activity was measured as the distance traveled in a circular corridor (outer/inner diameter, 30/10 cm; video tracking system, Anymaze, Stoeling). After 3 days of habituation, locomotion was recorded following five consecutive days of saline or cocaine (20 mg/kg, i.p.; Sigma) administration. Behavioral recording took place for 80 min, which consisted of 20 min habituation and 60 min of saline or cocaine-induced locomotion (saline: $Vglut2^{lx/lx;eDAT\text{-}Cre\text{-}wt}$, $n = 3$; $Vglut2^{lx/lx;eDAT\text{-}Cre\text{-}tg}$, $n = 2$, cocaine: $Vglut2^{lx/lx;eDAT\text{-}Cre\text{-}wt}$, $n = 5$; $Vglut2^{lx/lx;eDAT\text{-}Cre\text{-}tg}$, $n = 5$, saline: $Vglut2^{lx/lx;txDAT\text{-}Cre\text{-}wt}$, $n = 4$; $Vglut2^{lx/lx;txDAT\text{-}Cre\text{-}tg}$, $n = 4$, cocaine: $Vglut2^{lx/lx;txDAT\text{-}Cre\text{-}wt}$, $n = 9$; $Vglut2^{lx/lx;txDAT\text{-}Cre\text{-}tg}$, $n = 8$).

After 10 days, coronal mouse brain slices were prepared in cooled artificial cerebrospinal fluid containing (in mM): 119 NaCl, 2.5 KCl, 1.3 MgCl, 2.5 $CaCl_2$, 1.0 Na_2HPO_4, 26.2 $NaHCO_3$ and 11 glucose, bubbled with 95% O_2 and 5% CO_2. Slices were kept at 32–34°C in a recording chamber superfused with 2.5 ml/min artificial cerebrospinal fluid. Visualized whole-cell voltage-clamp recording techniques were used to measure holding and synaptic responses of DRD1-MSNs of the NAc shell, identified by the presence of the DRD1a-EGFP reporter. Holding potential was maintained at −70 mV, and access resistance was monitored by a depolarizing step of −14 mV each sweep, every 10 s. The liquid junction potential was small (−3 mV); therefore, traces were not corrected. Experiments were discarded if the access resistance varied by more than 20%. Currents were amplified, filtered at 5 kHz and digitized at 20 kHz. All experiments were performed in the presence of picrotoxin (100 μm) to isolate excitatory transmission. The internal solution contained (in mM) 130 CsCl, 4 NaCl, 5 creatine phosphate, 2 $MgCl_2$, 2 Na_2ATP, 0.6 Na_3GTP, 1.1 EGTA, 5 HEPES and 0.1 mm spermine. Synaptic currents were electrically evoked by stimuli (50–100 μs) at 0.1 Hz through bipolar stainless steel electrode placed onto the tissue. To isolate AMPAR-evoked EPSCs, the NMDA antagonist D-AP5 (50 μm) was bath applied. The NMDAR component was calculated as the difference between the EPSCs measured in the absence and presence of D-AP5. The AMPAR/NMDAR ratio was calculated by dividing the peak amplitudes. The RI of AMPAR-mediated currents was calculated as the ratio of the chord conductance calculated at −70 mV, divided by chord conductance at +40 mV. In sample traces, stimulation artifacts were removed. RI saline: $Vglut2^{lx/lx;eDAT\text{-}Cre\text{-}tg}$, $n = 5$, $Vglut2^{lx/lx;eDAT\text{-}Cre\text{-}wt}$, $n = 4$; cocaine $Vglut2^{lx/lx;eDAT\text{-}Cre\text{-}tg}$, $n = 28$, $Vglut2^{lx/lx;eDAT\text{-}Cre\text{-}wt}$, $n = 12$; AMPA/NMDA ratio saline: $Vglut2^{lx/lx;eDAT\text{-}Cre\text{-}tg}$, $n = 5$, $Vglut2^{lx/lx;eDAT\text{-}Cre\text{-}wt}$, $n = 7$; cocaine: $Vglut2^{lx/lx;eDAT\text{-}Cre\text{-}tg}$, $n = 33$, $Vglut2^{lx/lx;eDAT\text{-}Cre\text{-}wt}$, $n = 15$. RI saline: $Vglut2^{lx/lx;txDAT\text{-}Cre\text{-}tg}$, $n = 6$, $Vglut2^{lx/lx;txDAT\text{-}Cre\text{-}wt}$, $n = 6$; cocaine $Vglut2^{lx/lx;txDAT\text{-}Cre\text{-}tg}$, $n = 7$, $Vglut2^{lx/lx;txDAT\text{-}Cre\text{-}wt}$, $n = 12$; AMPA/NMDA ratio saline: $Vglut2^{lx/lx;txDAT\text{-}Cre\text{-}tg}$, $n = 9$, $Vglut2^{lx/lx;txDAT\text{-}Cre\text{-}wt}$, $n = 9$; cocaine: $Vglut2^{lx/lx;txDAT\text{-}Cre\text{-}tg}$, $n = 8$, $Vglut2^{lx/lx;txDAT\text{-}Cre\text{-}wt}$, $n = 13$. Data were analyzed with two-way repeated measures ANOVA and Tukey's *post hoc* test (behavior) or two-way ANOVA and Sidak *post hoc* (electrophysiological recordings). All mice expressed the DRD1-EGFP transgene.

RESULTS

Vglut2 Gene Highly Expressed Throughout Mouse Midbrain With Restricted Expression Within the Dopaminergic Area

The presence of VGLUT2 molecules enables neurons to package the essential amino acid glutamate into presynaptic

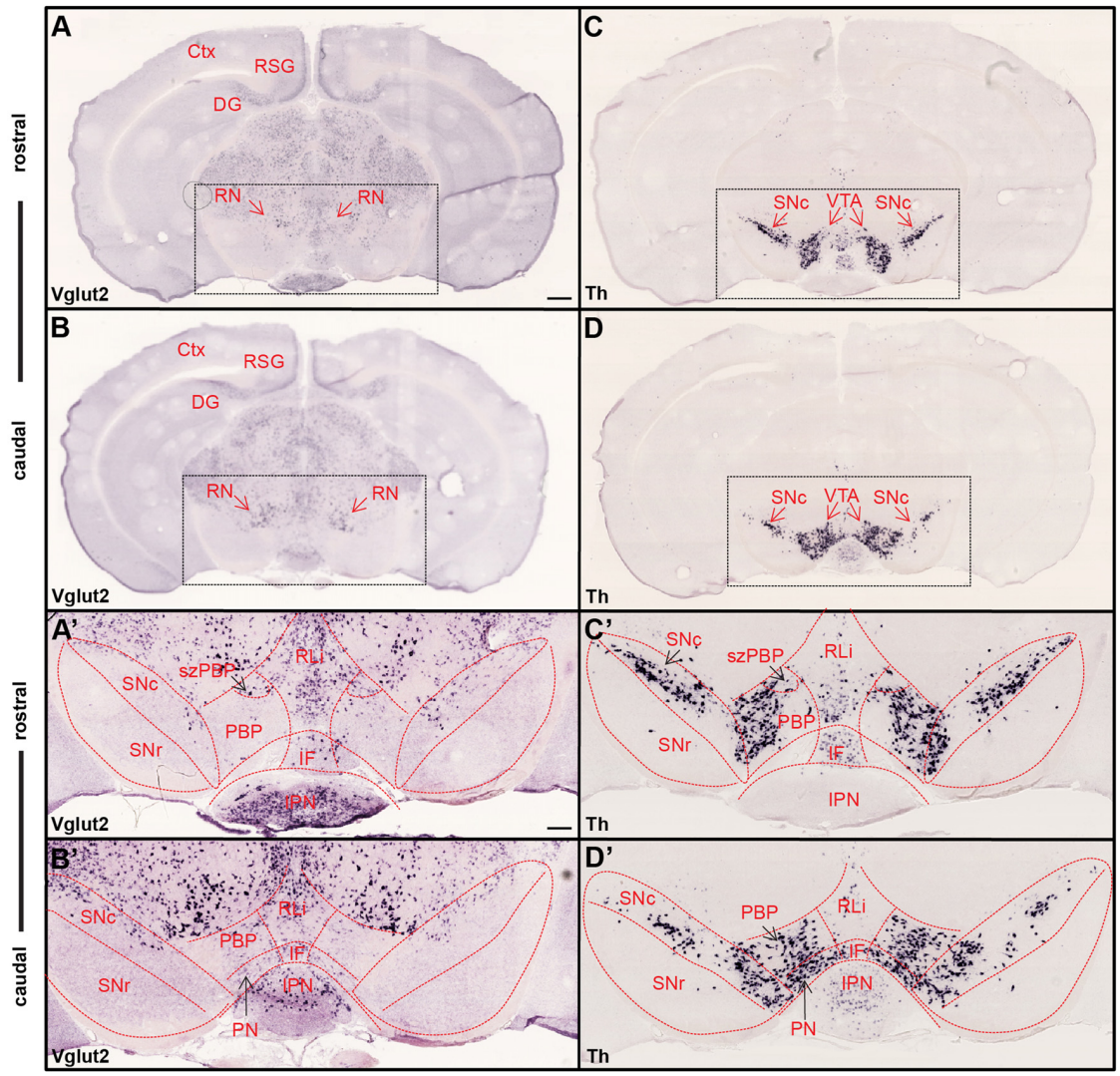

FIGURE 1 | Ample Vglut2 mRNA-positive cells throughout dorsal and ventral midbrain with more sparse expression within the dopaminergic area. Colorimetric *in situ* hybridization showing overview of Vglut2 **(A,B)** and Th **(C,D)** mRNA in midbrain section of wildtype adult mouse at two rostro-caudal levels. **(A,B)** Vglut2 mRNA is abundant throughout the midbrain with strong signal in e.g., the red nucleus (RN), RSG and dentate gyrus and weaker signal in the ventral tegmental area (VTA) and SNc areas. **(C,D)** Th mRNA is selectively localized in dopaminergic neurons of the VTA and SNc and its mRNA signal implemented to visualize these areas. Dotted square around the VTA and SNc (scale bar 500 μm) presented as closeups in (**A′–D′**; scale bar 200 μm). **(C′,D′)** SNc, SNr and subregions of VTA outlined in Th closeups and superimposed on Vglut2 closeups **(A′,B′)**. **(C′,D′)** Th mRNA was strongly localized in the SNc and within the parabrachial pigmented area (PBP) and paranigral nucleus (PN) of the VTA with weaker signal in the RLi and caudal IF. **(A′,B′)** Within the VTA, Vglut2 mRNA was detected in the PBP, PN, RLi and IF as well as within the medially located szPBP while no Vglut2 mRNA was detected in the GABAergic SNr area. Abbreviations: Ctx, Cortex; DG, Dentate gyrus; IF, interfascicular nucleus; IPN, interpeducular nucleus; PBP, parabrachial pigmented area; PN, paranigral nuclei; RLi rostral linear nucleus; RN, Red nucleus; RSG, Retrosplenial granular cortex; SNc, Substantia nigra *pars compacta*; SNr, Substantia nigra *pars reticulata*; szPBP, subzone of the parabrachial pigmented area; VTA, Ventral tegmental area.

vesicles for fast synaptic neurotransmission upon depolarization (Fremeau et al., 2001; Herzog et al., 2001; Takamori et al., 2001; Varoqui et al., 2002). While this protein is localized in the presynaptic axonal terminals within projection target areas, Vglut2 mRNA locates to the cell soma. Thus, VGLUT2 immunohistochemistry will detect VGLUT2 molecules in the presynaptic terminal and Vglut2 mRNA-selective *in situ* hybridization will visualize the cell body from which these axons originate. By implementing *in situ* hybridization in adult mouse midbrain, we first confirmed previous findings in rat that Vglut2 mRNA is abundant throughout the midbrain (Kawano et al., 2006; **Figures 1A,B**). Using a probe detecting Th mRNA, the VTA and substantia nigra *pars compacta* (SNc) area containing midbrain DA neurons, were visualized in adjacent sections (**Figures 1C,D**). While DA neurons in the midbrain are confined within the VTA/SNc area, *Vglut2* gene expression was sparse within this area compared to its expression throughout the other areas of the midbrain, for

example the red nucleus (RN) located immediately dorsal of the VTA. Th mRNA was used to illustrate the outline of the subareas of the VTA and the SNc (**Figures 1C′,D′**) which was subsequently superimposed on the images of Vglut2 mRNA (**Figures 1A′,B′**). Previous analyses of mouse and rat (Kawano et al., 2006; Yamaguchi et al., 2011, 2015) have shown that Vglut2 mRNA-positive cells are scattered throughout the mouse VTA and SNc with more frequent appearance medially than laterally and Vglut2/Th co-localization in the VTA but not in the SNc. Within the VTA, Vglut2 mRNA was detected in the parabrachial pigmented area (PBP) and paranigral nucleus (PN) as well as the medially located nuclei, rostral linear nucleus (RLi) and interfascicular (IF). As expected, no Vglut2 mRNA was detected in the GABAergic SNr area. We could show that within the VTA, highest density of Vglut2 mRNA is found in the RLi. Vglut2 mRNA was also prominent within the medially located subzone of the PBP (szPBP; described in Viereckel et al., 2016), but similar as RLi, less Th mRNA is found in this area (**Figures 1A′,B′**). These histological mRNA analyses confirmed previous findings of scattered Vglut2 mRNA in the VTA/SNc of the adult mouse midbrain. To address the spatio-temporal distribution of glutamate-DA co-releasing neurons in more detail, we next turned to double-labeling experiments.

Number of *Vglut2*/*Dat* Co-expressing VTA Neurons Highest Around Birth and Reduced in Adulthood

Combinatorial neurons expressing both *Vglut2* and *Th* genes comprise a minority of the total cells in the adult VTA expressing either *Th* or *Vglut2* genes (Yamaguchi et al., 2011, 2013; Morales and Root, 2014). In previous studies, we have shown that Vglut2 mRNA can be detected within TH-positive DA neurons of the developing midbrain already at E12.5 in the mouse embryo (Birgner et al., 2010; Nordenankar et al., 2014), while other studies have demonstrated an age-dependent decrease in Vglut2 mRNA in TH neurons from birth into adulthood (Yamaguchi et al., 2007; Mendez et al., 2008). TH is a crucial enzyme in the biosynthesis pathway for DA and hence, all DA neurons express its gene by definition. In contrast, medial VTA DA neurons have less DAT, the extracellular DA transporter, than DA neurons in the lateral VTA and SNc in the adult midbrain (Lammel et al., 2008; Li et al., 2013; Viereckel et al., 2016). To address and compare the temporal and spatial distribution of Vglut2 mRNA in Th- and Dat-positive neurons, we implemented double fluorescent *in situ* hybridization to co-localize Vglut2 mRNA with Th and Dat mRNA, respectively. Th mRNA has been described around E11 and Dat mRNA has been reported to appear around E13 (Ang, 2006). We addressed E14.5 as youngest stage and prepared multiple sections throughout the midbrain at E14.5, post-natal day 3 (P3) and in adulthood (10 weeks). At E14.5, Th mRNA was readily detected in the ventral midbrain where Dat mRNA co-localized with Th ventrally (**Figures 2A,A′**). Sparse co-localization was detected between Th and Vglut2 mRNA (**Figures 2B,B′**) and between Dat and Vglut2 mRNA at this embryonal stage (**Figures 2C,C′**).

At P3, Th and Dat mRNA showed ample co-localization in the lateral VTA and SNc, while Th mRNA was stronger than Dat mRNA in the medial VTA, confirming previous finding using the same method (Viereckel et al., 2016; **Figure 2D**). Vglut2 mRNA showed prominent co-localization with both Th and Dat mRNA at this stage, however, the density of Vglut2/Th double-positive cells was higher than Vglut2/Dat double-positive cells (**Figures 2E,F** and **Supplementary Figure S1A**). Subareas within the VTA showed different amount of co-localization at P3. Primarily the PBP, but also the PN and IF, showed co-localization of Vglut2 with Th and Dat, while the more dorsally located RLi, which shows the highest level of Vglut2 mRNA, was almost devoid of co-localization with either Th or Dat mRNA (**Figures 2E,F**).

In the adult mouse, Th and Dat mRNA showed substantial overlap and also at this stage, the overlap was stronger in the lateral VTA and SNc than in the medial VTA where Th was stronger than Dat (**Figure 2G**). In contrast, the level of Vglut2/Th and Vglut2/Dat double-positive cells was low in all VTA subareas (**Figures 2H,I**). Vglut2 mRNA, more medially than laterally located, showed more overlap with Th than with Dat also at this stage (**Figures 2H,I** and **Supplementary Figure S1B**). Quantification showed 128 (±10) vs. 24 (±5) Vglut2/Dat mRNA-double-positive cells in P3 vs. adult VTA (**Supplementary Figure S1C**). Together, these histological results show that *Vglut2* gene expression in Dat-positive DA neurons of the VTA is highest around birth with temporal down-regulation towards adulthood. Areas with highest level of Vglut2 mRNA (RLi and szPBP) show only low levels of Dat mRNA and are sparse also in Th mRNA. The presence of Vglut2 mRNA in mature Dat mRNA-positive neurons, if yet very few, might be sufficient to account for previously observed glutamate co-release in the mesoaccumbal pathway. We decided to address the role of this sparse presence of VGLUT2 within DAT neurons of the adult mouse by implementing a temporally controlled targeting strategy.

Tamoxifen-Induced Targeting of VGLUT2 in DA Neurons of Adult Mice Causes No Gross Morphological Alteration of Midbrain DA System

To specifically dissociate the role of VGLUT2 in mature DA neurons, we next employed the DAT-Cre-ERT2 transgenic mouse line in which the DAT-Cre transgene is temporally restricted (Engblom et al., 2008). By coupling of the Cre transgene to a mutated form of the estrogen nuclear hormone receptor (ERT2), the CreERT2 recombinase in these mice has been placed under control of the DAT promoter but the protein only translocates into the nucleus upon binding to the synthetic ligand tamoxifen. Recombination of any floxed alleles by the CreERT2 recombinase is thereby controlled by the time of injection of tamoxifen, which is delivered to the mouse by intraperitoneal injection (Engblom et al., 2008).

Since we have implemented and compared two different Cre-driver lines under control of the DAT promoter in this study, we will use the following abbreviations from now

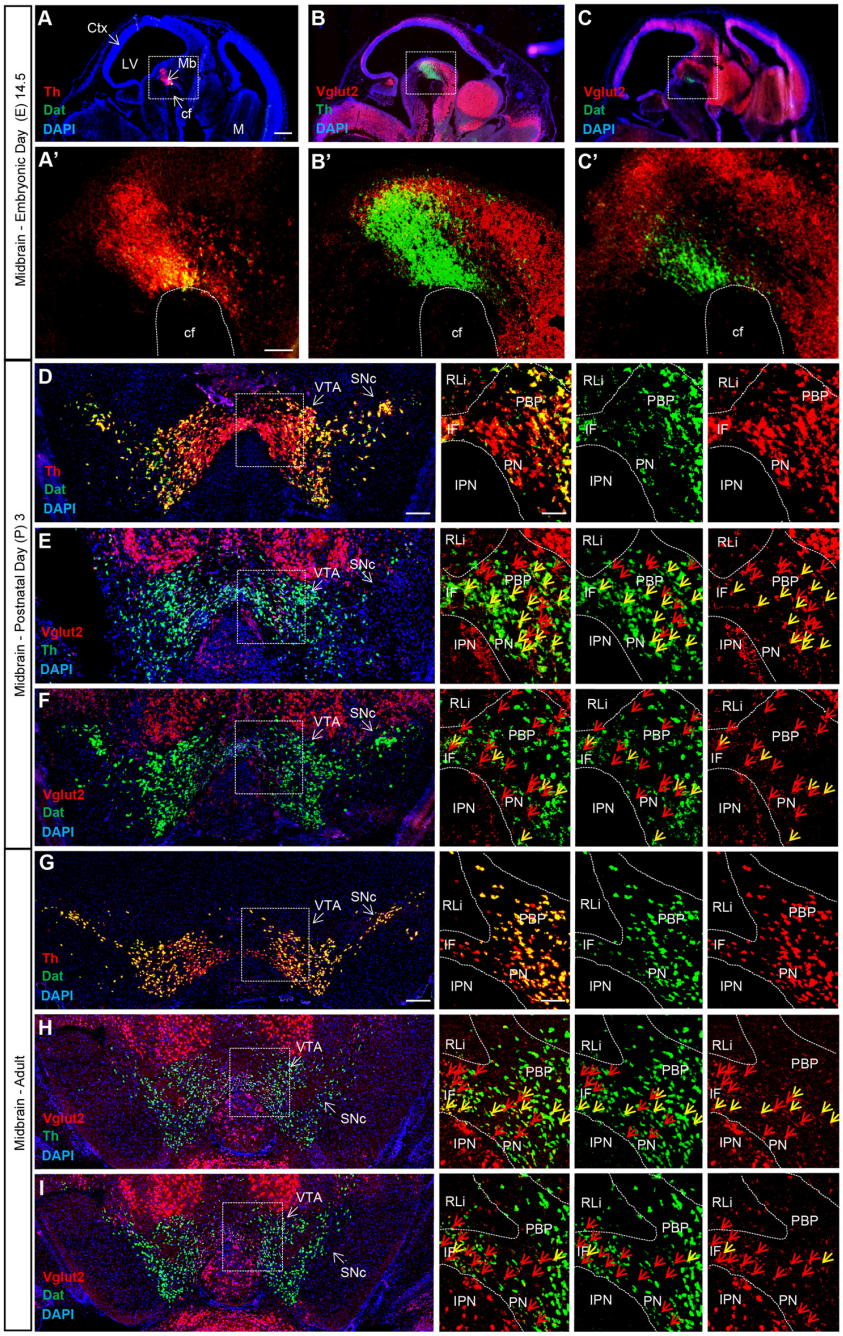

FIGURE 2 | Vglut2, Th and Dat mRNA co-localization within certain VTA dopamine (DA) neurons is sparse at E14.5, peaks around birth and is subsequently down-regulated in adulthood. Double fluorescent *in situ* hybridization for Th (red), Dat (green) and Vglut2 (red) mRNA, respectively, on wildtype mouse midbrain sections. **(A–C)** Sagittal sections of E14.5 embryo. Dotted square around the area of developing midbrain DA neurons **(A–C)** with close-ups in **(A′–C′)**. **(A)** Th and Dat mRNA show co-localization (yellow) in the ventral midbrain (scale bar 500 μm). **(A′)** higher magnification of insets (scale bar 100 μm); **(B,B′)** Th and Vglut2 mRNA expression in the midbrain. **(C,C′)** Dat and Vglut2 mRNA show sparse detection in the midbrain. **(D–F)** Coronal sections of ventral midbrain in pups of postnatal day (P) 3. **(D)** Th and Dat show ample co-localization (yellow) in the lateral VTA and SNc (scale bar 250 μm, inset 100 μm). **(E)** Th and Vglut2 mRNA and **(F)** Dat and Vglut2 mRNA prominently co-localize (yellow) at this age in the IF, PBP and PN areas (arrows) but not in the RLi of the VTA. **(G–I)** Coronal sections of the adult midbrain (10 weeks; scale bar 250 μm, inset 100 μm). **(G)** Th and Dat mRNA co-localization (yellow) remains strong; whilst the level of co-localization between **(H)** Th and Vglut2 and **(I)** Dat and Vglut2 mRNAs is lower than at P3 (arrows). Yellow arrows show co-localization green (Dat) and red (Vglut2) channel, red arrows show red (Vglut2) channel (Postnatal Day (P) 3 $n = 3$; adult $n = 3$). Abbreviations: cf, cephalic flexture; Ctx, cortex; IF, interfascicular nucleus; IPN, interpeducular nucleus; LV, lateral ventricle; M, medulla; Mb, midbrain; PBP, parabrachial pigmented area; PN, paranigral nuclei; RLi rostral linear nucleus; SNc, Substantia nigra *pars compacta*; VTA, Ventral tegmental area. See **Supplementary Figure S1** for low-magnification images of entire sections at P3 and adult as well as quantification Vglut2/Dat mRNA co-localization.

onwards: The embryonically active DAT-Cre (Ekstrand et al., 2007) has been abbreviated as eDAT-Cre while the tamoxifen-inducible DAT-Cre-ERT2 (Engblom et al., 2008) has been abbreviated as txDAT-Cre (see also "Materials and Methods" section). Throughout the study, txDAT-Cre mice were injected with tamoxifen at 8–9 weeks of age. First, to verify and compare spatial and temporal specificity of the Cre drivers, eDAT-Cre and txDAT-Cre transgenic mice were both bred with tdTom reporter mice. Offspring, $tdTom^{eDAT\text{-}Cre\text{-}tg}$ and $tdTom^{txDAT\text{-}Cre\text{-}tg}$ mice, were analyzed with histological methods. When comparing $tdTom^{eDAT\text{-}Cre\text{-}tg}$ mice with the tamoxifen-induced $tdTom^{txDAT\text{-}Cre\text{-}tg}$ mice, similar expression patterns of tdTom reporter gene expression were seen in midbrain DA areas VTA (including subareas PBP, RLi, IF, PN) and SNc as well as in striatal target regions, the dorsal striatum (DStr) and the NAc (both core and shell subareas, NAcC and NAcSh) of the ventral striatum (**Figure 3A**). The eDAT-Cre and txDAT-Cre drivers thus seem to have similar efficiency in mediating recombination of LoxP sites. As expected, tdTom overlapped well with TH immunoreactivity (**Figure 3B**). A subset of tdTom cells within the VTA, mainly within the PBP subarea, showed co-localization between tdTom and Vglut2 mRNA (**Figure 3C**).

As presented above, we previously targeted the *Vglut2* gene in DA cells during development by breeding the eDAT-Cre transgenic mouse line (Ekstrand et al., 2007) with a floxed *Vglut2* allele (Wallén-Mackenzie et al., 2006) which generated the $Vglut2^{eDAT\text{-}Cre}$ KO mouse line (Birgner et al., 2010). Now, to direct VGLUT2 targeting to mature DA neurons, we bred the same *Vglut2* allele to the txDAT-Cre transgenic line, thus generating the new $Vglut2^{txDAT\text{-}Cre}$ inducible KO mouse line, in which *Vglut2* gene expression should be ablated by recombination upon tamoxifen treatment (**Figure 4A**). To verify recombination, nested RT-PCR analysis was performed on dissected VTA, which confirmed the presence of a Vglut2 KO band (smaller band as targeted *Vglut2* allele lacks exons 4–6) and a wildtype Vglut2 band in the $Vglut2^{lx/lx;txDAT\text{-}Cre\text{-}tg}$ (txKO) midbrain, while the control $Vglut2^{lx/lx;txDAT\text{-}Cre\text{-}wt}$ (txCtrl) midbrain only contained a wildtype Vglut2 band (**Figure 4B**). Having verified successful targeting of the *Vglut2* gene in adult mice using the txDAT-Cre-driver, immunohistochemistry for TH was used to assess the integrity of the DA system. No difference in histological appearance of TH immunofluorescence in the midbrain or in the striatal target areas was seen between txKO and txCtrl midbrain (**Figures 4C,D**). Last, we compared the appearance of the tdTom reporter in the VTA and SNc area between the previously published embryonal $Vglut2^{eDAT\text{-}Cre}$ transgenic line (Birgner et al., 2010; Alsiö et al., 2011) and the newly generated tamoxifen-induced $Vglut2^{txDAT\text{-}Cre}$ transgenic line. txCtrl and txKO midbrains of the $Vglut2^{txDAT\text{-}Cre}$ transgenic line were strikingly similar to each other (**Figure 4E**) and they were both similar to the eCtrl and eKO midbrains of the $Vglut2^{eDAT\text{-}Cre}$ transgenic mouse line (**Figures 4E,F**). Taken together, these results demonstrate that tamoxifen-induced activation of txDAT-Cre leads to successful ablation of *Vglut2* gene expression in the VTA and that this removal does not cause a prominent morphological phenotype in the mesostriatal DA system.

Excitatory Postsynaptic Responses in Adult NAc Are Dependent on VGLUT2

Using an optogenetic patch clamp approach, we and others have verified that embryonal targeting of VGLUT2 using the eDAT-Cre driver abolishes post-synaptic EPSCs in the NAc (Stuber et al., 2010; Hnasko et al., 2012; Wang et al., 2017). We now implemented similar methodology to confirm the loss of post-synaptic EPSCs in NAc upon txDAT-Cre-driven VGLUT2 targeting. One week after the last tamoxifen injection, $Vglut2^{wt/wt;txDAT-Cre-tg}$ or $Vglut2^{wt/lx;txDAT-Cre-tg}$ (txCtrl) and $Vglut2^{lx/lx;txDAT\text{-}Cre\text{-}tg}$ (txKO) mice were stereotaxically injected into the VTA with AAV5-EF1a-DIO-ChR2(H134)-eYFP for subsequent *ex vivo* slice patch clamp recordings of blue light-evoked responses in the NAc (**Figure 5A**). Histological analysis following the patch clamp recordings verified that ChR2-eYFP expression in cell bodies was restricted to the VTA and co-localized with TH immunoreactivity (**Figures 5A,B**). ChR2-eYFP expression was detected in mesoaccumbal projections and within the NAc (**Figure 5B**). During electrophysiological patch clamp recordings in NAc slice preparations (**Figures 5C,D**), light-evoked EPSCs with short onset latencies (2.8 $\pm$ 0.1 ms) were detected in most recorded neurons. Response amplitudes were significantly smaller in txKO mice compared to compared to txCtrl mice (Mann-Whitney U test, p = 0.005; **Figures 5E,F**). Bath application of AMPA antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX) reduced light-evoked EPSCs by 71%, suggesting EPSCs were predominantly glutamatergic (**Figure 5F** and **Supplementary Figures S2A,B**). In some cases, we subsequently applied the NMDA antagonist (2R)-amino-5-phosphonovaleric acid (AP5) and observed a further decrease, but not complete abolishment, of light-evoked EPSCs (**Supplementary Figure S2A**). These experiments confirm the significant reduction of glutamatergic neurotransmission when *Vglut2* is gene-targeted in mature DA neurons.

Amphetamine-Induced Locomotor Sensitization Unaffected by Tamoxifen-Induced VGLUT2 Targeting in Adulthood

To address if the midbrain DA system has been functionally affected by the targeted deletion of VGLUT2 in mature DA neurons, an amphetamine-sensitization paradigm was implemented. In this experiment, we included both the embryonal $Vglut2^{eDAT\text{-}Cre}$ and the tamoxifen-induced $Vglut2^{txDAT\text{-}Cre}$ transgenic lines to enable a direct comparison between these mouse lines (**Figures 6A,A′**). All mice were given saline 1 day prior to initiation of four consecutive days of amphetamine injections. Upon 2 weeks of rest, the mice were given a challenge of one amphetamine dose (day 19). In accordance to our previous findings (Birgner et al., 2010), eKO mice showed a blunted response to amphetamine compared to eCtrl mice, that showed a potent psychostimulant-induced locomotion (two-way

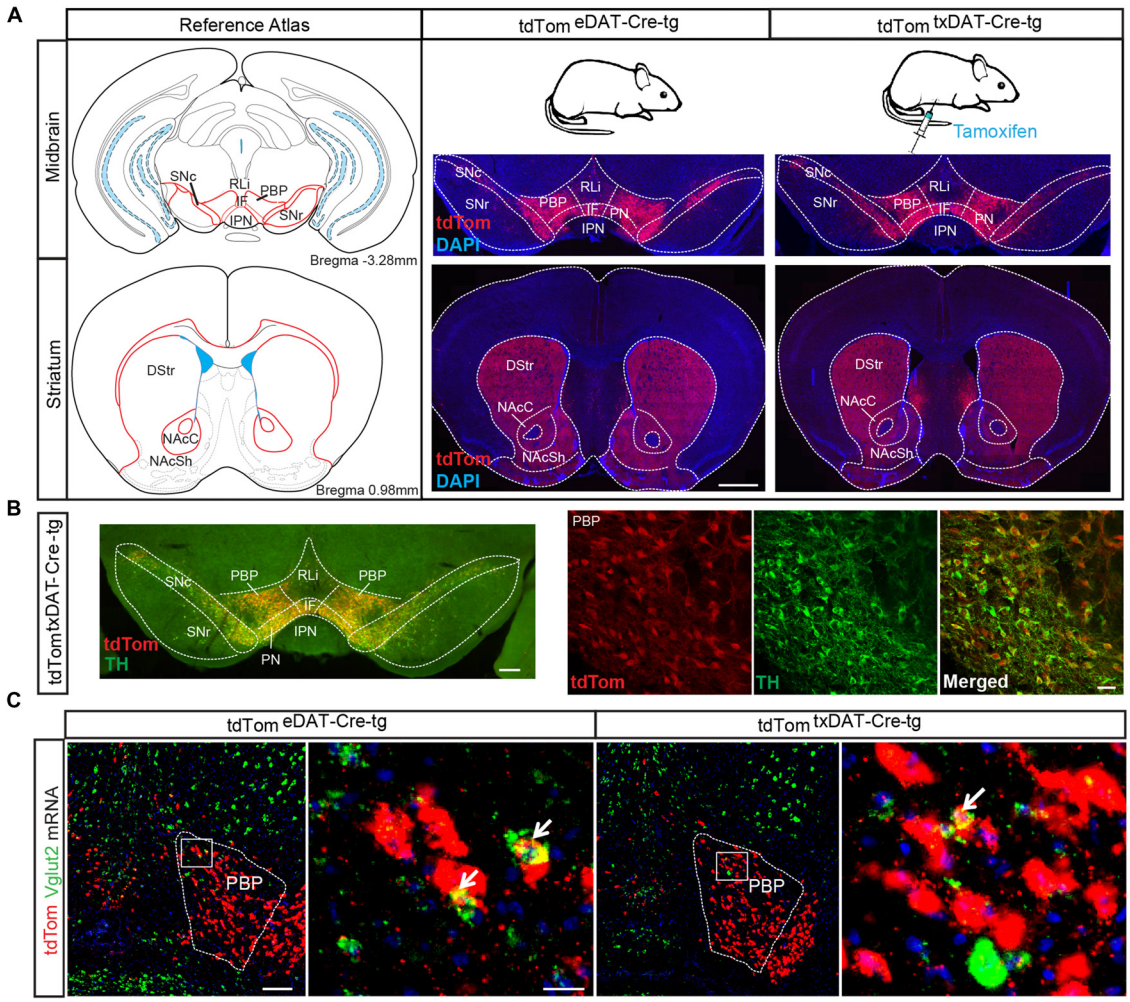

FIGURE 3 | Validation of tamoxifen-inducible DAT-Cre-mediated targeting via tdTom reporter. **(A)** tdTom-immunohistochemical analysis of midbrain and striatal sections from *tdTom*$^{eDAT\text{-}Cre\text{-}tg}$ and *tdTom*$^{txDAT\text{-}Cre\text{-}tg}$ mice show similar extent of labeling in both mouse lines, verifying a similar recombination efficiency of LoxP sites (scale bar 2 mm). **(B)** tdTom-positive labeling (red) within the VTA and SNc co-localizes with tyrosine hydroxylase (TH; green) immuno-labeling verifying selectivity to DA neurons (scale bar 250 μm and 50 μm). **(C)** Low and high magnification of fluorescent *in situ* hybridization for Vglut2 (green) and tdTom (red) mRNA in *tdTom*$^{eDAT\text{-}Cre\text{-}tg}$ and *tdTom*$^{txDAT\text{-}Cre\text{-}tg}$ mice showing co-localization in the PBP, albeit at low level (scale bar 200 μm; inset 25 μm). Abbreviations: DStr, Dorsal striatum; IF, interfascicular nucleus; IPN, interpeducular nucleus; NAcC, Nucleus accumbens core; NAcSh, Nucleus accumbens shell; PBP, parabrachial pigmented area; PN, paranigral nuclei; RLi, rostral linear nucleus; SN, Substantia nigra; SNc, Substantia nigra *pars compacta*; SNr, Substantia nigra *pars reticulata*; VTA, Ventral tegmental area; tdTom, tdTomato.

repeated measures ANOVA, treatment effect $F_{(5,20)} = 9.87$ $p < 0.001$, genotype effect $F_{(1,4)} = 99.1$ $p = 0.001$, interaction $F_{(5,20)} = 9.79$, $p < 0.001$). The degree of response did not differ between repeated or acute administration of amphetamine (**Figure 6B**). In contrast to the *Vglut2*$^{eDAT\text{-}Cre}$ transgenic line, loss of VGLUT2 from mature DA neurons did not seem to affect amphetamine-induced sensitization, as both txCtrl and txKO of the *Vglut2*$^{txDAT\text{-}Cre}$ transgenic line showed a similar increase in locomotion in response to amphetamine compared to saline (two-way repeated measures-ANOVA, treatment effect $F_{(5,80)} = 4.99$ $p < 0.001$, genotype effect $F_{(1,16)} = 0.868$ $p = 0.37$, interaction $F_{(5,80)} = 0.32$, $p = 0.90$; **Figure 6B′**).

To ascertain the behavioral response upon amphetamine sensitization further, we decided to perform a second round of amphetamine injections in the same mice. All mice therefore received amphetamine during four additional days (days 27–30), after which they were given one challenge 1 week (day 37) and then another challenge yet another week later (day 44; **Figures 6A,A′**). The results were similar to those seen during the first round of injections. There was a strong and significant difference between eCtrl and eKO mice, with eKO displaying a blunted response to amphetamine (eDAT-Cre two-way repeated measures ANOVA, treatment effect $F_{(6,24)} = 6.64$, $p < 0.001$, genotype effect $F_{(1,4)} = 16.6$ $p = 0.02$, interaction $F_{(6,24)} = 6.33$, $p < 0.001$). In contrast,

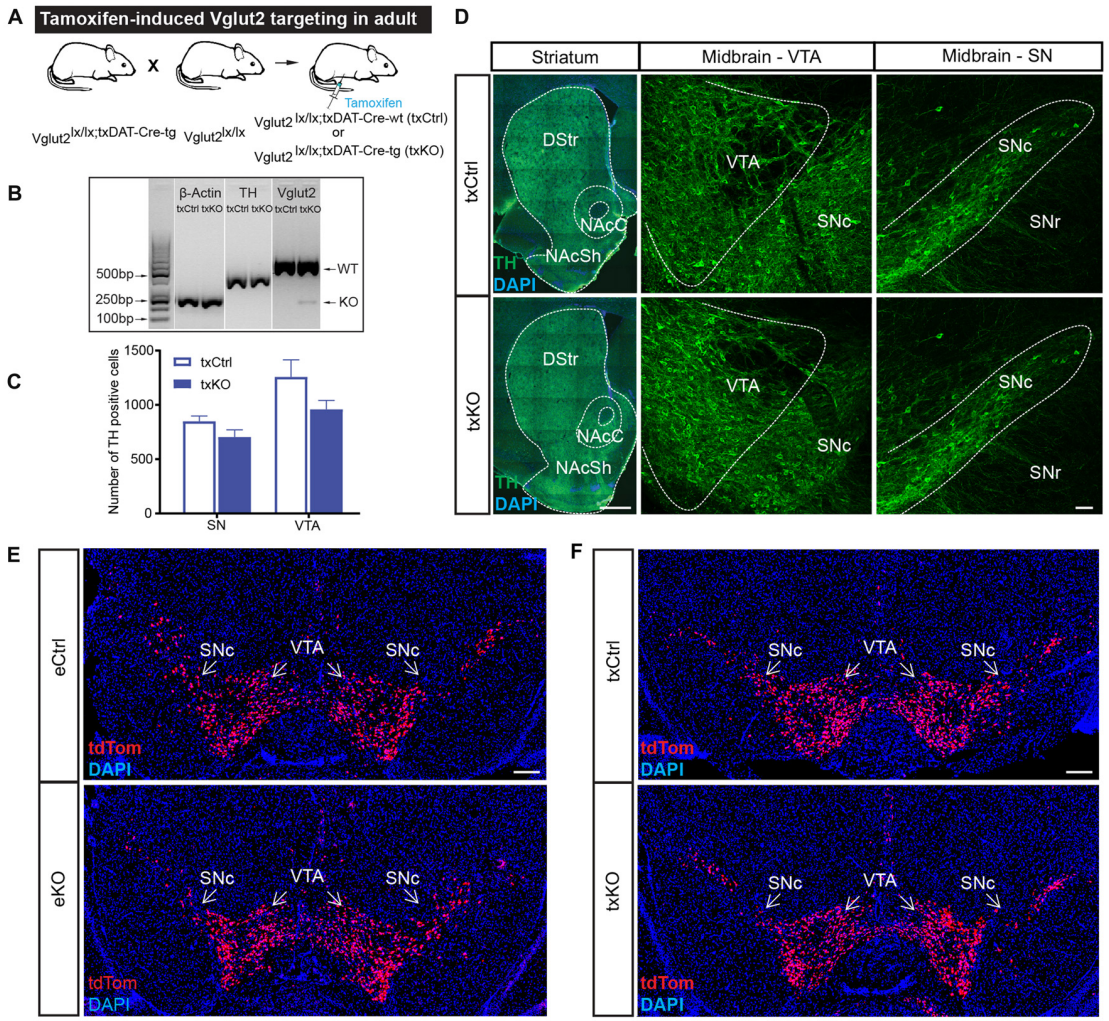

FIGURE 4 | Confirmation of tamoxifen-induced targeting of the *Vglut2* gene using tamoxifen-inducible DAT-Cre transgene and validation of intact midbrain DA system. **(A)** Schematic illustration of breeding strategy to generate $Vglut2^{lx/lx;txDAT\text{-}Cre\text{-}wt}$ (txCtrl) and $Vglut2^{lx/lx;txDAT\text{-}Cre\text{-}wt}$ (txKO). **(B)** Confirmation of *Vglut2* gene targeting using the tamoxifen-inducible DAT-CReERT2 (txDAT-Cre) mouse line. Nested RT-PCR for β-actin, TH and Vglut2 from dissected VTA. Vglut2 wildtype band (500 bp) was observed in both txCtrl and txKO midbrain, while knockout (KO) band (KO; 250 bp) was only found in the gene targeted midbrain. β-actin and TH served as controls. **(C)** Number of TH positive neurons in the SN and VTA did not differ between txCtrl and txKO. Two-way ANOVA with Sidak *post hoc* for SN and VTA along three rostro-caudal section (txCtrl $n = 3$; txKO $n = 3$). **(D)** TH immunoreactivity in striatum and midbrain in txCtrl and txKO mice (scale bar 500 μm (striatum) and 50 μm (midbrain). **(E,F)** Cre-driven tdTom expression in ventral midbrain of eCtrl (**E**, top), eKO (**E**, bottom), txCtrl (**F**, top) and txKO (**F**, bottom) (scale bar 250 μm). Abbreviations: DStr, Dorsal striatum; NAcC, Nucleus accumbens core; NAcSh, Nucleus accumbens shell; SNc, Substantia nigra *pars compacta*; SNr, Substantia nigra pars reticulata; VTA, Ventral tegmental area; tdTom, tdTomato.

no significant difference was seen between txCtrl and txKO (txDAT-Cre two-way repeated measures ANOVA treatment effect $F_{(6,96)} = 6.62$, $p < 0.001$, genotype effect $F_{(1,16)} = 0.088$ $p = 0.77$, interaction $F_{(6,96)} = 0.25$, $p = 0.96$), similarly to the first round of sensitization. The degree of locomotion did not differ between the first and last day of repeated amphetamine treatment or following acute injections of amphetamine on day 37 (challenge 1) and day 44 (challenge 2; **Figures 6B,B′**). These findings illustrate that DAT-Cre-driven targeting of *Vglut2* gene expression causes completely different behavioral effects in eKO (VGLUT2 targeting in embryogenesis) and txKO (VGLUT2 targeting in adulthood) mice, thus firmly demonstrating that the temporal onset of the VGLUT2 targeting event in DA neurons is crucial for behavioral outcome of relevance to drug addiction.

Maintained Cocaine-Induced Locomotor Sensitization and Elevated Baseline AMPA/NMDA Ratio

Repeated exposure to cocaine causes lasting adaptations of excitatory synaptic transmission onto DRD1-MSNs in the NAc (Engblom et al., 2008; Mameli et al., 2009; Pascoli et al., 2011, 2014). With repeated cocaine exposure, calcium-permeable

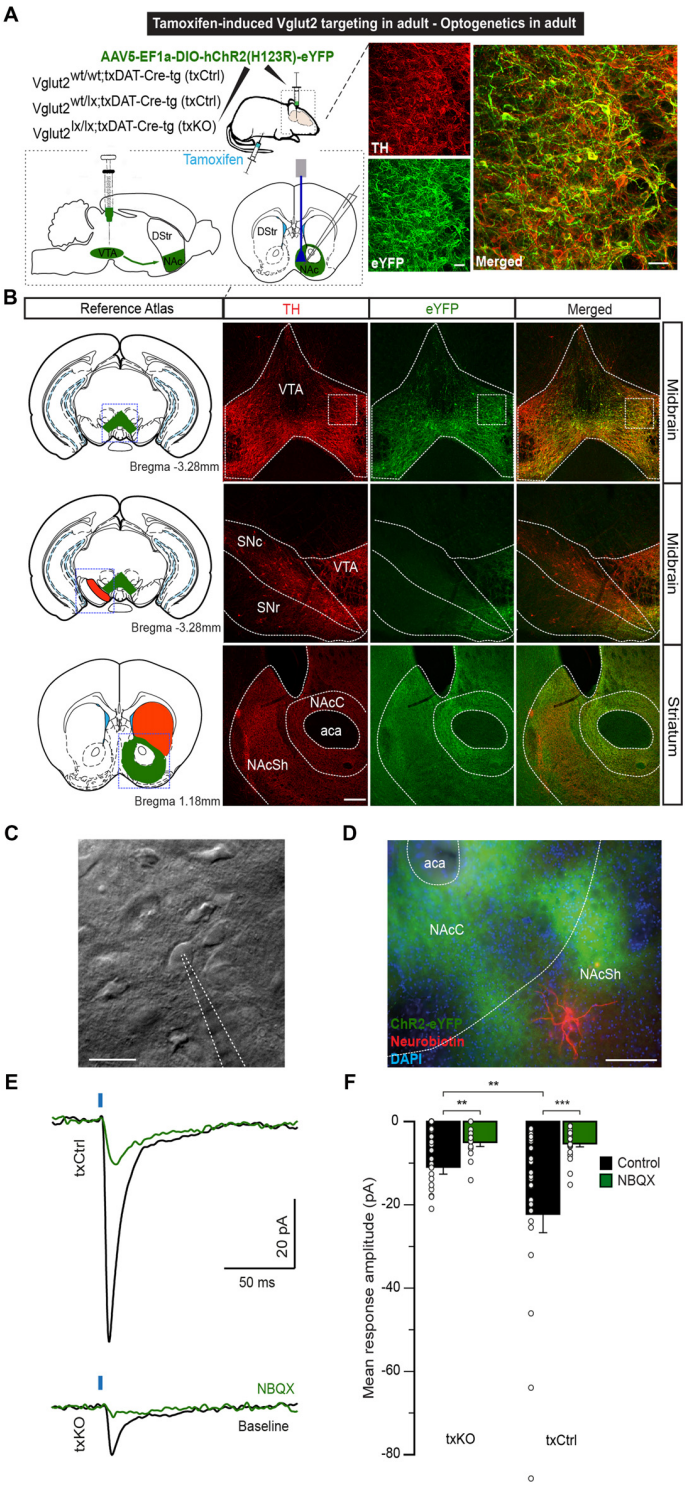

FIGURE 5 | Optogenetics-driven stimulation of dopaminergic fibers in the NAcSh evokes VGLUT2-dependent responses. **(A)** Eight-week-old txCtrl and txKO mice were tamoxifen-treated and 1 week later stereotaxically injected with of AAV5-EF1a-DIO-hChR2(H123R)-eYFP into the VTA. Glutamate release was recorded in the NAcSh upon optical stimulation. **(B)** ChR2 expression was restricted to the VTA and colocalized with TH immunoreactivity. Inset: eYFP and TH immunofluorescence showing ample co-localization (scale bar 50 μm). No eYFP expression was detected in the SNc or SNr. ChR2 expression was detected in the projecting fibers to the NAc of the ventral striatum (scale bar 200 μm). **(C)** Representative example of accumbal cell imaged under IR-DIC and patched with 4–6 MΩ patch pipettes (scale bar 20 μm). **(D)** Patched neurons were filled with neurobiotin and stained with Cy5-conjugated streptavidin. Blue: DAPI, Green: eYFP, Red: Cy5 (scale bar 200 μm).
(*Continued*)

FIGURE 5 | Continued
(E) Representative average traces of light-induced responses in NAc neurons in txCtrl and txKO, before and after bath application of NBQX. **(F)** Light-evoked response amplitude for txKO and txCtrl mice. txKO exhibited smaller responses compared to txCtrl in control, but not under NBQX conditions. Unpaired Mann-Whitney test, $^{**}p < 0.01$, $^{***}p < 0.001$ (txCtrl $n = 25$, 3 mice; txKO $n = 24$, 3 mice). Abbreviation: aca; anterior commissure, anterior part, DStr, Dorsal striatum; NAc, Nucleus accumbens; NAcC, Nucleus accumbens core; NAcSh, Nucleus accumbens shell; SNc, Substantia nigra pars compacta; SNr, Substantia nigra pars reticulata; VTA, Ventral tegmental area.

AMPA receptors are inserted into accumbal DRD1-MSNs, which is reflected by an increase in the AMPA to NMDA ratio and rectifying AMPAR-mediated currents in these cells. The prefrontal cortex, amygdala and hippocampus provide glutamatergic input to these neurons and mediate behavioral features of drug-adaptive behavior, including locomotor sensitization (Creed et al., 2015; Pascoli et al., 2015). The putative contribution of glutamate co-release from glutamate-DA neurons in the VTA to alterations in cocaine-evoked plasticity in DRD1-MSNs and subsequent behavior has never been investigated. To enable the analysis of putative contribution from VGLUT2-mediated co-release during development and in adulthood, we included both the embryonal *Vglut2*$^{eDAT\text{-}Cre}$ and the tamoxifen-induced *Vglut2*$^{txDAT\text{-}Cre}$ transgenic lines. To identify DRD1-MSNs in accumbal slice preparations and to enable the comparison between developmental and adult VGLUT2 targeting, both the *Vglut2*$^{eDAT\text{-}Cre}$ and the *Vglut2*$^{txDAT\text{-}Cre}$ mouse lines were bred with a DRD1-EGFP reporter line generating eCtrl, eKO, txCtrl and txKO mice expressing the DRD1-EGFP reporter (referred to as eCtrl-DRD1, eKO-DRD1, txCtrl-DRD1, txKO-DRD1). Mice were subjected to a cocaine-induced sensitization paradigm and following 10 days of cocaine withdrawal, patch clamp recordings were performed in DRD1-MSNs of the NAc (**Figures 7A,A′**). Cocaine administration resulted in increased locomotion with significantly higher activity compared to saline seen already on day 3 in both mouse lines irrespective of genotype (eDAT-Cre two-way repeated measures ANOVA Effect of, time effect $F_{(7,77)} = 7.43$, $p < 0.001$, treatment effect

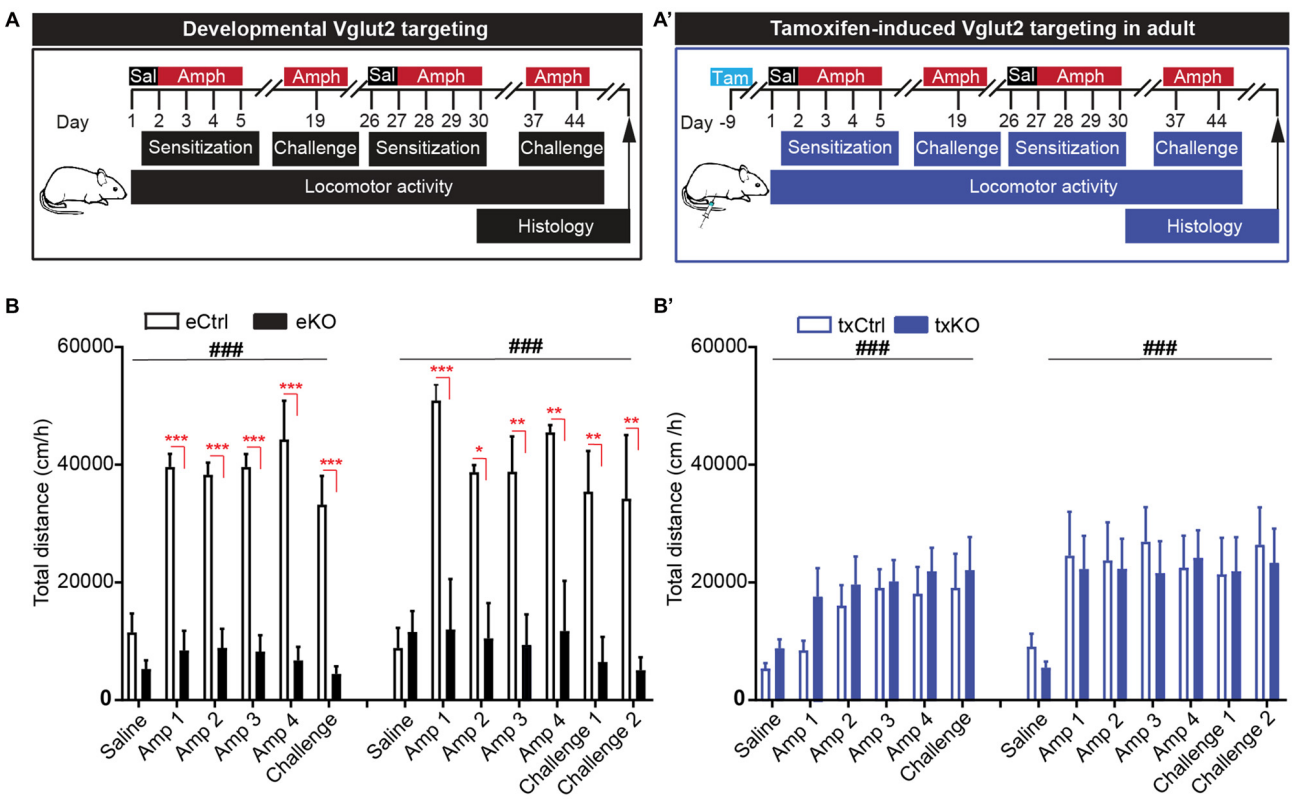

FIGURE 6 | Amphetamine-induced locomotor sensitization is impeded upon VGLUT2 targeting from developing, but not mature, DA neurons. **(A,A′)** Schematic illustration of amphetamine-induced sensitization paradigm in **(A)** eCtrl and eKO or **(A′)** txCtrl and txKO mice. Animals received a single dose of saline (Day 1) followed by four consecutive injections of amphetamine (3 mg/kg; Days 2–5). Acute dose of amphetamine (Challenge) was administered after a washout period of 14 days. A week later (Day 26), the protocol was repeated and included two acute challenges (Challenge 1 and Challenge 2) on days 37 and day 44, respectively. **(B)** eKO show blunted response to amphetamine compared to eCtrl during both rounds of sensitization. There was no difference in locomotion between last days of repeated injection (Amp 4) and acute challenges. **(B′)** Both txCtrl and txKO showed an increase in amphetamine-induced locomotion compared to saline and no difference was observed between genotypes. Two-way repeated measures ANOVA with Sidak *post hoc* test (ANOVA $^{###}p < 0.001$; *post hoc* between genotype $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; eCtrl $n = 3$; eKO $n = 3$; txCtrl $n = 7$; txKO $n = 11$).

$F_{(3,11)}$ = 3.93, p = 0.04, interaction $F_{(21,77)}$ = 3.81, $p < 0.001$; txDAT-Cre two-way repeated measures ANOVA, time effect $F_{(7,147)}$ = 10.1, $p < 0.001$, treatment effect $F_{(3,21)}$ = 5.59, p = 0.006, interaction $F_{(21,147)}$ = 3.17, $p < 0.001$; **Figures 7B,B′**). However, whilst both eKO-DRD1 and txKO-DRD1 covered greater distance compared to their equivalent controls following cocaine treatment, a significant difference between genotypes was only detected on day 5 between eKO-DRD1 and eCtrl-DRD1 (Tukey's *post hoc* p = 0.01) and not between txKO-DRD1 and txCtrl-DRD1 (Tukey's *post hoc* p = 0.47; **Figures 7B,B′**).

Electrically-evoked EPSCs from DRD1-MSNs were subsequently recorded at different holding potentials (−70 mV, 0 mV, +40 mV) under bath-application of 100 μm picrotoxin and 50 μm APV to isolate AMPA currents. Whole-cell patch clamp recordings *ex vivo* did not reveal differences in inward rectification between treatment nor genotype (two-way ANOVA, treatment effect $F_{(1,45)}$ = 2.25, p = 0.14; genotype effect $F_{(1,45)}$ = 0.03, p = 0.86, interaction $F_{(1,45)}$ = 0.02, p = 0.90) with RI of 1.00 ± 0.08 for eCtrl-DRD1 and 0.99 ± 0.07 for eKO-DRD1 under baseline conditions and 1.39 ± 0.14 for eCtrl-DRD1 and 1.32 ± 0.14 for eKO-DRD1 following cocaine (**Figure 7C** with representative trace). In contrast, patched cells from the inducible DAT-Cre mouse line did not differ between the genotypes after saline administration but showed an inward rectification following cocaine administration for the txCtrl-DRD1 compared to saline with rectification indices (RI) of 1.03 ± 0.13 for txCtrl-DRD1 and 1.03 ± 0.06 for txKO-DRD1 under saline, 1.37 ± 0.09 for txCtrl-DRD1 1.34 ± 0.11 for txKO-DRD1 with cocaine (two-way ANOVA treatment effect $F_{(1,27)}$ = 10.1, p = 0.004, genotype effect $F_{(1,27)}$ = 0.02, p = 0.90, interaction $F_{(1,27)}$ = 0.02, p = 0.89; Sidak *post hoc* saline vs. cocaine txCtrl p = 0.04; txKO p = 0.10; **Figure 7C′** with representative trace).

The NMDA current was obtained by subtraction of evoked current at +40 mV before and after the application of 50 μm of D-AP5. Targeted deletion of VGLUT2 from DA neurons during development did not affect the AMPA/NMDA ratio under saline or cocaine conditions between or within genotype with ratios of 0.96 ± 0.12 and 1.19 ± 0.28 for eCtrls-DRD1 and eKO-DRD1, respectively, under saline and ratios of 1.47 ± 0.08 (eCtrl-DRD1) and 1.19 ± 0.09 (eKO-DRD1) under cocaine (two-way ANOVA treatment effect $F_{(1,56)}$ = 2.59, p = 0.11, genotype effect $F_{(1,56)}$ = 0.03, p = 0.87, interaction $F_{(1,56)}$ = 2.58, p = 0.11 (**Figure 7D**). A significant effect of cocaine treatment and genotype was, however, detected in the inducible line (two-way ANOVA treatment effect $F_{(1,35)}$ = 6.28, p = 0.017, genotype effect $F_{(1,35)}$ = 5.08, p = 0.03, interaction $F_{(1,35)}$ = 2.57, p = 0.118). A significant higher AMPA/NMDA ratio was observed in saline-treated txKO-DRD1 mice (1.38 ± 0.13) with respect to saline-treated txCtrls-DRD1 (0.9 ± 0.15; Sidak *post hoc* p = 0.023). As expected cocaine treatment significantly increased the AMPA/NMDA ratio in txCtrl-DRD1 mice (Sidak *post hoc* p = 0.008; **Figure 7D′**). Interestingly, and in contrast to the txCtrl-DRD1 mice, the elevated AMPA/NMDA ratio detected at baseline conditions in txKO-DRD1 mice prevented cocaine treatment to lead to significantly higher AMPA/NMDA ratio in those mice (Sidak *post hoc* p = 0.80; **Figure 7D′**). Finally, no difference was detected between cocaine-treated txCtrl-DRD1 and txKO-DRD1 mice with ratios of 1.41 ± 0.09 and 1.49 ± 0.13, respectively (Sidak *post hoc* p = 0.871; **Figure 7D′**). In summary, while no significant effects were observed upon VGLUT2 targeting in embryogenesis, removal of VGLUT2 in mature DA neurons seems sufficient to increase AMPA/NMDA ratio in accumbal MSNs to such a level that cocaine-evoked plasticity is occluded further. This experiment reveals that VGLUT2-mediated glutamate co-release from mature VTA DA neurons is crucial for maintaining baseline AMPA/NMDA ratio of MSNs in the mesoaccumbal projection.

DISCUSSION

In this study, we created a tamoxifen-inducible *Vglut2*$^{txDAT\text{-}Cre}$ mouse line to selectively probe the role of VGLUT2 in mature DA neurons. We show that this temporally restricted *Vglut2* gene ablation in DAT-Cre-expressing DA neurons causes a significant decrease in glutamatergic neurotransmission in the NAc, thus demonstrating that mesoaccumbal glutamate release from VTA DA neurons is mediated via VGLUT2. The main behavioral findings we present are that *Vglut2*$^{txDAT\text{-}Cre}$ txKO mice showed the same elevated level of locomotor response upon injections of the addictive substances amphetamine and cocaine as observed in control animals, a finding which is strikingly different from the blunted phenotype observed in *Vglut2*$^{eDAT\text{-}Cre}$ eKO mice, in which VGLUT2 is deleted from embryogenesis. Finally, we demonstrate that glutamate release from mature VTA DA neurons significantly affects synaptic plasticity of MSNs in the NAc. By measuring the AMPA/NMDA ratio of DRD1-expressing MSNs, we identified a strongly elevated baseline AMPA/NMDA ratio in txKO compared to control mice. This effect was sufficiently potent to occlude any further effect on this measure of synaptic plasticity by cocaine. It is known that MSNs receive glutamatergic terminals from forebrain neurons onto the head of the dendritic spine, while VTA-derived DA reaches the neck region and can modulate the responsiveness of MSN spines to excitatory transmission (Russo et al., 2010; Yetnikoff et al., 2014). This interaction between midbrain dopaminergic and forebrain glutamatergic neurons has been established as a core feature for several stages of addiction, including drug-seeking and relapse (Lüscher and Malenka, 2011; Pascoli et al., 2015). The current results show for the first time that VGLUT2-mediated glutamatergic transmission from mature VTA DA neurons contributes to this critical interaction on NAc MSNs.

The role of VGLUT2 in mature DA neurons has been a matter of debate not least since the use of different experimental approaches in different laboratories has made it difficult to establish the amount of VTA DA neurons that express this transporter (El Mestikawy et al., 2011; Trudeau et al., 2014). VTA DA neurons develop in the embryonal midbrain from midgestation with TH immunoreactivity appearing in post-mitotic neurons already around E11 followed by DAT

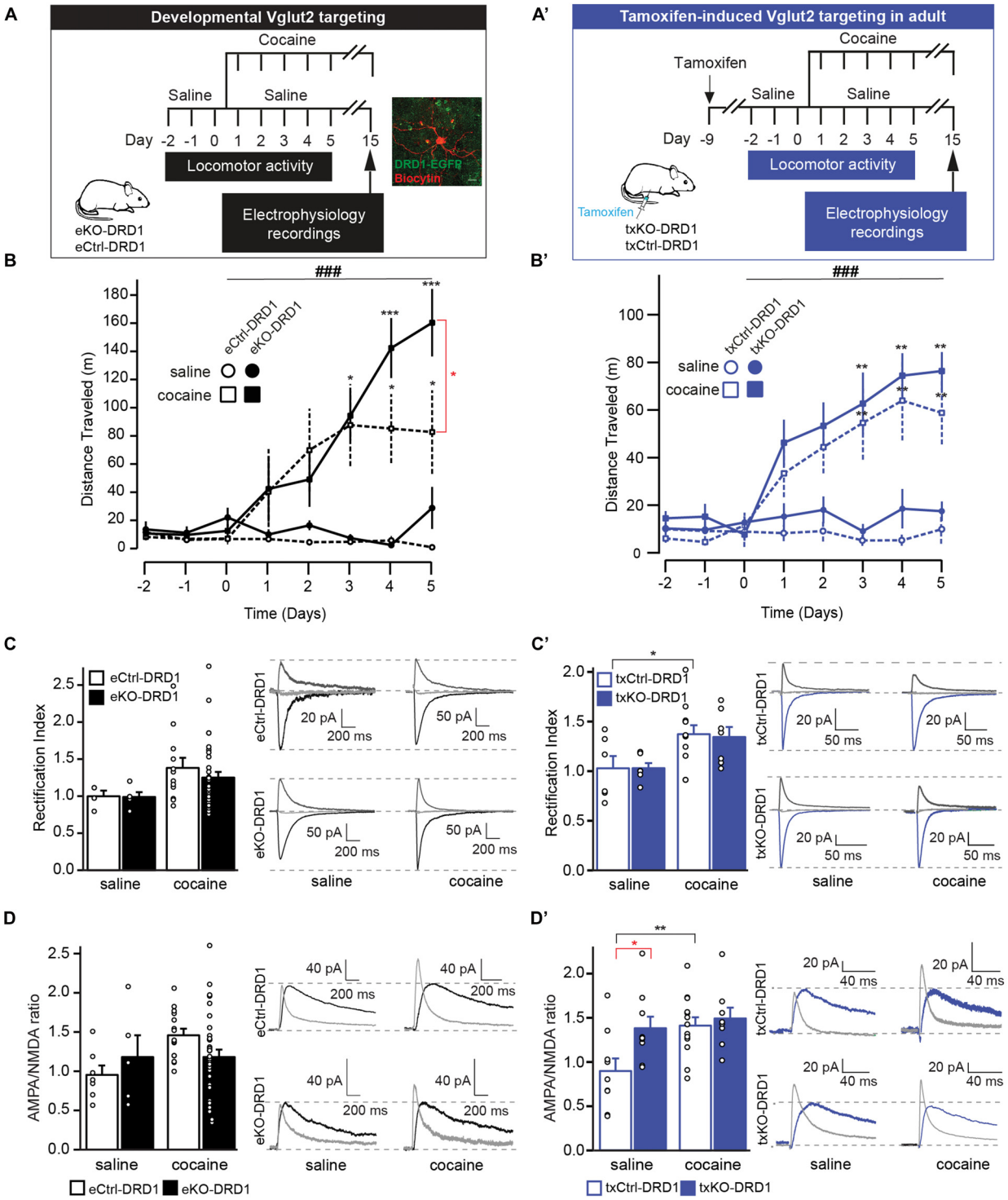

FIGURE 7 | VGLUT2 targeting in mature, but not developing, DA results in maintained cocaine-induced sensitization and elevated baseline AMPA/NMDA ratio. **(A,A')** Schematic timeline of cocaine-induced behavioral locomotor sensitization and subsequent electrophysiological recordings for **(A)** eKO-DRD1 and eCtrl-DRD1 and **(A')** tamoxifen-induced txKO-DRD1 and txCtrl-DRD1 mice. All mice were habituated to the arena for 3 days during which they received saline injections (i.p.) Starting from "Day 1," mice received either cocaine (20 mg/kg i.p.) or saline injections (i.p.) before behavioral testing for 5 days, then kept for additionally 10 days in their home cage. On Day 15, mice were sacrificed and whole-cell patch clamp experiments performed on brain slices. In **(A)** is also shown a fluorescent image of DRD1-expressing cells (EGFP; green) used in whole-cell patch clamp experiments filled with biocytin and stained with streptavidin (red). **(B,B')** Cocaine-induced locomotor sensitization measured as distance travelled following saline or cocaine injections, respectively. **(B)** eKO-DRD1 and eCtr-DRD1. **(B')** txKO-DRD1 and

(*Continued*)

FIGURE 7 | Continued
txCtrl-DRD1. **(C)** Rectification index (RI) and raw traces recorded cells from eKO-DRD1 and eCtrl-DRD1 mice treated with saline or cocaine at −70 mV (black), 0 mV (light gray) and +40 mV (dark gray). **(C')** RI and raw traces recorded cells from txKO-DRD1 and txCtrl-DRD1 mice treated with saline or cocaine at −70 mV (blue), 0 mV (light gray) and +40 mV (dark gray). **(D)** AMPA/NMDA ratio and raw traces of cells from eKO-DRD1 and eCtrl-DRD1 mice treated with saline or cocaine for NMDA current (black); AMPA current (light gray). **(D')** AMPA/NMDA ratio and raw traces of cells from txKO-DRD1 and txCtrl-DRD1 mice treated with saline or cocaine for NMDA current (blue); AMPA current (light gray). Two-way repeated measures ANOVA Tukey's *post hoc* (behavior) and two-way ANOVA Sidak *post hoc* (electrophysiology) (ANOVA $^{###}p < 0.001$; *post hoc* between genotype $^{*}p < 0.05$ and *post hoc* between treatment of same genotype: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; Behavior: saline: eCtrl, $n = 3$; eKO, $n = 2$, cocaine: eCtrl, $n = 5$; eKO, $n = 5$, saline: txCtrl, $n = 4$; txKO, $n = 4$, cocaine: txCtrl, $n = 9$; txKO, $n = 8$; electrophysiology: RI saline: eCtrl, $n = 4$, eKO, $n = 5$; cocaine: eCtrl, $n = 12$,: eKO, $n = 28$; saline txCtrl, $n = 6$, txKO, $n = 6$; cocaine txCtrl, $n = 12$: txKO, $n = 7$; AMPA/NMDA ratio saline: eCtrl, $n = 7$, eKO, $n = 5$; cocaine: eCtrl, $n = 15$; eKO, $n = 33$; saline: txCtrl, $n = 9$, txKO, $n = 9$; cocaine: txCtrl, $n = 13$, tKO, $n = 7$). All mice expressed the DRD1-EGFP transgene.

at E13 (Ang, 2006). Vglut2 mRNA has been observed within a subset of post-mitotic TH-positive DA neurons as early as E12.5 (Birgner et al., 2010; Nordenankar et al., 2014). Using fluorescent *in situ* hybridization for Vglut2, Th and Dat mRNAs, we now show that the mouse *Vglut2* gene has a peak of co-expression with endogenous Th and Dat around birth, with only sparse co-expression detected in embryonal (E14.5) and adult DA neurons. Notably, as thoroughly described in the rat (reviewed in Morales and Margolis, 2017), and confirmed in the present study, Vglut2 mRNA is stronger in the medial than lateral aspect of the VTA. However, Dat mRNA, but not Th mRNA, shows the opposite expression pattern with stronger lateral than medial expression. These inverse expression patterns between *Vglut2* and *Dat* genes help explain the current observation that the extent of co-localization between Vglut2 and Th mRNAs is higher than between Vglut2 and Dat mRNAs. The histological spatio-temporal mapping of Vglut2, Th and Dat mRNA performed in the present study should be useful to increase the understanding of the glutamate-DA co-releasing cells and their role in mesoaccumbal neurocircuitry.

It is noteworthy that not all midbrain DA neurons co-express the *Th* and *Dat* genes. This is of particular interest in the transgenic context when using their promoters to drive Cre-recombinase for gene targeting of floxed alleles (Lammel et al., 2015; Pupe and Wallén-Mackenzie, 2015). For example, expression of the *Vglut2* gene in Th-positive cells lacking *Dat* gene expression is of interest since not only different DAT-Cre-drivers (Birgner et al., 2010; Hnasko et al., 2010; Alsiö et al., 2011; Fortin et al., 2012), but also a TH-Cre-driver has been used to target *Vglut2* gene expression in mice (Nordenankar et al., 2014). This targeting event resulted in somewhat different phenotypes than obtained upon DAT-Cre-mediated targeting and based on an observed promiscuity of the TH-Cre driver in non-monoaminergic cells, the obtained phenotype was difficult to pinpoint (Nordenankar et al., 2014).

Despite the low amount of VTA DAT neurons containing Vglut2 mRNA observed in the histological analyses of the adult mouse midbrain, our electrophysiological data corroborate previous findings showing that optogenetic stimulation of mature DAT-Cre neurons in the VTA causes EPSCs in MSNs of the NAc (Stuber et al., 2010; Tecuapetla et al., 2010; Mingote et al., 2015). We can show that these currents significantly decrease in amplitude upon VGLUT2 ablation in mature VTA DA neurons. This finding is similar, but not identical, to previous analyses where optogenetic stimulations were performed in the embryonal $Vglut2^{eDAT\text{-}Cre}$ eKO mice, in which glutamate co-release was found completely abolished (Hnasko et al., 2010; Stuber et al., 2010; Wang et al., 2017). The results we present in the current study using tamoxifen-induced VGLUT2 targeting in adulthood demonstrate that glutamate co-release in mature DA neurons is to a large part dependent on VGLUT2.

When comparing previous reports of developmental $Vglut2^{eDAT\text{-}Cre}$ targeting (Birgner et al., 2010; Hnasko et al., 2010; Alsiö et al., 2011; Fortin et al., 2012) with the phenotypes obtained in the new $Vglut2^{txDAT\text{-}Cre}$ targeting of VGLUT2 in mature DA neurons presented here, a major difference is the locomotor response to psychostimulants. By comparing $Vglut2^{eDAT\text{-}Cre}$ eKO and $Vglut2^{txDAT\text{-}Cre}$ txKO and relevant control mice within the same experimental sensitization setup, we can firmly demonstrate that adult VGLUT2 targeting does not affect amphetamine-induced locomotor response while, as previously shown (Birgner et al., 2010), developmental targeting causes a strongly blunted response. This observation suggests that down-regulation of *Vglut2* gene expression levels from embryogenesis might pre-dispose to addictive-like behavior. Indeed, in a study of common haplotype tag-single nucleotide polymorphism, SNP, in the VGLUT genes in individuals suffering from substance use disorder, one SNP of the VGLUT2 gene (rs2290045) showed significant association with severe alcoholism (Comasco et al., 2014).

Synaptic plasticity, such as long-term potentiation and long-term depression (Malinow and Malenka, 2002; Kessels and Malinow, 2009) is also implicated in behavioral sensitization, in which repeated exposure to cocaine results in an increased locomotor response (Thomas et al., 2001). Cocaine administration has been shown to result in an increase of AMPA-mediated currents in DRD1-MSNs of the NAc (Pascoli et al., 2011; Creed et al., 2015) and replacement of GluR2-containing AMPA receptors to ones lacking the GluR2 subunit in VTA DA neurons (Bellone and Lüscher, 2006; Mameli et al., 2007). GluR2-lacking AMPA receptors exhibit higher peak conductance, permeability for Ca^{2+} and, as a result, inward-rectifying properties (Conrad et al., 2008; Wolf and Ferrario, 2010). In the present study, DRD1-MSNs in the NAc of $Vglut2^{txDAT\text{-}Cre}$ txKO mice exhibited a higher AMPA/NMDA ratio than txCtrl mice at saline conditions, which was, however, not matched by inward-rectification, indicating the maintained presence of GluR2-containing AMPA receptors in txKO under baseline conditions. Following cocaine administration, DRD1-MSN of both txKO and txCtrl mice exhibited an increase in RI. The shift coincided with

an increase in AMPA/NMDA ratio in the txCtrl, while the AMPA/NMDA ratio was not further altered in *Vglut2*$^{txDAT\text{-}Cre}$ txKO animals, suggesting that the synapses in the txKO are already potentiated due to disrupted glutamate co-release. This enhanced baseline level of AMPA/NMDA ratio may mask cocaine-induced plasticity as seen in txCtrl, and in turn explain the absent differences between the two genotypes in cocaine-induced behavioral sensitization. Based on these new observations, we propose that disruption of glutamate co-release in mature dopaminergic neurons of the VTA leads to alterations in baseline AMPA and NMDA currents in DRD1-MSN.

Taken together with our observations of significantly reduced glutamatergic post-synaptic currents in the MSN, our study shows that ablation of VGLUT2 in the mature mesoaccumbal DA circuitry leads to a measurable effect on both glutamatergic neurotransmission and synaptic plasticity of MSNs in the NAc. Since no study has addressed the consequence of VGLUT2-mediated glutamate co-release on AMPA/NMDA ratio in accumbal MSNs before, further studies will be required to fully explain the mechanism on molecular level. Clearly, ablation of VGLUT2 in adulthood followed through by behavioral and electrophysiological measurements within a restricted time span has the added benefit of a substantially restricted level of compensatory adaptations that might occur when ablation takes place during embryonal brain development. Indeed, we have previously shown that eKO mice have higher baseline levels of immediate early genes *c-fos* and *Nur77* in the striatal complex, to such a level that cocaine sensitization failed to increase them further (Alsiö et al., 2011). The current txKO line should be of particular use in the context of co-release in behavioral and molecular plasticity.

In this study, we pin-point that ablation of VGLUT2-mediated co-release from midbrain DA neurons during adulthood has a significant effect on AMPA/NMDA ratio but leaves amphetamine- and cocaine induced locomotion intact. While *Vglut2*$^{txDAT\text{-}Cre}$ txKO mice show normal locomotor sensitization in the cocaine paradigm, we had expected, based on previous studies (Hnasko et al., 2010), a blunted cocaine-induced locomotor response in the *Vglut2*$^{eDAT\text{-}Cre}$ eKO mice. However, in this experiment, there was no overall difference between genotypes in the *Vglut2*$^{eDAT\text{-}Cre}$ line, apart from the last day of cocaine administration in which eKO mice had higher locomotion than controls. This discrepancy is likely due to different genetic backgrounds between mice used in different laboratories, which highlights a need for common animal pools to enable direct comparison in terms of behavior and circuitry function. Together with our current observation, these findings support the need for genetic models implementing targeting in adulthood when attempting to study effects of manipulated glutamatergic neurotransmission. In addition, a recent study has demonstrated circadian-dependance in DA-related behavior influenced by VGLUT3, a sister molecule of VGLUT2 which is present in striatal cholinergic interneurons (Divito et al., 2015), showing that also wake-sleep cycle should be considered when addressing the role of VGLUTs in behavior, and also when comparing data obtained in different laboratories. Further, in contrast to targeting at a distinct time-point during adulthood, developmental targeting of VGLUT2 in DA neurons likely causes compensatory adaptations which may help explain the lack of post-synaptic phenotype in recorded MSNs. However, the relatively low amount of recording points might also contribute to the lack of statistical significance in this experiment. Further analyses would be crucial in order to fully understand the post-synaptic circuitry effects by glutamate-DA co-release.

Despite first described some two decades ago, the lack of selective experimental approaches has made it difficult to clarify the putative role of glutamate co-release from midbrain DA neurons in behavioral reinforcement and addiction (Pupe and Wallén-Mackenzie, 2015; Morales and Margolis, 2017). The current implementation of temporally controlled targeting in adulthood advances the knowledge of VGLUT2 in DA neurons and complements previous gene targeting studies performed in the developing mouse brain. To summarize, in the absence of VGLUT2 in mature DA neurons at adulthood, mice show normal psychostimulant-induced locomotion, while on a molecular level, the loss of VGLUT2 causes a significant decrease of mesoaccumbal glutamatergic neurotransmission and an elevated baseline AMPA/NMDA ratio, sufficiently high to occlude any further effect on neurotransmission by the addictive drug cocaine. Together with previously published data, our present results point towards a dual role for *Vglut2* gene expression within midbrain DA neurons. During development, VGLUT2 is essential for establishing and maintaining the mesoaccumbal DA system, as its loss causes severe dysfunction in DA release and abnormal behavioral response to addictive substances as well as sugar (Birgner et al., 2010; Hnasko et al., 2010; Alsiö et al., 2011; Fortin et al., 2012). While *Vglut2* expression levels in the *Dat*-expressing population of VTA DA neurons are high around birth, we show that there is a substantial down-regulation of Vglut2 mRNA in mature DA neurons. However, VGLUT2 in VTA DA neurons is still crucial in adulthood for maintaining mesoaccumbal glutamate transmission as well as synaptic strength in accumbal MSNs. Synaptic plasticity has never been studied in the context of mesoaccumbal glutamate co-release before, but the current study highlights VGLUT2 as an emerging player in the complex mechanisms of substance addiction that should be investigated further.

AUTHOR CONTRIBUTIONS

MP planned experiments, performed histological experiments, analyzed data and wrote the manuscript. MC performed cocaine experiments and analyzed data. MD performed glutamate recordings and analyzed data. ZB performed stereotaxic injections and analyzed data. SD performed *in situ* hybridization experiments and analyzed data. HP performed amphetamine sensitization experiments. CB performed cocaine experiments and analyzed data. GS supervised glutamate recordings. CL supervised cocaine experiments. ÅW-M conceived and planned all experiments, analyzed data and wrote the manuscript. All authors reviewed the manuscript.

FUNDING

This work was supported by Uppsala University and by grants to ÅW-M from the Swedish Research Council (Vetenskapsrådet 2011-4747, 2013-4657, 2017-02039), the Swedish Brain Foundation (Hjärnfonden), Parkinsonfonden, the Research Foundations of Bertil Hållsten (Bertil Hållsten forskningsstiftelse), Zoologiska föreningen and Åhlén (Åhlén-stiftelsen) (Åhlén Foundation). MP's post-doctoral grant was funded by Olle Engkvist Byggmästare Foundation (SOEB 2015/586). Work in GS lab was supported by Knut and Alice Wallenberg Foundation, the Swedish Research Council and the Swedish Brain Foundation.

ACKNOWLEDGMENTS

We thank Professors Günther Schütz (Heidelberg University) and David Engblom (Linköping University) for the DAT-CreERT2 (txDAT-Cre) transgenic mice, Professors Lars Olson and Nils-Göran Larsson (Karolinska Institutet) for the eDAT-Cre transgenic mice and Professor Bruno Giros (McGill University) for the DRD1-EGFP transgenic mice. Further, we thank Dr. Chadanat Noonin, Uppsala University, for expert technical assistance and Ms. Bianca Vlcek, Dr. Gian Pietro Serra and Dr. Niclas König, Uppsala University, for constructive comments on the manuscript. Previous and present members of the Mackenzie lab are thanked for technical assistance and constructive comments on the manuscript. BioVis, Uppsala University, is acknowledged for providing confocal imaging services. Image platform at Institute de la Vision (Paris, France) is acknowledged for performing slide scanning.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fncir.2018.00064/full#supplementary-material

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Conflict of Interest Statement: SD is the owner of Oramacell.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling Editor declared a shared affiliation, though no other collaboration, with several of the authors MP, ZB, HP, ÅW-M.

frontiers in Neural Circuits

REVIEW
published: 21 December 2018
doi: 10.3389/fncir.2018.00106

Neuropeptide and Small Transmitter Coexistence: Fundamental Studies and Relevance to Mental Illness

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Edited by:
Miguel Angel Morales, Universidad Nacional Autónoma de México, Mexico

Reviewed by:
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Received: *28 June 2018*
Accepted: *05 November 2018*
Published: *21 December 2018*

Citation:
Hökfelt T, Barde S, Xu Z-QD, Kuteeva E, Rüegg J, Le Maitre E, Risling M, Kehr J, Ihnatko R, Theodorsson E, Palkovits M, Deakin W, Bagdy G, Juhasz G, Prud'homme HJ, Mechawar N, Diaz-Heijtz R and Ögren SO (2018) Neuropeptide and Small Transmitter Coexistence: Fundamental Studies and Relevance to Mental Illness. Front. Neural Circuits 12:106. doi: 10.3389/fncir.2018.00106

Neuropeptides are auxiliary messenger molecules that always co-exist in nerve cells with one or more small molecule (classic) neurotransmitters. Neuropeptides act both as transmitters and trophic factors, and play a role particularly when the nervous system is challenged, as by injury, pain or stress. Here neuropeptides and coexistence in mammals are reviewed, but with special focus on the 29/30 amino acid galanin and its three receptors GalR1, -R2 and -R3. In particular, galanin's role as a co-transmitter in both rodent and human noradrenergic locus coeruleus (LC) neurons is addressed. Extensive experimental animal data strongly suggest a role for the galanin system in depression–like behavior. The translational potential of these results was tested by studying the galanin system in *postmortem* human brains, first in normal brains, and then in a comparison of five regions of brains obtained from depressed people who committed suicide, and from matched controls. The distribution of galanin and the four galanin system transcripts in the normal human brain was determined, and selective and parallel changes in levels of transcripts and DNA methylation for galanin and its three receptors were assessed in depressed patients who committed suicide: *upregulation* of transcripts, e.g., for galanin and GalR3 in LC, paralleled by a *decrease* in DNA methylation, suggesting involvement of epigenetic mechanisms. It is hypothesized that, when exposed to severe stress, the noradrenergic LC neurons fire in bursts and release

galanin from their soma/dendrites. Galanin then acts on somato-dendritic, inhibitory galanin autoreceptors, opening potassium channels and inhibiting firing. The purpose of these autoreceptors is to act as a 'brake' to prevent overexcitation, a brake that is also part of *resilience* to stress that protects against depression. Depression then arises when the inhibition is too strong and long lasting – a maladaption, allostatic load, leading to depletion of NA levels in the forebrain. It is suggested that disinhibition by a galanin antagonist may have antidepressant activity by restoring forebrain NA levels. A role of galanin in depression is also supported by a recent candidate gene study, showing that variants in genes for galanin and its three receptors confer increased risk of depression and anxiety in people who experienced childhood adversity or recent negative life events. In summary, galanin, a neuropeptide coexisting in LC neurons, may participate in the mechanism underlying resilience against a serious and common disorder, MDD. Existing and further results may lead to an increased understanding of how this illness develops, which in turn could provide a basis for its treatment.

Keywords: allostatic load, epigenetics, galanin, locus coeruleus, major depression disorder, neuropeptides, resilience

INTRODUCTION

The first evidence for chemical signaling in the central nervous system was reported by Eccles et al. (1954), when they demonstrated that acetylcholine is the transmitter released from motor neuron collaterals onto Renshaw cells in the spinal cord. Some 10 years later the Canadian electrophysiologist Hugh McLennan in his monograph "Synaptic transmission" (McLennan, 1963) reviewed in some detail the evidence for a number of molecules being transmitters: "Acetylcholine," "Catecholamines," "5-Hydroxytryptamine," "Substance P," "Factor I and the Inhibitory Transmitter," "GABA and Glutamic Acid," and "Cerebellar Excitatory Factor" were the chapter sub-headings. Some further compounds were mentioned, like other amino acids. A detailed table of the regional distribution of these molecules was included. In the "Conclusions" McLennan stated "With the exception of a number of cholinergic and rather fewer adrenergic systems, the data supporting a certain type of chemical mediation in any given situation are quite inadequate, and in spite of the inherent difficulties the number of problems to be solved are of great interest." Indeed, many efforts in the following years rapidly expanded the number of candidates and 'certified' their transmitter status – work still ongoing. However, to identify a molecule as a transmitter was at that time often a difficult process with strong pro and contra arguments. More recently completely different molecules have appeared on the scene, not stored in vesicles and thus not exocytosed, like nitric oxide (NO) and hydrogen sulfide (H_2S), sometimes called "gasotransmitters" (Paul and Snyder, 2015). Subsequently, substance P, mentioned already by McLennan, was identified as a member of the by far most diverse group of signaling molecules (>100) in the nervous system, the neuropeptides (Burbach, 2010).

The purpose of the present article is to review data on one of these peptides, galanin, which was discovered by Tatemoto et al. (1983) at Karolinska Institutet, a peptide that is a co-transmitter in many systems. In particular, focus is on recent results describing the distribution of galanin and it three receptors GalR1-3 in the 'normal' human brain by studying post mortem tissue samples (Le Maitre et al., 2013). More importantly, results are discussed showing significant changes in expression of the galanin family 'members' in post mortem brains from depressed patients having committed suicide, as compared to controls (Barde et al., 2016). A hypothesis is presented on a possible role of galanin, coexisting in noradrenergic neurons in the locus coeruleus (LC), in the development of depression and in resilience. This hypothesis is based on results from extensive animal experiments, so discussion of the human studies is preceded by an overview of "neuropeptides" with some comments on "methodological approaches," of "neuropeptide – small transmitter molecule coexistence," of the neuropeptide "galanin," followed by a summary of the critical and relevant animal experiments.

NEUROPEPTIDES

The concept of neuropeptide transmitters was introduced by the late Dutch scientist David de Wied and colls. (see De Wied and De Kloet, 1987). Neuropeptides are different from classic transmitters in several ways (Strand, 1991). In brief, neuropeptides are ribosomally synthesized as large precursor molecules in cell soma and dendrites (Noda et al., 1982; Mains et al., 1987), and the bioactive peptide(s) is excised from prepropeptide precursors by convertase enzymes (Seidah and Chretien, 1999). Packed in storage vesicles the peptides are axonally transported and released by exocytosis from nerve terminals, and also from dendrites and soma.

Neuropeptides in the nervous system encompass > 100 members (Burbach, 2010), almost always acting via one or more of a correspondingly large number of 7-transmembrane, G protein-coupled receptors (GPCRs) (>200). Much research is ongoing in the neuropeptide field. A search on PubMed with the

terms "neuropeptides, review" (August 1, 2018) generated 35.579 hits. However, work on neuropeptides has not been without controversies. Already in the 1990'ies doubts were expressed with regard to functional significance [see for example the article entitled "Superfluous neurotransmitters" (i.e., *neuropeptides*) by Bowers (1994)]. The recent statement by Sudhof (2017) still reflects a cautious attitude: "At the forefront of early molecular neuroscience was the identification of neuropeptide precursors and neuropeptide receptors (Noda et al., 1982), but since then the question of neuropeptide signaling has largely faded from view with a few exceptions."

However, peptides have an important and well accepted physiological function, when they are expressed in neurosecretory systems (Scharrer and Scharrer, 1937; Bargmann, 1949; Bargmann and Scharrer, 1951; Swaab et al., 1975; Vandesande and Dierickx, 1975; Brownstein and Mezey, 1986; Swanson et al., 1986; Ceccatelli et al., 1989; Meister, 1993; Morris et al., 1998; Gainer et al., 2002; Landgraf and Neumann, 2004; Jurek and Neumann, 2018), releasing their peptides into the general circulation (e.g., vasopressin, oxytocin) (Acher and Chauvet, 1954; Du Vigneaud, 1954), or into the hypothalamic portal circulation [thyrotropin releasing hormone (TRH), luteinizing releasing hormone (LHRH), somatostatin (a.k.a. growth hormone release-inhibiting hormone, GHR-IH), corticotropin releasing factor/hormone (CRF/CRH), and growth hormone releasing hormone (GHRH)] (Guillemin, 1978; Schally et al., 1978; Spiess et al., 1981, 1983; Vale et al., 1981; Brazeau et al., 1982; Rivier et al., 1982).

It is fair to say that many of the initial, high expectations of neuropeptides were not met. Examples are: (i) the discovery of the first endogenous ligands met- and leu-enkephalin for the morphine receptor (Hughes et al., 1975), present in dorsal horn interneurons (Hokfelt et al., 1977b), was expected to lead to new efficacious medicines for fighting pain, without the serious side effects of morphine; and (ii) antagonists to substance P, present in sensory neurons and the spinal dorsal horn (Lembeck, 1957; Hokfelt et al., 1975b; Takahashi and Otsuka, 1975) and acting as a transmitter (Otsuka et al., 1975; Henry, 1976) via NK1 receptors (Mantyh et al., 1995), were anticipated to represent a new type of painkiller.

These 'failures' have occurred in spite of considerable efforts from academia and pharmaceutical companies. For example, a substance P (neurokinin 1, NK1) antagonist was tested some 25 years later in the clinic but did not induce analgesia (Hill, 2000; Herbert and Holzer, 2002). However, and interestingly, it was also reported in a placebo-controlled trial in patients with moderate to severe major depression that the substance P (NK1) antagonist MK-869 (Aprepitant, EMEND), has robust antidepressant activity (Kramer et al., 1998). Moreover, the improvement was similar to that observed (in the same study) with the widely used antidepressant serotonin reuptake inhibitor (SSRI) paroxetine (Paxil, Seroxat) and essentially without (the common sexual) side effects seen with SSRIs (Kramer et al., 1998). However, a phase 3 trial failed to reproduce the antidepressant effects of MK-869 (Keller et al., 2006). Reasons for the failure in the treatment of depression have recently been analyzed (Rupniak and Kramer, 2017), and psychiatric studies of NK1 are still ongoing (e.g., Frick et al., 2016; Schank and Heilig, 2017). Neuropeptides and pharmacotherapy for depression will be discussed further below.

There is, however, one 'sphere' where neuropeptides have achieved a significant 'status,' and that is as markers for specific neuron populations, in particular in cortex and hippocampus [1], without defining their functional role. This said, there *are* interesting examples, where a neuropeptide is essential for particular mouse behaviors. For example, in the lateral amygdaloid nucleus gastrin releasing peptide (GRP) regulates fear via the GRP receptor (Shumyatsky et al., 2002), and the same peptide and receptor modulate sighing in the preBötzinger complex in the ventrolateral medulla oblongata (Li et al., 2016). Arcuate AgRP neurons projecting to i.a. the parabrachial nucleus (Broberger et al., 1998) represent another example. These neurons are GABAergic and also express and release NPY, thus a good example of peptide and small molecule co-transmission. Alhadeff et al. (2018) have now shown that, of these three molecules, NPY via its NPY Y1 receptor is selectively responsible for a pain-inhibiting effect. Finally, based on a Drosophila study (Asahina et al., 2014), Zelikowsky et al. (2018) use a battery of the most recent methodologies to conduct a landmark study that demonstrates a key role for the neuropeptide tachykinin 2/neurokinin B and its receptor NK3 in chronic isolation stress, opening up for a new treatment strategy of this serious mood disorder.

The therapeutic potential of neuropeptide signaling has been extensively discussed based on animal experiments. These experiments also consider a possible role of neuropeptides in behaviors related to stress and mood regulation, and explore their receptors as possible targets for antidepressant drug development, a main theme of this review (Herbert, 1993; Maubach et al., 1999; Hokfelt et al., 2003; Holmes et al., 2003; Sajdyk et al., 2004; Nemeroff and Vale, 2005; Millan, 2006; Steckler, 2008; Wu et al., 2011; Griebel and Holsboer, 2012; Griebel and Holmes, 2013).

LOCALIZATION AND FUNCTION OF NEUROPEPTIDES: METHODS

Four methods are of crucial importance for the exploration of neuropeptides and their coexistence with small molecule transmitters: Immunohistochemistry (IHC), radioimmunoassay (RIA), *in situ* hybridization (ISH) and real-time (quantitative)

[1]There are many examples: interneurons in neocortex are partly defined by (five) neuropeptides (Somogyi and Klausberger, 2005). For example, somatostatin-positive cortical interneurons are associated with gamma-rhythms (Veit et al., 2017), with the development of neuropathic pain (Cichon et al., 2017) and possibly with mental illness (Hamm and Yuste, 2016); and galanin-immunoreactive neurons in the medial preoptic area govern parental behavior (Wu et al., 2014), and in the ventrolateral preoptic nucleus they are sleep active (Gaus et al., 2002). However, in none of these studies is a functional role assigned to the peptide. Neuropeptides as phenotype marker are thus similar to calcium-binding proteins (such as parvalbumin) (Baimbridge et al., 1992; Andressen et al., 1993), which e.g., in neocortex label subpopulations of interneurons, often in combination with neuropeptides (e.g., Somogyi and Klausberger, 2005).

polymerase chain reaction (qPCR).[2] These methods allow not only studies of the localization and levels of various neuropeptides but also give a hint toward functionality.

Neuropeptides released from nerve endings have to be replaced by ribosomal synthesis in cell soma followed by axonal transport. Thus, replacement can take a considerable time, of course especially in neurons with long projections, and especially in large brains like the human brain. However, here dendritic release is special as the distance between site of release and site of synthesis is short and allows for rapid replacement. In fact, dendritic release is associated with distinct features: peptide release (see below) via exocytosis is stimulated by depolarization-induced Ca2+ entry through voltage-gated calcium channels, whereby the SNARE proteins in the dendrites may partly differ from those in nerve endings (Ludwig and Leng, 2006; Kennedy and Ehlers, 2011; Ovsepian and Dolly, 2011; van den Pol, 2012; Ludwig et al., 2016).

Neuropeptide dynamics distinctly contrast those of classic transmitters: the latter are enzymatically produced also at release sites (in the nerve endings), and they have a membrane reuptake mechanism (transporters) at both the cell and storage vesicle membrane (Kanner, 1994; Liu and Edwards, 1997; Chen et al., 2004; Eiden et al., 2004; Hahn and Blakely, 2007; Torres and Amara, 2007). These transporters allow rapid replacement at the site of release, i.e., no axonal transport is needed. Such transporters have not been demonstrated for neuropeptides. This said, there is evidence that galanin after intraventricular injection can accumulate in a small number of neurons, e.g., in the hippocampus (Jansson et al., 2000).

Monitoring peptide mRNA levels with ISH provides a measure of activity of specific neurons. If analyzed in an experimental paradigm, one may even associate involvement of a peptide with a certain function. For example, an increase in galanin transcripts in dorsal root ganglion (DRG) neurons, after peripheral nerve injury, has been interpreted as a defense against pain (Xu et al., 2008) and as a signal for repair (Hobson et al., 2010).

However, reporting of mRNA levels alone always raises the issue of translation: Can the presence of transcript really equal the presence of protein (peptide)? Many studies suggest this to be the case in DRGs, for example. Also, the experiments on human *postmortem* brains, where transcript (qPCR) and peptide (RIA) were analyzed in the same samples (Barde et al., 2016) support this view (see below). Ideally this issue can be solved by double-labeling of individual cells: ISH for transcript and IHC for neuropeptide (Grabinski et al., 2015). Contrasting ISH it is, however, difficult to quantify peptide levels at the microscopic level with IHC. Also, IHC requires fixed tissues, whereas snap-frozen fresh tissue is used for ISH. Nevertheless, these histochemical/biochemical approaches have been applied in countless animal experimental studies to explore a possible functional role of neuropeptides in specific neuronal populations.

NEUROPEPTIDE AND SMALL TRANSMITTER COEXISTENCE

In the 1970's several groups reported that a neuron may release more than one transmitter. These findings were often considered to violate "Dale's principle," a rule generally thought to state that a neuron only produces and releases one neurotransmitter. This was subsequently clarified as a misunderstanding (e.g., Eccles, 1986). Several of the early studies on transmitter co-existence focused on *invertebrates*, and only on classic transmitters and not neuropeptides (Kerkut et al., 1967; Brownstein et al., 1974; Hanley et al., 1974; Cottrell, 1976). Since then the analysis of co-transmission in this class of animals has been extremely informative. Thanks to in-depth analyses of the comparatively easily accessible and well-characterized systems in invertebrates using front-line methods, detailed knowledge of the mechanisms underlying co-transmission, and of its functional consequences has been generated (as reviewed in, e.g., Kupfermann, 1991; Bargmann, 1993; Nusbaum et al., 2017; Nassel, 2018). In the present article, the focus is on transmitter coexistence in *mammalian* systems.

In mammals, co-existence of noradrenaline (NA) and serotonin (5-hydroxytryptamine, 5-HT) in the same synaptic vesicle of sympathetic nerves in the pineal gland was reported (Jaim-Etcheverry and Zieher, 1973); but, serotonin presumably originated from pinealocytes and had been translocated into the storage sites with the help of cell and vesicular membrane transporter molecules. At that time, evidence was also presented for a developmental transmitter "switch" from a cholinergic to a noradrenergic transmitter phenotype in sympathetic neurons *in vitro*, with some neurons temporarily expressing both acetylcholine and noradrenaline (Furshpan et al., 1976); later work revealed that this also occurred *in vivo* (Landis and Keefe, 1983). Furthermore, several groups, in particular Burnstock and coworkers, provided evidence that ATP is a transmitter and co-transmitter (Burnstock, 1972), at that time a controversial view (Burnstock, 2012).

This was also the period when attention started to focus on peptides/neuropeptides in the brain. David de Wied and colleagues in the Netherlands studied the effects of pituitary hormones on behavior (de Wied and Bohus, 1966). Guillemin and Schally's groups discovered that the hypothalamic

[2]IHC is based on antibodies and allows demonstration of the cellular and ultrastructural localization of peptide/proteins in the microscope. The method was introduced already in the early 1940s by Coons et al. (1942) but was not applied to the nervous system until almost 30 years later (Geffen et al., 1969). Since peptides are rapidly transported out from the cell body after synthesis, the mitosis inhibitor and axonal transport-blocker colchicine is often needed to visualize cell bodies in the brain with this method (Barry et al., 1973; Ljungdahl et al., 1978). Using RIA, also based on (actually often the same) antibodies, developed by Yalow and Berson (1959) almost 60 years ago, concentrations/levels of peptides/proteins can be quantified in tissues and fluids. ISH, also a histochemical technique, detects nucleic acid sequences in tissue sections (Brahic and Haase, 1978; Gee et al., 1983). Since transcripts (mRNAs) are detected, the signal labels cell soma (and to some extent dendrites). The PCR method was invented by Mullis et al. (1987). A note of concern: In addition to specificity problems, especially associated with IHC and GPCRs, histochemical techniques often lack sensitivity to detect low-abundance molecules. Evidence for this view is provided by single cell analysis (Eberwine and Bartfai, 2011). This is particularly true for receptor transcripts, since these proteins have a low turnover (in any case compared to releasable molecules like neuropeptides). And only few receptor molecules are needed for signaling. The present review may 'underestimate' the number of molecules that coexist in a neuron and its signaling.

thyrotropin-releasing hormone is a tripeptide (Boler et al., 1969; Burgus et al., 1970), and several new peptides were isolated from the intestine and brain (Tatemoto and Mutt, 1980; Mutt, 1989). Also substance P was isolated from the intestine (von Euler and Gaddum, 1931), but only after 40 years (!) was it chemically identified as an undecapeptide (Chang and Leeman, 1970; Chang et al., 1971). Last but not least, a very large number of important peptides were isolated from the skin of various frog species by Erspamer et al. (1978). In a visionary review, Burnstock raised the question "Do some nerve cells release more than one transmitter?" with focus on ATP and also mentioning neuropeptides (Burnstock, 1976).

At that time the neuropeptide somatostatin was, surprisingly, localized to peripheral sympathetic neurons (Hokfelt et al., 1977a) already known to signal via NA, the transmitter of sympathetic neurons (von Euler, 1948; Hamberger and Norberg, 1963) (**Figures 1A,B**). Somatostatin had been discovered as an inhibitor of growth hormone release from the anterior pituitary (Brazeau et al., 1973; Vale et al., 1975; Guillemin, 2008). However, it turned out that somatostatin was not only present, as expected, in neurosecretory nerve endings in the hypothalamic median eminence (Dubois et al., 1974; Hokfelt et al., 1974a; Pelletier et al., 1975), but also in many other brain nuclei (Hokfelt et al., 1974a, 1975a; Brownstein et al., 1975; Dubé et al., 1975; Elde and Parsons, 1975). This indicated roles far beyond that of a hypothalamic hormone controlling pituitary growth hormone release. Then somatostatin was shown to have a depressant action on cortical neurons (Renaud et al., 1975). So somatostatin in noradrenergic neurons was the first example of coexistence of a neuropeptide transmitter with a classic neurotransmitter in mammals (Hokfelt et al., 1977a).

Other early examples of this type of coexistence were vasoactive intestinal polypeptide with acetylcholine (Lundberg et al., 1979), and the neuropeptide Y (NPY) with NA (Lundberg et al., 1982). In the brain substance P was found in 5-HT (serotonin) neurons (Chan-Palay et al., 1978; Hokfelt et al., 1978), and cholecystokinin (CCK) in dopamine neurons (Hokfelt et al., 1980b), followed by many more combinations.

Regarding function, it could be shown, for example, that VIP contributes to the atropine-resistant vasodilation in exocrine glands (Lundberg et al., 1980), that NPY interacts with NA in sympathetic functions (Allen et al., 1982; Lundberg et al., 1982; Ekblad et al., 1984), and that CCK affects dopamine release (Kovacs et al., 1981; Starr, 1982), binding (Fuxe et al., 1981; Murphy and Schuster, 1982) and behavior (Crawley et al., 1984). In an elegant landmark study on a frog sympathetic ganglion Jan and Jan demonstrated that cholinergic presynaptic fibers express and release an LHRH-like peptide that is responsible for the late, slow excitatory post-synaptic potential via 'volume transmission' (Jan and Jan, 1982).

Taken together, these findings suggested a new principle: co-transmission - the release of a neuropeptide and a classic (small molecule) transmitter from the same neuron. In fact, the view emerged that neuropeptides *always* 'co-exist' with small molecule transmitters. Moreover, many groups, using IHC at the ultrastructural level, found that peptides are stored in large dense core vesicles (LDCVs) (diameter ∼1,000 Å) (Goldsmith and Ganong, 1975; Swaab et al., 1975; Vandesande and Dierickx, 1975; Castel and Hochman, 1976; Dube et al., 1976; Krisch, 1976; Pelletier et al., 1981; Merighi, 2002) (**Figures 1C,E**), whereas monoamines like NA are present both in synaptic vesicles (diameter ∼500 Å) and LDCVs as shown with potassium permanganate fixation (KMnO4) (**Figure 1D**) (Richardson, 1966; Hokfelt and Jonsson, 1968). The number of LDCVs in a nerve ending is mostly low compared to synaptic vesicles, indicating a lower content of peptide molecules vs. classic transmitters. However, the affinity at peptide receptors is in the low nanomolar range, allowing efficacious signaling even by low numbers of peptide molecules in the extracellular space.

It was not clear, whether IHC could exclude that peptides are stored in synaptic vesicles. Pelletier et al. (1981) incubated adjacent, ultrathin sections with antibodies against substance P and 5-HT, respectively, but in both cases *only* LDCVs were stained, not synaptic vesicles. This in spite of the fact that monoamines are (mainly) stored in synaptic vesicles (**Figure 1D**). Thus, it did not seem possible to visualize the main transmitter (5-HT) in the synaptic vesicles with IHC, contrasting, e.g., the KMnO4 method for NA (**Figure 1D**). So perhaps IHC also failed to demonstrate *neuropeptides* in synaptic vesicles? Therefore, subcellular fractionation studies were carried out, strongly suggesting lack of peptide in the synaptic vesicle pool but presence of NPY in the fraction with many LDCVs (**Figures 2A–E**) (Lundberg et al., 1981; Fried et al., 1985)[3]. In contrast to monoaminergic neurons, in sensory glutamatergic neurons the amino acid appears to be exclusively stored in synaptic vesicles (Merighi, 2002) (**Figures 1C,E**).

Furthermore, peptides are in general released when neurons fire at high frequency or in bursts (Lundberg et al., 1980; Andersson et al., 1982; Bondy et al., 1987; Bartfai et al., 1988; De Camilli and Jahn, 1990; Verhage et al., 1991; Consolo et al., 1994; Xia et al., 2009), and often extrasynaptically (Zhu et al., 1986) (**Figure 3**). The latter was already indicated in a pioneering study on the presynaptic structure of the synapse, showing docking sites for the synaptic vesicles which, however, are not spacious enough to leave room for LDCVs which are twice-the-size (1,000 Å) (Pfenninger et al., 1969) (**Figure 3**)[4]. This is of course not valid for somato-dendritic release and where true synapses do not exist, nor for the peripheral autonomic nervous system, where there is a considerable distance between the nerve ground plexus (Hillarp, 1949; Falck, 1962) and the smooth

[3]The preparation used in Fried et al. (1985) was very suitable for the purpose: The muscle layer of rat vas deferens contains a dense network of noradrenergic nerve terminals storing NPY (**Figures 2A–C**). However, the very thick, compact smooth muscle layer makes isolation of nerves/storage vesicles difficult. This obstacle was circumvented by castrating rats which leads to muscle atrophy. Thus, fairly pure fractions containing, respectively, synaptic and large vesicles, many of the latter with a visible dense core (**Figure 2E**), could be obtained (Fried et al., 1985).

[4]Of note, Figure 24–4A in Chapter 24 by A. I. Basbaum and T. M. Jessell shows an electron micrograph of an afferent C fiber nerve ending making a type 1 synapse with a dendrite in the *monkey* superficial dorsal horn. Here a string of LDCVs are seen close to the presynaptic membrane opposite to the postsynaptic density. It is not possible to definitely decide, if the LDCVs reach the presynaptic membrane. Nevertheless, the 'rule' of extrasynaptic release of LDCVs may not be without exceptions. The micrograph is by courtesy of H. J. Ralston, III. [from the Fourth Edition of the Textbook "Principles of Neural Science" (2000), edited by E. R Kandel, J. H. Schwartz and T. M. Jessell.]

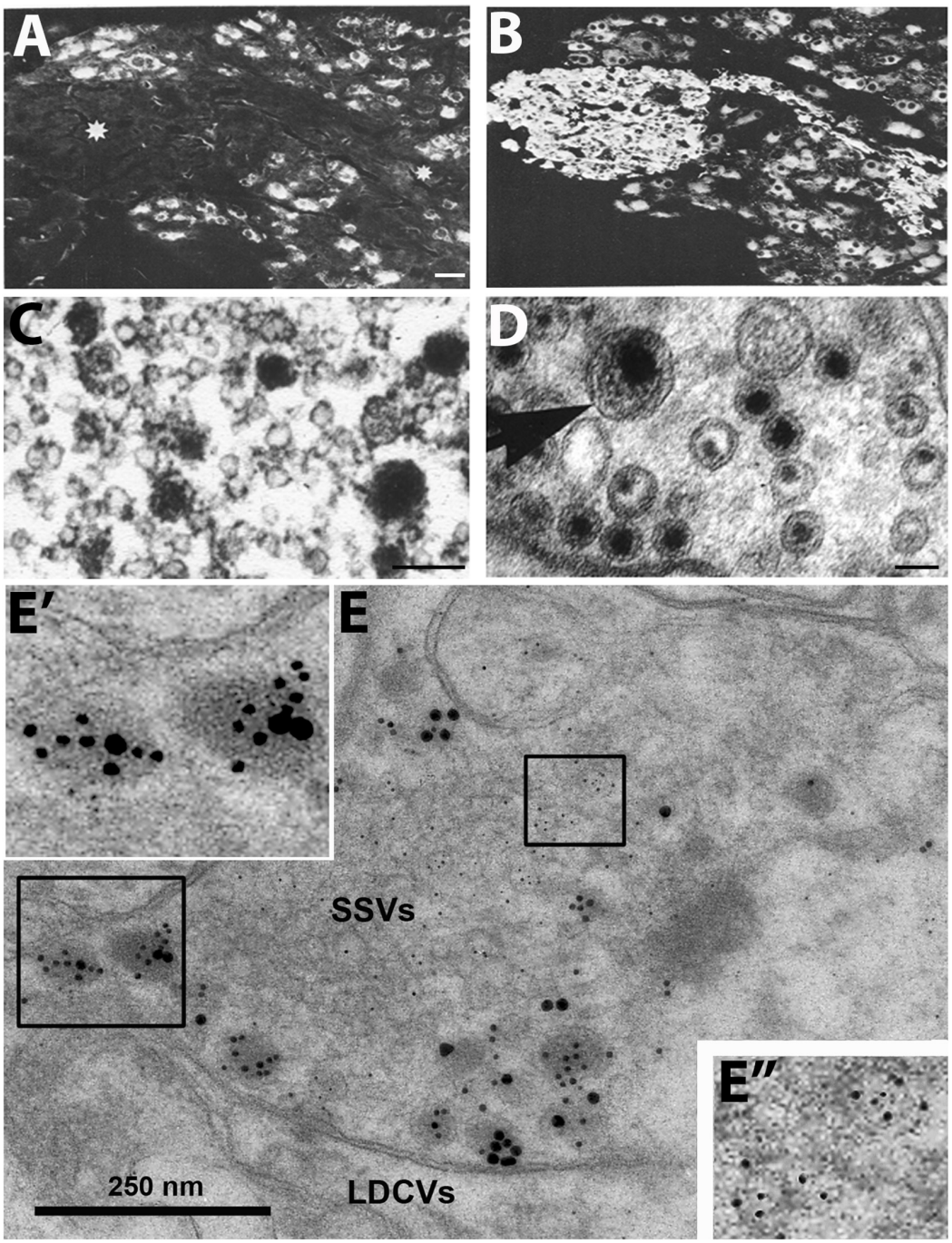

FIGURE 1 | Immunofluorescence micrographs of the guinea-pig inferior mesenteric ganglion **(A,B)** and electron micrographs from different types of nerve endings **(C–E)**. **(A,B)** Two adjacent sections incubated with antibodies to somatostatin **(A)** and the noradrenaline (NA) synthesizing enzyme dopamine ß-hydroxylase (DBH) **(B)**. The majority of the principal ganglion cells are somatostatin-positive, whereas the small intensely fluorescent (SIF) cells (asterisk) lack the peptide. Virtually all ganglion cells and the SIF cells are DBH-positive, i.e., are noradrenergic. **(C–E)** Examples of transmitter storage in nerve endings based on or immunohistochemistry **(C,E)** or potassium permanganate fixation **(D)**. **(D)** In sympathetic nerve endings NA (black precipitate) is stored in both (small) synaptic vesicles and large dense core vesicle (LDCVs) (arrow). Note that content varies between vesicles, both in the synaptic and LDCVs. **(C)** Substance P, a neuropeptide (black precipitate), in a sensory nerve ending in the monkey dorsal horn, is stored exclusively in LDCVs, all synaptic vesicles are empty. **(E)** Peptide and glutamate co-storage and coexistence in the dorsal horn of the rat spinal cord based on immunogold immunohistochemistry. Substance P/CGRP is detected with 10/20 nm gold particles and glutamate with 5 nm gold particles. Note that substance P and CGRP can be stored within the same LDCV (left box, magnified in **E'**). Staining for glutamate is restricted to synaptic vesicles (right box, magnified in **E"**). The results suggest that glutamate, a small molecule transmitter, is *not* stored in LDCVs in sensory nerve endings, and release of peptide and amino acid may be separate events. This contrasts NA (see **D**). Bars: 40 μm, for **(A,B)**; 100 nm for **(C,D)**; 250 nm for **(E)**. **(A,B)** From Hokfelt et al. (1977a). **(C)** From DiFiglia et al. (1982), with permission. **(D,E)** Courtesy of Dr. A. Merighi (cf., Merighi, 2002).

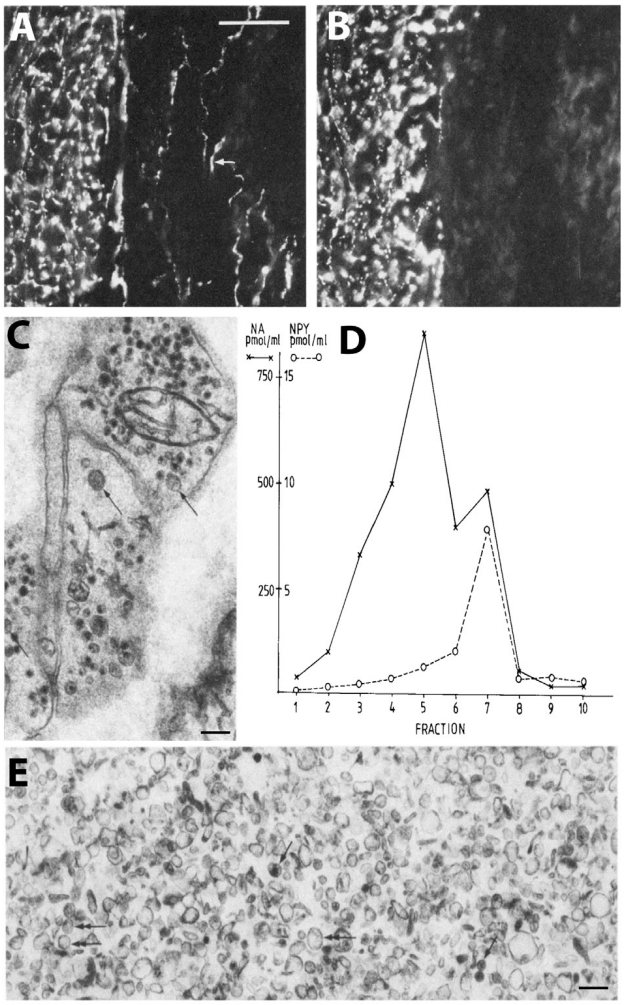

FIGURE 2 | Coexistence and subcellular distribution of neuropeptide Y (NPY) and noradrenaline (NA) in the rat vas deferens. **(A,B)** Immunohistochemical visualization of NPY- **(A)** and tyrosine hydroxylase (TH)-**(B)** positive nerve terminals in adjacent sections. Overlapping, dense NPY and noradrenergic networks are seen in the muscle layer. Note sparse NPY-only positive nerves (arrow) in the subepithelial region, possibly cholinergic nerves. **(C)** Electron microscopic micrograph of several nerve terminal profiles in the muscle layer after potassium permanganate (KMnO4) fixation, showing small synaptic vesicles with a dense core and LDCVs. The dense core indicates presence of NA both in the synaptic and LDCVs (cf. **Figure 1D**). No profiles without small vesicle with a dense core are seen, suggesting a pure adrenergic innervation of the muscle layer. **(D,E)** Subcellular distribution of NA (x) and NPY (o) in a density gradient of rat vas deferens. There is only one peak for NPY (fraction 7; **E**), whereas there are two peaks for NA (fraction 5 and 7), tentatively representing synaptic vesicles and LDCVs, respectively. Note many LDCVs (arrows), as well as many vesicles of the same size but without dense core (double-headed arrow). The peptide is only present in the heavy fraction (in agreement with **Figures 1C,E**), whereas NA is present also in the light one (in agreement with **Figure 1D**). On the abscissa, totally recovered sedimentable substance is given as picomoles per milliliter after centrifugation at 145,000 × g_{max} for 45 min. On the ordinate, density gradient fractions 1–10 are given, corresponding to the following sucrose molarities: 1 (0.26 M), 2 (0.32 M), 3 (0.47 M), 4 (0.56 M), 5 (0.69 M), 6 (0.74 M), 7 (0.84 M), 8 (0.91 M), 9 (0.98 M), 10 (1.2 M). Recoveries of NA = 70%, of NPY = 65%, and of protein = 87%. Reprinted from Fried et al. (1985), with permission.

muscle cells, as shown with electron microscopy combined with electrophysiology (Merrillees et al., 1963). Furthermore, in the brain, extrasynaptically released neuropeptides may diffuse over long distances, so called volume transmission (Fuxe et al., 2010).

The exocytotic machinery underlying neurotransmitter release has been thoroughly characterized with regard to release of small molecule transmitters stored in synaptic vesicles (De Camilli and Jahn, 1990; Sudhof, 2014). However, the exocytotic neuropeptide release from LDCVs is less well defined. In early studies on synaptosomes it was shown that CCK release from LDCVs is triggered by small elevation of Ca^{2+} concentration in the bulk cytoplasm, whereas glutamate release from the synaptic vesicles requires the higher concentrations produced close to Ca^{2+} channels in the active zone (Verhage et al., 1991). This is in agreement with the localization of the two types of vesicles consistently observed in electron microscopic micrographs of the nerve endings: many synaptic vesicles with some close to the presynaptic membrane, versus a few LDCVs virtually always distant from the synapse (**Figure 3**).

There is evidence for involvement of SNAREs [soluble *N*-ethyl maleimide (NEM)-sensitive factor attachment protein receptor protein family] (Sudhof, 2014) also in dendritic release from magnocellular dendrites (Schwab et al., 2001; de Kock et al., 2003; Ovsepian and Dolly, 2011). The calcium-dependent activator protein for secretion (CAPS) (Walent et al., 1992) has been identified as a priming factor for exocytosis of LDCVs (Stevens and Rettig, 2009; James and Martin, 2013). Thus CAPS2, but not CAPS1, is required for LDCV exocytosis as shown in cerebellar granule cells and hippocampal interneurons (Sadakata et al., 2004; Shinoda et al., 2011).

Taken together, these early findings suggested that neuropeptides were not the main neuronal messengers. Moreover, when neuropeptides are released, the fast small molecule transmitters are already active in the synaptic cleft – i.e., no peptide release without release of classic transmitters. The discovery of coexistence and co-transmission was summarized in several books/reviews (Burnstock, 1978; Hokfelt et al., 1980a, 1986, 1987a; Cuello, 1982; Chan-Palay and Palay, 1984; Jaim-Etcheverry, 1994; Merighi, 2002), and since then further efforts have been made to understand co-signaling involving neuropeptides, including co-release of both an excitatory and an inhibitory neuropeptide. For an up-to-date overview of many aspects on neuropeptide signaling (see e.g., Salio et al., 2006; van den Pol, 2012; Ludwig et al., 2016).

More recently it has become clear that coexistence of small molecule transmitters, encompassing various combinations of GABA, glycine, glutamate, dopamine and acetylcholine (e.g., Guiterrez, 2009; Hnasko and Edwards, 2012; Trudeau et al., 2014) (**Figure 3**). For example, coexistence of GABA and glycine was first reported in the cerebellum (Ottersen et al., 1988), and then in the spinal cord (Todd and Sullivan, 1990; Ornung et al., 1994), where evidence for GABA-glycine co-transmission was obtained in the dorsal horn, and possible co-release from the same synaptic vesicles (Jonas et al., 1998) (**Figure 3**). Moreover, mesencephalic dopamine neurons can also release glutamate (Hnasko et al., 2010) and GABA (Tritsch et al., 2012), whereby GABA is

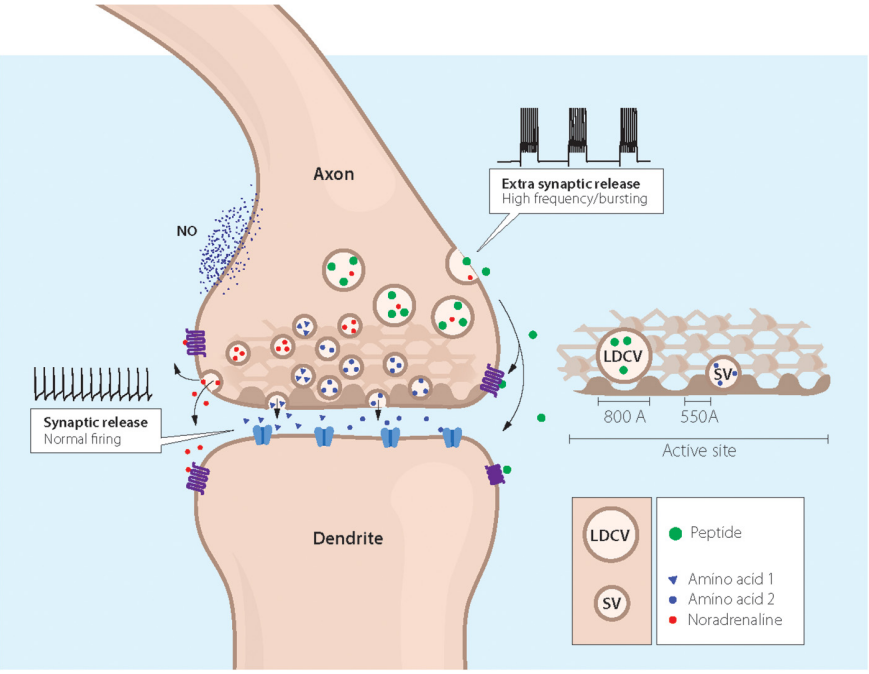

FIGURE 3 | Cartoon showing coexistence of a neuropeptide with classic and 'unconventional' neurotransmitters in a nerve ending synapsing on a dendrite. Two types of storage vesicles are shown: synaptic vesicles (diameter 500 Å) storing classic transmitters (e.g., 5-HT, NA, GABA or glutamate), mainly released at synapses; large dense core vesicles (LDCVs) storing neuropeptides and, in amine neurons NA or 5-HT. The peptides are in general released extrasynaptically ("volume transmission"), when neurons fire with high frequency or in bursts. Peptide receptors are essentially extrasynaptic or presynaptic, whereas ligand-gated receptors are mostly localized in the postsynaptic membrane. 'Gaseous' (e.g., nitric oxide, NO) and other non-conventional transmitters are not stored in vesicles, but are generated upon demand (Snyder and Ferris, 2000). The presynaptic grid, an egg basket-like structure, originally described by Pfenninger et al. (1969), is indicated in the nerve ending and high-lighted to the right. Note that the LDCV does not fit into the grid and thus cannot attach to the presynaptic membrane for release. In contrast, there is room for the synaptic vesicle. This supports the concept that peptides are mostly not released into the synaptic cleft. Drawing by Mattias Karlen. Modified from Pfenninger et al. (1969),Lundberg and Hokfelt (1983), and Lang et al. (2015).

not synthesized via the classic enzyme glutamate decarboxylase (GAD) but via aldehyde dehydrogenase 1a1 (Kim et al., 2015).

Thus, the number and combinations of transmitters present in a nerve ending (and/or dendrites) virtually seem endless, and it is difficult to define rules according to which neurotransmitters co-exist and are involved in co-transmission, as is discussed further in this Frontiers special topic. Furthermore, neurotransmitter switching, the gain of one and loss of another transmitter in the same, mammalian neuron, can occur not only during development but also in adult animals (Spitzer, 2017).

There is an increasing interest in neuropeptide/neurotransmitter coexistence and a need to explore transcriptional changes in defined healthy and diseased brain circuitries (Akil et al., 2010). In fact, there are many interesting results from *animal* disease models, suggesting involvement of neuropeptides and neuropeptide coexistence in patho-physiological processes with potential therapeutic implications. However, information on the significance of transmitter and neuropeptide *coexistence* in the normal and diseased *human* nervous system is limited. In this article, the focus is on galanin co-existing in noradrenergic neurons in the LC, and on galanin receptor expression in *postmortem* brains from normal subjects and depressed patients who committed suicide (Le Maitre et al., 2013; Barde et al., 2016). This is in line with previous extensive work carried out on *postmortem* brains from depressed humans, showing changes in transcripts related to neurotransmitters/neuropeptides and their receptors and to transporters, growth factors in nerve cells, and in glia, in cortical, limbic, hypothalamic and lower brain stem regions (Evans et al., 2004; Iwamoto et al., 2004; Aston et al., 2005; Choudary et al., 2005; Kang et al., 2007; Anisman et al., 2008; Kozicz et al., 2008; Tochigi et al., 2008; Klempan et al., 2009; Sequeira et al., 2009, 2012; Sibille et al., 2009; Poulter et al., 2010; Bernard et al., 2011; Bloem et al., 2012; Kerman et al., 2012; Zhurov et al., 2012; Labonte et al., 2013, 2017; Li et al., 2013; Du et al., 2014; Lopez et al., 2014a,b; Hayley et al., 2015; Maheu et al., 2015; Torres-Platas et al., 2016; Roy et al., 2017).

GALANIN

Galanin was originally isolated from porcine intestine as a 29-amino acid (30 in humans) neuropeptide (Tatemoto et al., 1983; Schmidt et al., 1991) (**Figure 4A**) with a wide distribution in the rat brain as shown with RIA (Skofitsch and Jacobowitz, 1986), IHC (Rokaeus et al., 1984; Melander et al., 1985, 1986b,c,d; Skofitsch and Jacobowitz, 1985; Merchenthaler et al., 1993), and ISH (Gundlach et al., 1990b; Jacobowitz and Skofitsch, 1991;

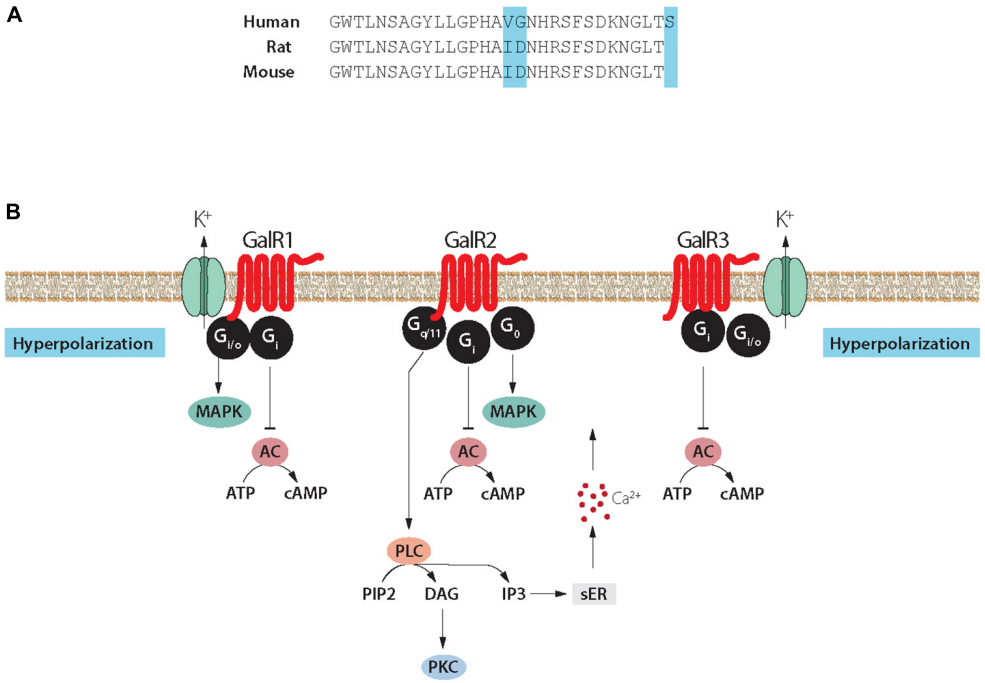

FIGURE 4 | (A) Structure of galanin in three species. Galanin is composed of 29 amino acids in most species, except humans (30 amino acids). Note conservation of N-terminal portion. **(B)** Signaling pathways of galanin receptor subtypes. Galanin, via GalR1 and GalR3, opens potassium channels leading to membrane hyperpolarization. Galanin can via GalR2 activate PLC resulting in generation of IP3, release of Ca^{2+} from the smooth endoplasmic reticulum, opening of Ca^{2+} channels and eventually transmitter release. AC, adenylate cyclase; cAMP, 3′, 5′-cyclic adenosine monophosphate; DAG, diacylglycerol; K^+, G-protein-regulated inwardly rectifying potassium channel; sER, smooth endoplasmic reticulum; IP_3, inositol triphosphate; PIP_2, phosphatidylinositol bisphosphate; PKC, protein kinase C; PLC, phospholipase C. Modified from Iismaa and Shine (1999) and Lang et al. (2015). Drawing by Mattias Karlén.

Jacobowitz et al., 2004). The distribution of galanin in the mouse brain is similar to that in rat, both with regard to galanin peptide (Perez et al., 2001) and to its mRNA (Cheung et al., 2001; Lein et al., 2007). The galanin system has also been characterized in the monkey brain (Melander and Staines, 1986; Kordower and Mufson, 1990; Walker et al., 1991) (for human brain, see below).

For many years galanin was considered as the sole endogenous ligand for GalR1-3 but more recently additional ligands were described (Lang et al., 2015)[5]. Currently, three galanin receptors, GalR1-3, have been cloned, all three belonging to the family of seven transmembrane-spanning GPCRs, with different transduction mechanisms, with GalR1 and -R3 having distinct similarities (Habert-Ortoli et al., 1994; Fathi et al., 1997; Howard et al., 1997; Wang et al., 1997; Ahmad et al., 1998; Smith et al., 1998; Iismaa and Shine, 1999; Branchek et al., 2000; Lang et al., 2007, 2015) (**Figure 4B**). The three galanin receptors are present in most parts of the rat brain, but could not be detected e.g., in dorsal cortical areas and the hippocampal formation (HiFo) in early autoradiographic ligand binding studies (Skofitsch et al., 1986; Melander et al., 1986a, 1988).

Galanin receptors have also been mapped in the mouse brain using 125I-galanin binding autoradiography (Jungnickel and Gundlach, 2005). A direct comparison with results in rat in the study by, e.g., O'Donnell et al. (2003) reveals an overall similar distribution but with some remarkable, apparently qualitative species differences. Thus, mouse shows, i.a., a strong signal in two important regions, the striatum and the cerebellum (Jungnickel and Gundlach, 2005) which both lack binding in the rat (Skofitsch et al., 1986; Melander et al., 1988; O'Donnell et al., 2003). To our knowledge, no attempts have been made to identify the cellular localization and origin of, e.g., the structures binding galanin in the mouse striatum.

The cloning of the receptors allowed localization with ISH and qPCR, which revealed that the transcripts for GalR1 and GalR2 are widely distributed in the rat brain, primarily in the brain stem and in ventral cortical areas (Landry et al., 1998; Mitchell et al., 1999; O'Donnell et al., 1999, 2003; Burazin et al., 2000; Waters and Krause, 2000; Mennicken et al., 2002). However, the GalR2 transcript is transiently highly expressed in neocortex

[5]First to be identified was the galanin message-associated peptide (GMAP), a product generated from the same precursor as galanin (Rokaeus and Brownstein, 1986). In brain it was also recognized that the N-terminal fragment galanin (1–16), conserved throughout species, is recognized by high affinity receptor sites in the forebrain (Fisone et al., 1989), and subsequently other fragments have been identified in the brain (Sillard et al., 1992; Ihnatko and Theodorsson, 2017). Almost 20 years ago the galanin-like peptide (GALP) was discovered in the porcine hypothalamus and shown to be an endogenous ligand of GalR2 (Ohtaki et al., 1999). GALP (9–21) is identical to galanin (1–13) with a high sequence homology among species. In the analysis of ganglioneuroma tissues Santic and colleagues discovered a splice variant of GALP mRNA, a 25 amino acid peptide and named it Alarin (Santic et al., 2006). This peptide, however, does not bind to any of the three galanin receptors, but still is considered a member of the galanin family (Lang et al., 2015). More recently it was found that spexin, a 14-amino acid peptide, is a ligand at the GalR2 and -R3 receptors (Kim et al., 2014).

during the first week after birth (Burazin et al., 2000). The distribution of GalR3 is limited (Mennicken et al., 2002). Only the GalR1 transcript has been mapped with ISH in the mouse brain (Hohmann et al., 2003; Lein et al., 2007). Thus, The Allen Brain Atlas (Lein et al., 2007) lacks results on GalR2 or GalR3, suggesting that they are expressed at low levels. This is also supported by the demonstration that the 125I-galanin binding sites are absent in a GalR1 knock-out mouse (Jungnickel and Gundlach, 2005). Taken together, these results suggest that GalR1 is the predominant receptor in the mouse brain, and that distinct species differences exist between mouse and rat.

GalR3 has emerged as a complex receptor (Lang et al., 2015), not present in all mammals (Liu et al., 2010). Its signaling properties are still not well defined, even though GalR3-transfected cell lines have now been generated (Lu et al., 2005b; Robinson et al., 2013). However, these cells could so far not be used for stable signaling experiments (see Lang et al., 2015). Still, GalR3 presumably acts via a PTX sensitive $G_{i/o}$-type G protein which in turn regulates inwardly rectifying K^+ channels (Smith et al., 1998), as do GalR1 receptors (Smith et al., 1998) (**Figure 4B**). This lack of knowledge contrasts the substantial information about GalR1 and GalR2 (see Lang et al., 2015). The cloning of the receptors was useful, also because it has been difficult to raise specific antibodies to GalR1-3 (Lu and Bartfai, 2009; Brunner et al., 2018). A similar situation exists for other GPCRs (Michel et al., 2009). Detailed tables on the distribution of galanin and GalR1-3 in rodent brain are found in O'Donnell et al. (1999, 2003), Burazin et al. (2000), Hohmann et al. (2003), and Jungnickel and Gundlach (2005).

Early research on galanin was initiated because of its strong reaction to nerve injury. Transection of the sciatic nerve in rat causes an >100-fold increase in galanin synthesis (mRNA and peptide levels) in the corresponding somata of DRG somata (Hokfelt et al., 1987b). Upregulation could also be detected in the brain after various types of injury/manipulations (Cortes et al., 1990a,b; Villar et al., 1990; Agoston et al., 1994; Palkovits, 1995). In fact, galanin meets the criteria of a neurotransmitter/-modulator, but also has trophic functions, as shown both in brain and the peripheral nervous system (Hobson et al., 2010). Galanin has, in fact, many characteristics similar to the brain-derived neurotrophic factor (BDNF), including storage in, and exocytotic release from LDCVs and both transmitter and trophic functions (Barde, 1994). For example, galanin affects spine density (Sciolino et al., 2015), and it is well-known that BDNF influences dendritic morphology (Bennett and Lagopoulos, 2014). Thus, trophic functions of galanin are potentially interesting but will not be discussed here.

A further early finding in the rat was the coexistence (**Figures 5A,B"**) of galanin (**Figure 5B**) in both noradrenergic neurons in the LC (**Figure 5B'**) (Rokaeus et al., 1984; Skofitsch and Jacobowitz, 1985; Melander et al., 1986b,c; Holets et al., 1988; Moore and Gustafson, 1989) and in serotonergic neurons in the dorsal raphe nucleus (DRN) (Melander et al., 1986c; Fuxe et al., 1990; Priestley et al., 1993; Xu and Hokfelt, 1997), two systems associated with mood-related behavior. The LC neurons also express transcripts for both GalR1 and -R2 (O'Donnell et al., 1999; Burazin et al., 2000).

Thereafter galanin biology has since the early 1990's been regularly summarized in books/journal from meetings (Hökfelt et al., 1991, 1998; Hökfelt and Crawley, 2005; Hokfelt, 2010; Hokfelt and Tatemoto, 2010); and in peer-reviewed articles focusing on the nervous system (only such published after 2004, and not included in the books/journals cited above, are listed here) (Lundstrom et al., 2005; Holmes and Picciotto, 2006; Karlsson and Holmes, 2006; Ogren et al., 2006, 2007, 2010; Robinson et al., 2006; Walton et al., 2006; Wrenn and Holmes, 2006; Lu et al., 2007; Tortorella et al., 2007; Picciotto, 2008; Robinson and Brewer, 2008; Butzkueven and Gundlach, 2010; Picciotto et al., 2010; Webling et al., 2012; Diaz-Cabiale et al., 2014; Freimann et al., 2015; Weinshenker and Holmes, 2016; Millon et al., 2017a; Genders et al., 2018a); and in some major comprehensive reviews (Lang et al., 2007, 2015).

GALANIN INHIBITS RAT LOCUS COERULEUS NEURONS

Locus coeruleus is a small, compact bilateral nucleus in the pons located in the gray matter close to the lateral aspect of the 4^{th} ventricle (Maeda, 2000). Dahlstrom and Fuxe first reported that NA is a transmitter in the rat LC, a.k.a. the A6 group (Dahlstrom and Fuxe, 1964). They used the formaldehyde, or Falck-Hillarp, fluorescence method that allows microscopic visualization of catecholamines and serotonin in tissue sections (Carlsson et al., 1962; Falck, 1962; Falck et al., 1962).

In the rat, the LC contains 2,800–3,600 neurons (both sides) (with an additional 260 neurons in the subcoeruleus area, the vast majority of which are noradrenergic with wide projections to virtually all parts of the central nervous system (Ungerstedt, 1971; Descarries and Saucier, 1972; Swanson and Hartman, 1975; Swanson, 1976; Morrison et al., 1978; Moore and Bloom, 1979; Goldman and Coleman, 1981; Foote et al., 1983; Aston-Jones et al., 1995). NA nerve terminals are also extensively present in primate cortex (Lewis et al., 1986).

When explored with electrophysiological methods galanin has effects on the membrane potential of several neuron systems (see Xu et al., 2005). Galanin hyperpolarizes noradrenergic LC neurons in a slice preparation (Seutin et al., 1989; Sevcik et al., 1993; Pieribone et al., 1995), mediated via GalR1 (Ma et al., 2001) (**Figure 5C**). However, the GalR2 (R3) agonist ARM-1986 (Liu et al., 2001; Lu et al., 2005b) does not cause any effect on the membrane potential (Ma et al., 2001) (**Figure 5C**). GalR2 may instead have a presynaptic role in the projection areas of LC neurons, perhaps mainly acting as an autoreceptor (Ma et al., 2001). In agreement, galanin is present in noradrenergic [dopamine ß-hydroxylase (DBH)]-positive nerve terminals in cortex and the hippocampus (Melander et al., 1986d; Xu et al., 1998). Galanin activation of GalR1, but not -R2 or R3, has been shown also in other studies on the rat and mouse LC (Hawes et al., 2005; Mitsukawa et al., 2009). In addition to this direct effect, galanin at *low* concentrations (10^{-9}M) enhances the autoinhibitory effect of NA on LC neurons via alpha-2A receptors

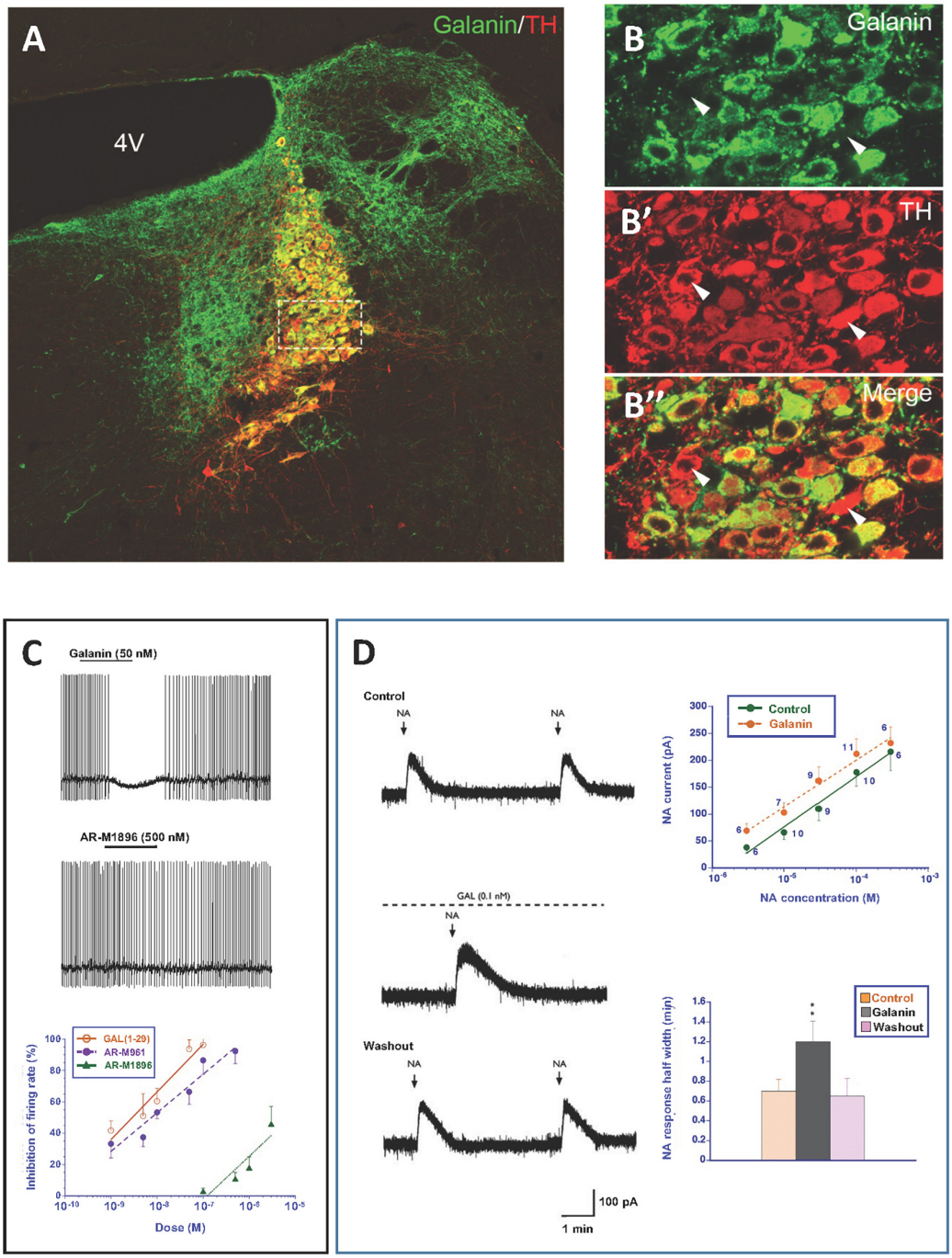

FIGURE 5 | (A–B") Immunofluorescence micrographs of the dorsal pontine periventricular region of mouse after double-staining of a section with antibodies to galanin (green) and tyrosine hydroxylase (TH) (red), the rate-limiting enzyme for catecholamine synthesis and thus a marker for NA neurons. Note that both antibodies stain neurons in the locus coerulus (LC) **(B,B')**, whereby many (yellow, **B"**), but not all TH-positive neurons express galanin [arrowheads point to TH-only neurons (red), apparently lacking galanin] **(B')**. Galanin is also present in many structures outside the LC. Colchicine treated animal. Courtesy Joanne Bakker and Mingdong Zhang. Bar for **(A)** 200 μm, for **(B–B")** 20 μm. **(C)** Effect of galanin and the GalR2 agonist AR-M1896 on LC neurons (upper two traces), and the dose–response curves of galanin (red), the AR-M1896 (green) and the mixed GalR1-GalR2 M961 agonist (magenta) (lower trace). Note strong hyperpolarization of galanin and a less strong effect of M961, whereas that AR-M1896 hardly causes any effect at all. From Ma et al. (2001). (**D**, left panel) Effect of galanin on the response of LC neurons to NA. NA (applied from a pipette at the arrowhead) induces a persistent outward current (upper trace). When galanin (0.1 nM) is present, the NA-induced outward current is enhanced, and the duration is prolonged (middle trace). After wash out of galanin, the amplitude and duration of the NA response was similar to that seen before galanin administration (lower trace). (**D**, right panel) Effect of galanin on dose-response (upper figure) and duration (lower figure) of NA. The NA dose-response curve is shifted to the left, when galanin (0.1 nM) is present (upper figure). The duration of the NA-induced current is increased in the presence of galanin (lower figure). $^{**}P < 0.01$. From Xu et al. (2001) with permission.

(Xu et al., 2001) (**Figure 5D**). This may in fact be the primary action of galanin in controlling the firing of LC neurons. Thus, galanin can via different autoinhibitory mechanisms exert a two-step inhibition on LC neurons, at low concentrations enhancing the inhibitory alpha-2A receptor effect.

Autoinhibition of LC neurons, mediated by NA via alpha-2A receptors, was early discovered by Svensson et al. (1975) and Aghajanian et al. (1977). It is assumed that autoinhibition, both at NA and serotonin neurons, at least in part, is responsible for the delayed onset of the clinical effect of monoamine reuptake inhibitors (Artigas et al., 1996; Mongeau et al., 1997; Millan, 2006). Autoinhibition via NA in LC was originally suggested to be a consequence of the release from collaterals (Aghajanian et al., 1977). There is, however, evidence that NA can be released from soma/dendrites (Pudovkina et al., 2001; Pudovkina and Westerink, 2005), and more recently release was shown to occur from individual vesicles by combined measurements using amperometry and patch clamp methodologies (Huang et al., 2007). This is in agreement with electron microscopic analysis, showing synaptic vesicles with a dense core in LC dendrites (Shimizu et al., 1979). Thus, collaterals are not necessarily the only structure involved in the autoinhibition.

There is another source of catecholamine input to the LC neurons originating from one of the three C neuron groups in the medulla oblongata: adrenaline (epinephrine) containing afferents (**Figure 6**) (Hokfelt et al., 1974b, 1984; Howe et al., 1980; Armstrong et al., 1982), which synapse on LC dendrites (Milner et al., 1989). This was supported by early tracing experiments, although at that time no transmitter histochemical identification was performed (Cedarbaum and Aghajanian, 1978). One likely origin is C1 neurons, since they display a high degree of collateralization, including inputs to the LC (**Figure 6**) (Haselton and Guyenet, 1990).

Early studies suggested that the adrenaline (Cedarbaum and Aghajanian, 1976) and the C1 neurons (Aston-Jones et al., 1991) inhibit LC neurons. However, the more recent discovery that the C1 neurons are glutamatergic together with optogentic analysis demonstrated excitation as the primary effect (**Figure 6**) (Abbott et al., 2012). Released adrenaline may act as a modulator not only on postsynaptic but also presynaptic (Li et al., 1995) alpha-2A receptors, which will, respectively, directly and indirectly, dampen LC neuron activity (**Figure 6**) (Guyenet et al., 2013).

Taken together, galanin prevents overexcitation of LC, but is only one of several molecules performing this task (Aston-Jones et al., 1991; Singewald and Philippu, 1998; Van Bockstaele, 1998; Berridge and Waterhouse, 2003; Van Bockstaele and Valentino, 2013). This comprehensive network is perhaps a sign of how important it is to balance the activity of the noradrenergic LC neurons, which are involved in the control of many bodily functions (see below).

Kehr and colleagues have analyzed the effect of intracerebroventricularly administered galanin in freely moving rats and mice, monitoring several neurotransmitters using *in vivo* microdialysis (Ungerstedt, 1984) and a sensitive HPLC method. Their studies indicate that galanin reduces basal and desipramine-induced extracellular NA levels (Yoshitake et al., 2003, 2004). This effect is assumed to be exerted via GalR1 at the noradrenergic cell bodies/dendrites in the LC.

Galanin and Dendritic Release

Studies on the hypothalamic magnocellular hormones vasopressin and oxytocin have provided compelling evidence that these two peptides are not only released from nerve endings in the posterior pituitary but also, independently, from dendrites in the paraventricular and supraoptic nuclei (Morris et al., 1998; Landgraf and Neumann, 2004; Ludwig and Leng, 2006; Kennedy and Ehlers, 2011; Ovsepian and Dolly, 2011; Ludwig et al., 2016). There is evidence for involvement of SNAREs [soluble *N*-ethyl maleimide (NEM)-sensitive factor attachment protein receptor protein family] (Sudhof, 2014) in release from magnocellular dendrites (Schwab et al., 2001; de Kock et al., 2003; Ovsepian and Dolly, 2011). Results from studies on CAPS2-dependant neuropeptide release from soma of dorsal root ganglion neurons (Bost et al., 2017; Shaib et al., 2018) may also be relevant for dendritic/somatic release in the brain. Galanin may be released from soma and dendrites in the LC (Pieribone et al., 1995; Vila-Porcile et al., 2009) (**Figure 6**). Therefore, it has been hypothesized that stress-induced firing increases galanin release from nerve terminals in the forebrain and dendrites-soma of LC neurons. This could lead to activation of GalR1 autoreceptors and inhibition of firing of LC neurons, a possible mechanism involved in resilience and development of depression-like behavior in animals (Sciolino et al., 2015) (see below).

Other Co-transmitters in the LC

Neuropeptide Y is expressed in LC neurons in rat (Everitt et al., 1984; Chronwall et al., 1985; Yamazoe et al., 1985; Holets et al., 1988) and human (Chan-Palay et al., 1990). Recently it has been shown in mice that dopamine is co-released with NA in the hippocampus (Kempadoo et al., 2016; Takeuchi et al., 2016) and the paraventriculer *thalamic* nucleus (Beas et al., 2018) and is involved in memory consolidation and control of stress responsitivity, respectively.

GALANIN AND DEPRESSION-LIKE BEHAVIOR IN RODENTS

Galanin influences mood-related behavior in a region-specific way (Bing et al., 1993; Moller et al., 1999). Moreover, results from a number of rat experimental models suggest that galanin can be both prodepressive/anxiogenic and antidepressive (Fuxe et al., 1990, 1991, 1998; Weiss et al., 1998, 2005; Bellido et al., 2002; Khoshbouei et al., 2002; Barrera et al., 2005; Sergeyev et al., 2005; Lu et al., 2005a, 2007, 2008; Holmes and Picciotto, 2006; Karlsson and Holmes, 2006; Ogren et al., 2006; Kuteeva et al., 2008, 2010; Kozlovsky et al., 2009; Picciotto et al., 2010; Le Maitre et al., 2011; Sciolino et al., 2012, 2015; Weinshenker and Holmes, 2016).

In many of the early studies listed above on depressive-like behavior the receptor involved was not identified, or the site of action was not defined experimentally, but there was a general consensus that it is GalR1 that mediates the depressive behavior and that GalR2 may be prodepressive (summarized in

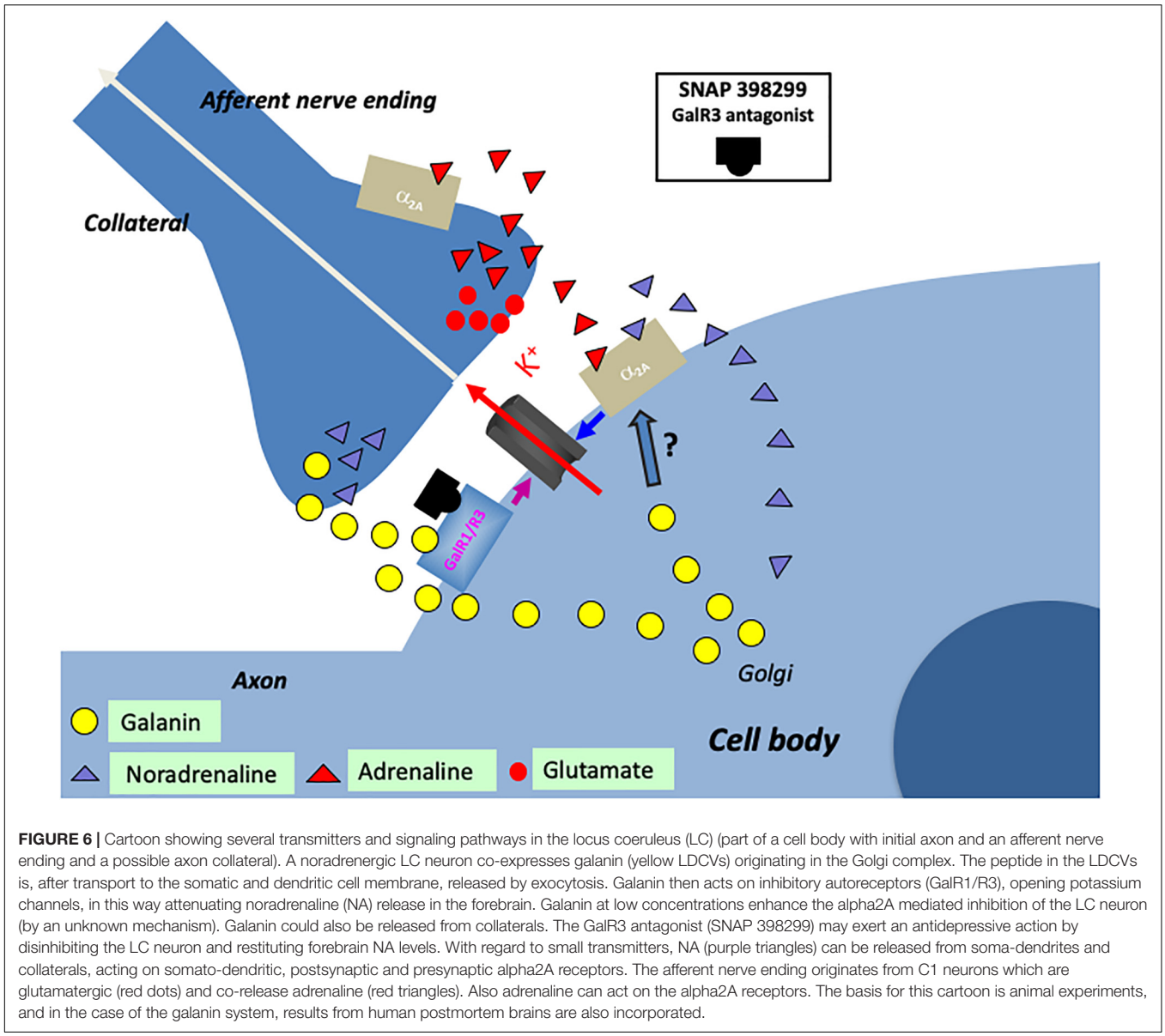

FIGURE 6 | Cartoon showing several transmitters and signaling pathways in the locus coeruleus (LC) (part of a cell body with initial axon and an afferent nerve ending and a possible axon collateral). A noradrenergic LC neuron co-expresses galanin (yellow LDCVs) originating in the Golgi complex. The peptide in the LDCVs is, after transport to the somatic and dendritic cell membrane, released by exocytosis. Galanin then acts on inhibitory autoreceptors (GalR1/R3), opening potassium channels, in this way attenuating noradrenaline (NA) release in the forebrain. Galanin at low concentrations enhance the alpha2A mediated inhibition of the LC neuron (by an unknown mechanism). Galanin could also be released from collaterals. The GalR3 antagonist (SNAP 398299) may exert an antidepressive action by disinhibiting the LC neuron and restituting forebrain NA levels. With regard to small transmitters, NA (purple triangles) can be released from soma-dendrites and collaterals, acting on somato-dendritic, postsynaptic and presynaptic alpha2A receptors. The afferent nerve ending originates from C1 neurons which are glutamatergic (red dots) and co-release adrenaline (red triangles). Also adrenaline can act on the alpha2A receptors. The basis for this cartoon is animal experiments, and in the case of the galanin system, results from human postmortem brains are also incorporated.

Mitsukawa et al., 2009; Kuteeva et al., 2010; Hoyer and Bartfai, 2012; Webling et al., 2012; Freimann et al., 2015).

Two recent studies support involvement of GalR1, and suggest the ventral periaqueductal gray as one likely site of action. Using a rat model of depression based on chronic mild stresses (CMS) (Willner et al., 1987; Moreau et al., 1992), behavior was evaluated in the open field test, the forced swim test (FST), and by monitoring sucrose consumption (Wang et al., 2016). Transcript levels of galanin and GalR1-3 in various, laser-dissected brain regions, including the hippocampal formation (HiFo), vPAG, the DRN and the LC were analyzed with quantitative real time PCR (qPCR) (Wang et al., 2016). Only GalR1 mRNA levels were significantly changed (increased), in a single region, the vPAG. Moreover, after knocking down GalR1 in the vPAG using siRNA, the depressive behavioral phenotypic parameters were similar to unstressed controls. This result suggested that the depression-like behavior in rats exposed to CMS is likely related to an elevated expression of GalR1 in the vPAG. The phenotype of the GalR1-positive neurons was not identified, despite comparing their distribution with serotonin, glutamate (vesicular glutamate transporter type 2, VGLUT2) and GABA (glutamic acid decarboxylase, GAD) neurons (Wang et al., 2016).

In another study, the galanin system was monitored with qPCR, ISH and RIA methodologies following mild blast-induced traumatic brain injury (mbTBI) (Kawa et al., 2016). Significant increases in galanin peptide and transcript were observed in the LC, at 1 day with qPCR, at 3 days with RIA and from 2 h to 7 days with ISH. The increases thus remained for 7 days (ISH) (the longest period studied). With regard to galanin receptors, GalR1 mRNA was significantly increased in vPAG at 1 and 7 days, likely in the same neuron population as seen in the CMS model

(Wang et al., 2016). These findings suggest a long-lasting role for the galanin system in the endogenous response to mbTBI. Again, the phenotype of these GalR1-positive neurons was not identified. Nevertheless, in both cases stress, and possibly depression-like behavior, are associated with increased levels of GalR1 transcript in the vPAG. Interestingly, the galanin system has also been shown to modulate stress-related responses related to mild TBI in a model of postraumatic stress disorder (PTSD) (Kozlovsky et al., 2009).

The robust and lasting effect of mbTBI on the expression of galanin (at least 7 days) not only in LC but also in 5-HT neurons (Kawa et al., 2016) is more sustained than the transient increase in tyrosine hydroxylase (TH) (3 days) and tryptophan hydroxylase 2 (1 day) seen in the same *mbTBI* model (Kawa et al., 2015). Thus, in mbTBI the coexisting peptide may have a more long-lasting and important effect than the small molecule transmitter.

In another study, i.p. injection (a stress by itself) and swim stress increased both galanin and TH mRNA levels in the LC, but not TPH2 or galanin transcripts in the DRN (Kuteeva et al., 2008), indicating that the serotonergic system is less sensitive to stress than the noradrenergic system. This has also been shown in other studies employing different types of stress (e.g., Wilkinson and Jacobs, 1988; Jordan et al., 1994; Kuteeva et al., 2008, 2010). The long lasting effects of stress can also be gauged against the fact that peptides can exert effects over long periods of time (Herbert, 1993; van den Pol, 2012). One example is a study on the lamprey locomotor network that revealed that a 10-min administration of substance P causes a long-lasting (>24 h) modulation of the frequency and regularity of NMDA-evoked locomotor bursts (Parker and Grillner, 1999).

GalR2 may also be involved, but here an opposite effect has been recorded, i.e., galanin actions via this receptor are antidepressive (Gottsch et al., 2005; Lu et al., 2005a, 2007, 2008; Kuteeva et al., 2008, 2010; Kinney et al., 2009; Le Maitre et al., 2011; Saar et al., 2013a,b; Kawa et al., 2016), in some cases associated with the vPAG. For example, in a neuropharmacological study (Kuteeva et al., 2008) the time of immobility (**Figure 7A**) and climbing (**Figure 7B**) were recorded in the FST. Galanin, the GalR1 receptor agonist M617, the GalR2(R3) agonist AR-M1896, the GalR2 antagonist M871 or aCSF were infused intracerebroventricularly. Galanin significantly increased immobility time, as did the GalR1 receptor agonist M617 (**Figure 7A**). In contrast, the GalR2(R3) agonist AR-M1896 decreased immobility, similar to fluoxetine, whereas the GalR2 antagonist M871 *increased* the time of immobility. Together these results support the view of GalR1 being pro- and GalR2 antidepressive. Moreover, the antidepressive effect of the GalR2 antagonist suggests there is an *in vivo*, tonic activation of this receptor under forced swimming (stress). This provides further evidence that galanin is released *in vivo* under stressful conditions. More recently it has been shown that an anxiolytic-/antidepressive effect of galanin injected directly into the DRN is mediated via GalR2 (Silote et al., 2013; de Souza et al., 2018).

Taken together, galanin receptors GalR1 and GalR2 play a differential role in regulation of depression-like behavior. Thus, galanin exerts a prodepressive effect, presumably via GalR1, while stimulation of GalR2 has an antidepressant-like effect.

While little interest has been paid to GalR3 in relation to mood, possibly due to its low expression in the rat (Mennicken et al., 2002) and mouse (Lein et al., 2007) brain, a GalR3 knockout mouse exhibits an anxiety-like phenotype (Brunner et al., 2014).

Following early studies on intra-membrane receptor-receptor interactions (Fuxe and Agnati, 1985), receptor di- and heteromerization have become a recognized mechanism for signaling through GPCRs (Bouvier, 2001; Devi, 2001; Agnati et al., 2003). Recent studies reveal that galanin receptor heteromers exist, introducing a further degree of complexity in interpreting galaninergic signaling in the brain (Fuxe et al., 2012), and in relation to mood control. Thus, in addition to GalR1 and 5-HT1A receptor heterodimers (Borroto-Escuela et al., 2010), the galanin (1–15) fragment alone induces strong depression- and anxiogenic-related effects and may regulate mood via binding to GalR1 and GalR2 heterocomplexes (Millon et al., 2014, 2017a,b). Interestingly, galanin (1-15) causes a dose-dependent hyperpolarization of a population of hippocampal CA3 neurons (Xu et al., 1999), and after iodination it binds to other regions including the dorsal hippocampus, as shown in autoradiographic studies (Hedlund et al., 1992). Taken together these results provide evidence for a functional role of galanin (1–15), perhaps unexpected in view of results showing a high affinity of the N-terminal galanin *(1-16)* fragment to galanin binding sites in the brain (Fisone et al., 1989).

GALANIN AND DEPRESSION-LIKE BEHAVIOR IN RODENTS – LC

The LC and NA have since the 1960's been a focus of clinical and preclinical monoamine research, because of their involvement in stress, mood control and treatment of mood disorders (Bunney and Davis, 1965; Schildkraut, 1965; Weiss et al., 1981, 1994; Svensson, 1987; Simson and Weiss, 1988; Page and Valentino, 1994; Schatzberg and Schildkraut, 1995; Aston-Jones et al., 1996; Bremner et al., 1996a,b; Harro and Oreland, 2001; Charney, 2004; Millan, 2006; Samuels and Szabadi, 2008; Seki et al., 2018).

There is a strong relationship between stress and the LC: stress increases NA turnover, as well as tyrosine hydroxylase activity and transcription in the LC (Korf et al., 1973; Zigmond et al., 1974; Abercrombie and Jacobs, 1987; Komori et al., 1990; Smith et al., 1991; Melia et al., 1992; Aston-Jones et al., 1996; Rusnak et al., 1998; Chang et al., 2000; McDevitt et al., 2009; Ong et al., 2014; Kawa et al., 2015). Moreover, stress activation of LC neurons results in release of NA in the forebrain (Abercrombie et al., 1988; Jordan et al., 1994; Vahabzadeh and Fillenz, 1994; Ihalainen et al., 1999; Yoshitake et al., 2004) and cortical EEG activation, i.e., arousal (Page et al., 1993). Here CRF (Vale et al., 1981) is an important mediator of the stress-induced LC activation (Valentino and Van Bockstaele, 2015).

Also galanin expression is upregulated in LC neurons in response to stress/exercise (Holmes et al., 1995; Sweerts et al., 1999; O'Neal et al., 2001; Sciolino et al., 2012; Weinshenker and Holmes, 2016), establishing a relation between stress, NA and galanin in LC. Similarly, a single dose of the monoamine-depleting drug reserpine (Pletscher et al., 1955; Carlsson, 1975)

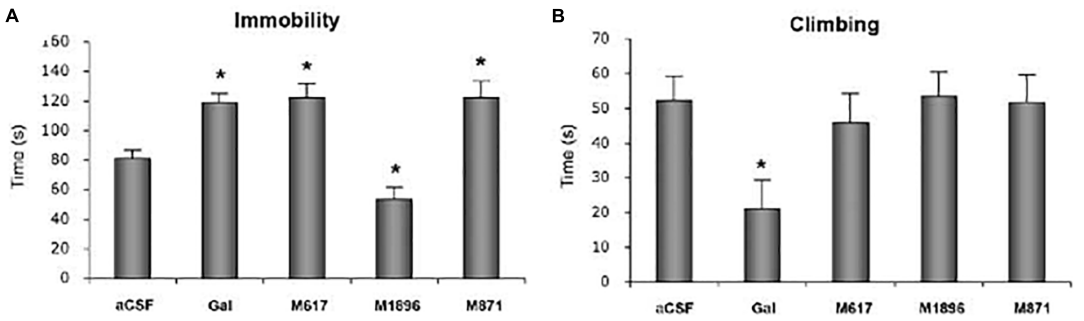

FIGURE 7 | Time of immobility **(A)** and climbing **(B)** in the forced swim test (FST). Rats received i.c.v. infusion of aCSF, galanin (Gal), the GalR1 receptor agonist M617, the GalR2(R3) agonist AR-M1896 or the GalR2 antagonist M871 (M871) 20 min prior to a 5 min test. Data presented as mean ± SEM. significant difference from the control swim group; *one-way ANOVA, Fisher's PLSD. Galanin, the GalR1 agonist and the GalR2 antagonist increase the immobility time versus a decrease after the GalR2(3) agonist. From Kuteeva et al. (2008), with permission.

causes an increase in galanin mRNA levels in LC neurons (Austin et al., 1990; Gundlach et al., 1990a). The same treatment results in a complete depletion of galanin in the noradrenergic cortical/hippocampal nerve terminals (Xu et al., 1998), that are the projections of the LC neurons: evidence for the view that release of a neuropeptide leads to increased synthesis of transcript and peptide. NPY expression in LC has, contrasting galanin, not been reported to be regulated by stress, but NPY mRNA is increased after reserpine administration (Gundlach et al., 1990a).

An involvement of LC in depression-like behavior has been studied by Weiss and colleagues focused on a link with the ascending mesencephalic dopamine system (Weiss et al., 1981, 1996, 1998, 2005). They based their experiments on the study by Grenhoff et al. (1993) showing that burst stimulation of LC inhibits DA neurons in the ventral tegmental area (VTA) (a.k.a the A10 group) (Dahlstrom and Fuxe, 1964). Weiss and colleagues have found that infusion of galanin into the VTA reduced exploratory behavior and increased immobility in the Porsolt test (a.k.a. Forced Swim Test, FST), an increase that was blocked by the galanin antagonist galantide. These findings link the LC-galanin system to studies showing involvement of the VTA and the reward system in stress and depression (Everitt and Robbins, 2005; Nestler and Carlezon, 2006; Thomas et al., 2008; Nestler, 2015; Pena et al., 2017).

The LC is involved in other mood-related behaviors such as addiction and reward (Maldonado and Koob, 1993), and galanin plays a role also in this context (Picciotto, 2008; Genders et al., 2018a). Thus, galanin binding and levels of GalR1 mRNA are increased in the LC during opiate withdrawal (Zachariou et al., 2003). Moreover, galanin-knockout mice exhibit more pronounced signs of opiate withdrawal, and galanin and the galanin ligand galnon both attenuate opiate reward and signs of withdrawal (Zachariou et al., 2003).

THE GALANIN SYSTEM IN THE NORMAL HUMAN BRAIN

The distribution of galanin in the 'normal' human brain has been studied with RIA (Bennet et al., 1991; Barde et al., 2016), IHC (Chan-Palay, 1988a,b, 1990; Gentleman et al., 1989; Kowall and Beal, 1989; Beal et al., 1990; Kordower and Mufson, 1990; Kordower et al., 1992; Gabriel et al., 1994) and ISH (Miller et al., 1999; Le Maitre et al., 2013). In addition, the receptor distribution was analyzed with autoradiographic ligand binding methodology (Kohler et al., 1989; Kohler and Chan-Palay, 1990). Here, recent results obtained with ISH, qPCR and RIA on the galanin system in the LC and some other regions are summarized (Le Maitre et al., 2013; Barde et al., 2016). These studies were based on the identified gene sequences of the *human* galanin peptide and receptors (Evans and Shine, 1991; Jacoby et al., 1997; Lorimer et al., 1997; Fathi et al., 1998; Kolakowski et al., 1998; Smith et al., 1998).

In situ Hybridization

The ISH analysis of the human LC (Le Maitre et al., 2013) revealed expression of TH, the rate-limiting enzyme for catecholamine synthesis in presumably all noradrenergic neurons (**Figures 8A,B**), galanin mRNA in many LC neurons (**Figures 8C,D**) and GalR3 mRNA in many, perhaps all neurons (**Figures 8E,F**), the latter two overlapping with the TH distribution. However, whereas the levels of TH and GalR3 mRNA are relatively similar in all cells, there was a large variation in the intensity of the galanin mRNA signal (c.f. **Figures 8A,B,E,F** with **Figures 8C,D**). This likely reflects the fact that galanin is a releasable molecule and that individual neurons are in different activity states. Note that the exposure time of the emulsion dipped slides is very different for the three markers (10 days for TH versus several months for GalR3), reflecting differences in mRNA levels (**Figures 8A–F**). Thus, GalR3 mRNA levels are very low, in agreement with low levels in rat (Mennicken et al., 2002) and potentially undetectable levels in mouse (Lein et al., 2007). In fact, GalR3 transcripts could only be visualized in human brains with very short post mortem delays prior to freezing (2–4 h).

RIA, qPCR and DNA Methylation

Barde and colleagues analyzed > 200 *postmortem* brain samples from 'normal' (and depressed, see below) female and male subjects, including the following regions: in addition to LC,

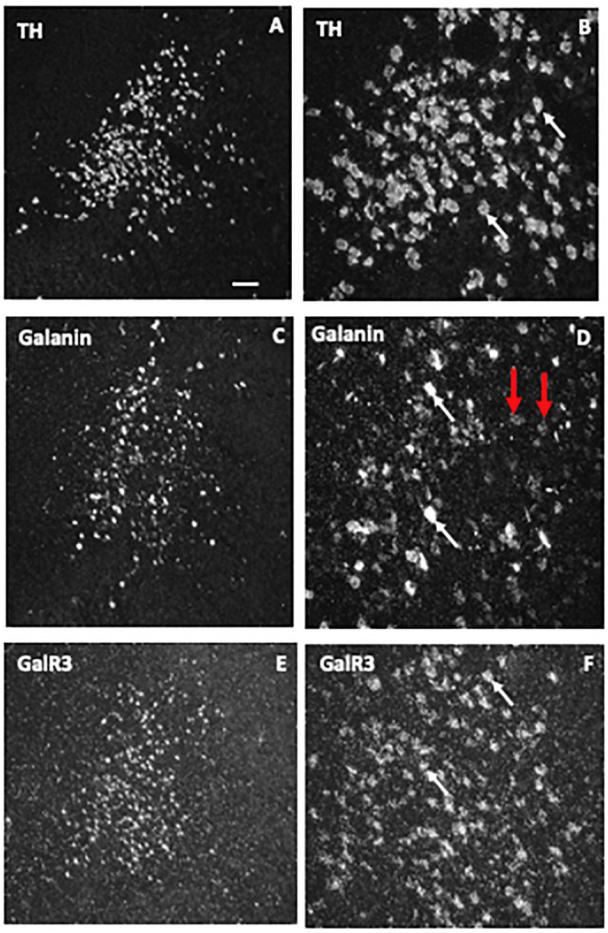

FIGURE 8 | Dark-field ISH photomicrographs showing the distribution of transcripts for tyrosine hydroxylase (TH), galanin, and GalR3 in the locus coeruleus. The three markers TH **(A,B)**, galanin **(C,D)**, and GalR3 **(E,F)** show overlapping distribution patterns. TH and GalR3 transcript levels seem approximately similar in all cells. In contrast, there is variability in the strength of the signal for galanin mRNA (white arrow points to neurons with a strong signal, red ones to such with a weak signal). Exposure time: TH, 10 days; galanin, 4 weeks; GalR3, 8 weeks. This difference in exposure time transcript reflects difference in transcript levels, that is GalR3 mRNA levels are very low. Reprinted from Le Maitre et al. (2013) [Scale bars: 200 μM for **(A,C,E)**; 50 μM for **(B,D,F)**].

dorsolateral prefrontal cortex (DLPFC), anterior cingulate cortex (ACC), DRN and the medullary raphe nuclei (MRN)[6] (Barde et al., 2016). Three methods were applied: RIA to monitor peptide levels, qPCR for transcript levels, and pyrosequencing to analyze DNA methylation. Comparable information is presented for ligand (galanin) (**Table 1A**) and transcripts (**Table 1B**) of galanin and GalR1-3 in the *normal* (control) and ('depressed') male and female brain (Barde et al., 2016).

When evaluating the results it should be noted that peptide levels in normal brains mostly reflect peptide present in nerve terminals and less so in cell bodies. Animal studies have shown that galanin peptide (like many other peptides) can best be detected in cell bodies after inhibition of axonal transport by colchicine (Rokaeus et al., 1984; Skofitsch and Jacobowitz, 1985; Melander et al., 1986c). In contrast, peptide transcripts are easily seen in cell bodies/dendrites, and often confined to these neuronal compartments.

With RIA, marked regional differences in galanin levels were observed, being highest in DRN (> LC > MRN > ACC = DLPFC), whereby DRN levels were 2 times higher than in LC and ∼12 times higher than in ACC/DLPFC. The qPCR analysis revealed the highest galanin mRNA levels in LC (reflecting the many positive cell bodies), about 4-fold higher than in DRN, and 6-fold higher than DLFPC, in agreement with the ISH results (Le Maitre et al., 2013) (**Table 1A**). Thus, the results from the LC suggest that there is a good translation from mRNA to peptide. The RIA results are generally in agreement with IHC studies on the primate brain (Kordower et al., 1992), and on the rat brain, when analyzed with RIA (Skofitsch and Jacobowitz, 1986) and IHC (Skofitsch and Jacobowitz, 1985; Melander et al., 1986b; Merchenthaler et al., 1993).

The results are also in line with the cited immunohistochemical results in the rat with a high density of galanin-positive nerve terminals in the DRN, and fewer in the LC (Skofitsch and Jacobowitz, 1985; Melander et al., 1986b; Merchenthaler et al., 1993). It is likely that galanin in cortical areas is present in thin and rather sparse afferents to the cortex, possibly originating in LC, as is the case in rat (Xu et al., 1998) and also in local neurons (see below).

With regard to receptors only transcripts and methylation were studied. This is partly due to a lack of specific antibodies for the galanin receptors, as discussed (Lu and Bartfai, 2009; Brunner et al., 2018), and there was no attempt to use Western blotting, Elisa or IHC for receptors. The strongest signal by far was noted for GalR1 mRNA, with the highest levels seen in DLPFC (> LC > MRN = DRN > ACC) (**Table 1B**). GalR1 levels in DLFPC were 2 times higher than in LC, and the GalR1 mRNA levels in DLFPC were *8-fold* higher compared to the 'adjacent' ACC. GalR2 mRNA levels were in general considerably lower in LC (64-fold lower than GalR1). The GalR3 mRNA levels were low, although four times higher in the lower brain stem (LC, DRN) than in cortical regions, in agreement with the ISH results showing that GalR3 mRNA is present in NA neurons in LC, and possibly in 5-HT neurons in the DRN (Le Maitre et al., 2013).

In summary, GalR1 mRNA is the most prominent galanin receptor transcript in the human brain, including cortical regions. This is in agreement with early studies on human *postmortem* brain with iodinated galanin and autoradiography that revealed a distinct cortical signal (Kohler et al., 1989; Kohler and Chan-Palay, 1990), thus likely representing GalR1. The

[6]Please note that the samples from the five regions contain a heterogeneous collection of neurons (and of course glia), where members of the galanin family are present to a varying percentage. (These are cell bodies, since transcripts are monitored. However, the efficacy of probes may vary, and false negatives are not unlikely.) The "LC" sample contains a fairly high percentage of NA neurons. In "DRN" the 5-HT neurons probably represent a small proportion of all neurons, also since the sample includes the vPAG, and more. In the "MRN" sample an even smaller percentage of the neurons are serotonergic. Knowledge of the localization of transcripts of the galanin family is available only for DRN and LC, and even here only to a limited extent.

TABLE 1 | (A,B) Concentration of galanin peptide (pmol/mg ± SEM) analyzed by RIA **(A)** and raw Ct values ± SEM monitored by qPCR **(B)** for male and female control and suicide samples from five brain regions.

	DLPFC	ACC	DRN	LC	MRN
(A) Region					
Male Con	3.9 ± 1.2	5.4 ± 1.2	61.6 ± 13.7	31.8 ± 7.5	15.6 ± 2.3
Male DS	2.7 ± 0.5	4.9 ± 1.0	68.1 ± 11.6	*37.4 ± 10.7*	18.7 ± 4.8
Female Con	1.7 ± 0.4	6.4 ± 1.8	80.6 ± 10.2	43.4 ± 6.3	15.5 ± 3.5
Female DS	1.3 ± 0.3	6.0 ± 1.3	81.6 ± 5.2	*67.7 ± 7.5*	13.5 ± 2.9
(B) Region					
Galanin	27.1 ± 0.2	27.5 ± 0.1	26.2 ± 0.4	24.6 ± 0.5	25.2 ± 0.4
GalR1	25.7 ± 0.2	28.6 ± 0.1	27.0 ± 0.4	26.4 ± 0.6	26.9 ± 0.4
GalR2	31.4 ± 0.2	31.6 ± 0.2	29.3 ± 0.2	32.3 ± 0.1	34.6 ± 0.2
GalR3	33.2 ± 0.1	33.5 ± 0.2	31.5 ± 0.4	31.4 ± 0.6	31.9 ± 0.4

From Barde et al. (2016). Con, controls, DS, depressed suicides; DLPFC, dorsolateral prefrontal cortex; ACC, anterior cingulate cortex; DRN, dorsal raphe nucleus; LC, locus coeruleus; MRN, medullary raphe nuclei; Gal, galanin. Italics indicate significantly lower galanin levels in male vs. female depressed suicides.

results differ from rat, since the early ligand binding studies in adult rat lacked binding in dorsal cortical areas (Skofitsch et al., 1986; Melander et al., 1988), and since GalR1 and -R2 mRNA levels are low (O'Donnell et al., 1999; Burazin et al., 2000). Thus, cortical receptor levels may represent another species difference not only between rat and human, but also between rat and mouse (Jungnickel and Gundlach, 2005). However, overall, GalR1 is also the most prominent galanin receptor in the rat brain (O'Donnell et al., 1999; Burazin et al., 2000) and likely also in mouse brain (Hohmann et al., 2003; Jungnickel and Gundlach, 2005; Lein et al., 2007). Waters and Krause (2000) monitored the levels of transcript for all three galanin receptors in the rat brain: GalR1 is highest in amygdala and spinal cord, whereas in cortex GalR2 > GalR1 > GalR3, and in hippocampus GalR2 > GalR1 = GalR3. In that study values were expressed as mean pg/25 μg total RNA.

The interpretation of the human qPCR results is not straight forward. For example, there is a lack of knowledge of the cellular localization of the transcripts in the prefrontal cortex regions, i.e., these areas have not been studied with ISH. It is likely that the transcripts are present in neurons, but a glial localization cannot be excluded (Butzkueven and Gundlach, 2010). Under certain circumstances galanin is expressed in specialized glial cells, e.g., after colchicine treatment alone or after spreading depression (Xu et al., 1992; Shen et al., 2003, 2005). The colchicine-induced signal was abolished by thyroidectomy (Calza et al., 1998). However, the results strongly suggest that galanin, and possibly all three galanin receptors, or at least GalR1, are expressed locally in cells, likely in cortical neurons. In contrast, ISH results *are* available for DRN and LC and reveal neuronal localization (Le Maitre et al., 2013). In particular, the results on the LC offer a possibility to form a hypothesis about the galanin system in this nucleus being involved in stress and genesis of depression, as discussed below.

Overall major differences exist between species, both with regard to galanin and galanin receptor expression, whereby galanin signaling seems to be more important for dorsal cortical functions in the human brain than in rodents. In contrast, in the rat ventral cortical areas, like entorhinal and piriform cortices, have abundant galanin receptor expression (Skofitsch et al., 1986; Melander et al., 1988; O'Donnell et al., 1999; Burazin et al., 2000), suggesting involvement in limbic processes.

DEPRESSIVE DISORDERS

Major depressive disorder (MDD) is a common and serious disease afflicting up to 2–5% (12-month prevalence; lifetime prevalence 10–15%) of the population worldwide, and women being more susceptible than men. Thus, MDD is a leading cause of disability worldwide associated with much suffering and major costs for society (Murray and Lopez, 1997; Kessler et al., 2003; Wittchen et al., 2011; Ferrari et al., 2013; World Health Organization [WHO], 2013). Adverse life events usually precede depression episodes, and experiences of physical and emotional abuse during early childhood and parental neglect are important predisposing vulnerability factors, strongly indicating that environmental psychosocial stressors are essential in pathogenesis (Kendler, 2012, 2013; Lutz et al., 2017; Tanti et al., 2017). The heritability is significant, about 35% (Sullivan et al., 2000). Interaction of genetic and environmental factors including stressful life events plays a major role in the development of MDD (Nestler et al., 2002; Akil, 2005; de Kloet et al., 2005; McEwen, 2007; McEwen et al., 2015). Epigenetic mechanisms through altered DNA methylation (Meaney and Ferguson-Smith, 2010; Zhang and Meaney, 2010) are probably involved, leading to stable changes in brain function that may underlie the psychopathology (Labonte et al., 2013; Vialou et al., 2013).

Over the last several decades two major hypotheses of the cause of unipolar depression have dominated, clinically associated with catecholamines (Bunney and Davis, 1965; Schildkraut, 1965; Schatzberg and Schildkraut, 1995) and with serotonin (Coppen, 1968; Maes and Meltzer, 1995). Pharmacological management of depression therefore often involves drugs that target the monoamine transporters, which include SSRIs, the transporter for noradrenaline (NA) (NRIs) or a combination of both (SNRIs) (Gardier et al., 1996; Mongeau et al., 1997; Millan, 2006), as well as a number of other medications (Berton and Nestler, 2006; Millan et al., 2015b). However, the therapeutic efficacy of these antidepressants is hampered by a

slow onset of action, a limited response rate and considerable side effects (Montgomery, 2006; Trivedi et al., 2006). Of particular importance is the treatment resistant depression which affects some 20% of afflicted subjects (Akil et al., 2018). These issues have led to an intensive search for novel therapeutic approaches for MDD (Berton and Nestler, 2006; Artigas, 2015; Akil et al., 2018) (and see below), including targeting receptors for neuropeptides (Maubach et al., 1999; Hokfelt et al., 2003; Holmes et al., 2003; Nemeroff and Vale, 2005; Griebel and Holsboer, 2012), the most diverse family of brain messenger molecules (Burbach, 2010).

THE GALANIN SYSTEM AND DEPRESSION IN HUMANS

The evidence from animal experiments described led us to explore to what extent galanin may be involved in MDD and other mood disorders, and whether results from the analysis of human brain can guide the search for new antidepressants.

Genetic Variations in the Galanin System in Depression

A candidate gene study of a cohort of European White ethnic origin totaling 2,361 from Manchester, United Kingdom and Budapest, Hungary was carried out (Juhasz et al., 2014) and revealed that variants in genes for galanin and its three receptors confer increased risk of depression and anxiety in people who experienced childhood adversity and/or recent negative life events (**Figure 9**). Genetic factors were only relevant in the moderate or high stress exposure groups when applying Bayesian multivariate analysis (Juhasz et al., 2014; Gonda et al., 2018). The rank order of the relevance of gene polymorphisms was GalR2 > GalR3 > GalR1 > galanin, with strong relevance for the first three in the moderately or highly exposed persons by recent negative life events in the last 12 months. All four were more relevant than the serotonin transporter gene-linked polymorphic region (5-HTTLPR) of the serotonin transporter gene. The effects were seen in the Manchester and the Budapest population, and in both males and females. This impact was seen only if taking stress into account, after medium and strong stress (the GalR2 gene) or strong stress (the GalR3, GalR2 and galanin genes), underlining the importance of environmental factors. In addition, the GalR2 gene polymorphism was more relevant than candidate gene polymorphisms of the genes for BDNF, the serotonin 1A receptor (HTR1A), the cannabinoid 1 receptor (CB1) and the serotonin 2A receptor (HTR2A) in the moderately stress exposed subjects (Gonda et al., 2018). The traditional analysis based on general linear models confirmed the gene-environment interaction; namely, no main effect of genes, but a significant modulatory effect of environment-induced development of depression were found.

Evidence for collaboration between small neurotransmitter and neuropeptide in the development of depression was also identified in this study, namely a gene–gene–environment interaction between the GalR2 and 5-HTTLPR genes in strongly exposed persons (Gonda et al., 2018). This could be of interest regarding antidepressant drug targets. The expression of the GALR2

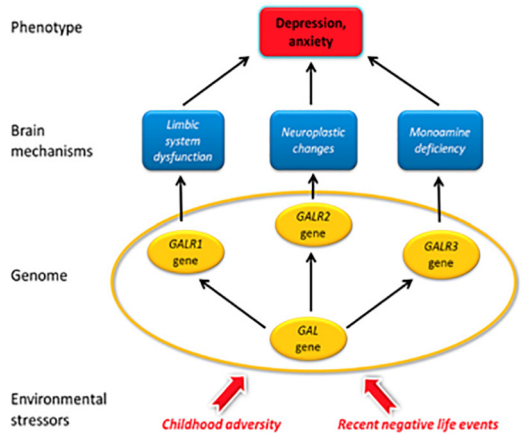

FIGURE 9 | Galanin mechanisms hypothetically involved in MDD in humans. Galanin and its receptors are colocalized in some monoaminergic neurons in the brain. The galanin system is highly sensitive to experimental and naturalistic stressors. Stress-induced activation of the galanin system represents the first phase in the development of depression. Recent analysis of human brain has shown that the Gi protein-coupled GalR3 (and not GalR1 as in rodents) is the main galanin receptor in noradrenergic neurons in the locus coeruleus and probably the dorsal raphe nucleus and that the Gi protein-coupled GalR1 is the main receptor in the forebrain. Antidepressive effects may be achieved by (i) GalR3 antagonists, by reinstating normal monoamine turnover in LC neurons in the lower brainstem projecting to the forebrain, and by (ii) GalR1 antagonists in the forebrain by normalization of limbic system activity, or by (iii) agonists at GalR2, a Gq protein-coupled receptor, promoting neuroprotection. A candidate gene analysis suggests that GalR1 risk variants may compromise galanin signaling during childhood, whereas GalR2 signaling may be influenced by recent negative life events. In addition, all four galanin system genes have relevant roles in the development of depression-related phenotypes in those persons who were highly exposed to life stressors. From Juhasz et al. (2014).

polymorphism is about 2.5 times higher compared to 5-HTTLPR. Currently, the most frequently used antidepressants are the SSRIs, NRIs and SNRIs. Preliminary pre-clinical results suggest that an SNRI (venlafaxine) does not alter the transcript levels of galanin and its receptors (Petschner et al., 2016). Other studies show that chronic treatment with SSRIs increases galanin mRNA levels in various brain regions (Christiansen et al., 2011; Rovin et al., 2012; Yamada et al., 2013). The higher relevance of the GalR2, GalR3 and GalR1 gene polymorphisms in stress-induced depression and the galanin system-independent effects of the currently used antidepressants suggest that novel antidepressants acting on GalR1-3 could be developed. Such compounds could perhaps be more effective in SSRI/SNRI non-responders.

Galanin Versus 5-HT Transporter in Depression

For a long time, it has been assumed that there is an interaction between stressful life events and a polymorphism in the promoter region of the 5-HT transporter (5-HTT) gene (SLCA4) (Lesch et al., 1996; Caspi et al., 2003). In the Newmood cohort this effect was weak and not significant in most comparisons, when corrections for multiple testing were applied (Juhasz et al., 2015). Furthermore, Bayesian relevance analyses consistently failed to

show relevance for 5-HTTLPR (Juhasz et al., 2014; Gonda et al., 2018). Parallel to these findings, a recent large meta-analysis could not confirm an interaction (Culverhouse et al., 2018). The findings on the galanin system provide evidence for a more robust and relevant effect of galanin system genes compared to 5-HTTLPR of the serotonin transporter gene (Juhasz et al., 2014; Gonda et al., 2018). The 5-HTT and GalR2 receptor act jointly in the development of depression (Gonda et al., 2018).

Other Genetic Studies

Involvement of galanin in depression is further supported by a gender-specific association of galanin polymorphisms with antidepressant treatment response (Unschuld et al., 2010) and by a study reporting an association of galanin and MDD in the Chinese Han population (Wang et al., 2013). In addition, the first large genome-wide association study (GWAS) obtained a suggestive association of *GAL* with MDD using a gene based test, which retained low association p-values in two additional independent cohorts (Wray et al., 2012). A very large GWAS failed to identify risk genes (Major Depressive Disorder Working Group of the Psychiatric Gwas Consortium, 2013), but recent advances in large MDD GWAS studies resulted in several SNPs being associated with MDD (e.g., Hyde et al., 2016; Okbay et al., 2016; Xiao et al., 2017; Wray et al., 2018), and provided further evidence that genetic risk for depression is a continuous measure that translate environmental adversities into depressive symptoms. Taken together, the genetic analysis of the four members of the galanin system genes are complemented by a study on *postmortem* brains from depressed suicides (Barde et al., 2016), strengthening an involvement of galaninergic mechanisms in depression, as discussed below.

Multiple Changes in the Galanin System in MDD

Differences in levels of galanin peptide, and of transcripts for, and DNA methylation of, galanin and GalR1-3 between MDD patients and matched controls were observed in an analysis of > 200 *postmortem* samples from five male and female brain regions (DLPFC, ACC, DRN, LC, and MRN). The significant and selective differences and changes in the galanin system in depressed versus control brains are summarized in **Table 2**. The most pronounced changes were observed for galanin and GalR3 in the DLPFC, and for galanin and GalR3 in the DRN and LC, in males and females (examples of results in **Figures 10A,B**). In DRN and LC there was an *upregulation* of the transcripts, paralleled by a *decrease* in DNA methylation. The decrease in methylation in galanin and GalR3 was most pronounced in female DRN and in male and female LC. In DLPFC, galanin mRNA levels were *decreased* in males and *increased* in females, the only distinct sex difference observed in the study. The changes in GalR1 were also increased and confined to three regions, DLPFC (male and female), DRN (male) and MRN (male), versus no change in LC. No differences were seen with regard to GalR2, except a decrease in MRN, included as a control region. The complete lack of changes in ACC contrasts the dramatic

TABLE 2 | Overview of mRNA and DNA methylation changes.

		Galanin				GalRI				GalR2				GalR3			
		IdNA methylation				gDNA methylation				gDNA methylation				gDNA methylation			
Regions	**Sex**	**mRNA**	**CpGI**	**CpG2**	**CpG3**	**mRNA**	**CpGI**	**CpG2**	**CpG3**	**CpG4**	**mRNA**	**CpGI**	**CpG2**	**CpG3**	**CpG4**	**mRNA**	**CpGI**	**CpG2**	**CpG3**
DLPFC	Males	↓↓	↑		↑↑	↑	–	–	–	–	–	–	–	–	–	↓↓	–	–	–
	Females	↑	↑	→	–	↑	–	–	–	–	–	–	–	–	–	–	–	–	–
ACC	Males	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Females	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
DRN	Males	↑↑	–	→	–	↑	–	–	–	–	–	–	–	–	–	↑	–	–	–
	Females	↑↑	–	–	–	–	–	–	–	–	–	–	–	–	–	↑↑	↓↓	↓↓	–
LC	Males	↑↑	↓↓	→	–	–	–	–	–	–	–	–	–	–	–	↑	→	–	–
	Females	↑↑	→	↓↓	–	–	–	–	–	–	–	–	–	–	–	↑↑	→	↓↓	–
MRN	Males	↑	–	–	–	↑	–	–	–	–	↓↓	–	–	–	–	↑	–	–	–
	Females	↑	–	–	–	–	–	–	–	–	–	–	–	–	–	↑	–	–	–

From Barde et al. (2016). The arrows represent statistical significance, where upward arrow signifies increase in gene expression and methylation status and vice-versa. One arrow signifies $P < 0.05$ and two arrows stand for $P < 0.01$. DLPFC, dorsolateral prefrontal cortex; ACC, anterior cingulate cortex; DRN, dorsal raphe nucleus; LC, locus coeruleus; MRN, medullary raphe nuclei.

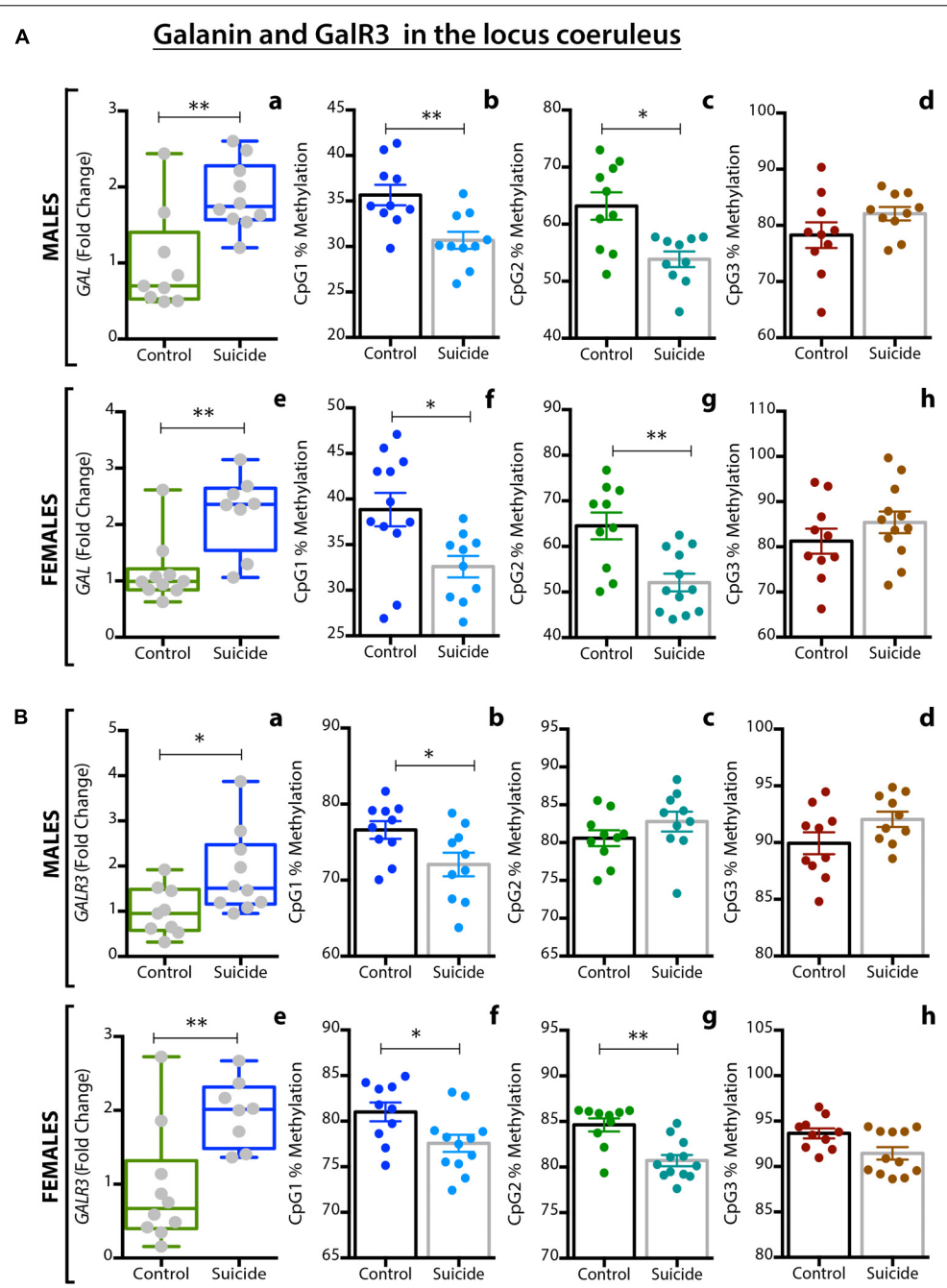

FIGURE 10 | (A,B) Alterations in galanin **(A)** and GalR3 **(B)** gene expression and DNA methylation in the locus coeruleus (LC) of male and female depressed subjects who committed suicide, as compared to matched controls. **(a,e)** Expression levels of the two genes in the LC of male **(a)** and female **(e)** controls and depressed suicide (DS) subjects. **(b–d,f–h)** Percentage of DNA methylation levels at individual CpG sites of the two genes in male **(b–d)** and female **(f–h)** controls and DS subjects. All data are presented as mean ± SEM; males: $n = 10$ controls, 10 DS subjects; females: $n = 12$ controls, 10 DS subjects. Significant differences between DS subjects and controls are indicated: $^{*}P < 0.05$, $^{**}P < 0.01$. CON, controls. From Barde et al. (2016).

alterations in DLPFC, both regions belonging to the prefrontal cortex complex.

With regard to methylation, changes were always opposite to those in transcript levels. This is in agreement with the general view that methylation suppresses transcript synthesis (Moore et al., 2013). The results lend further support for an involvement of epigenetic mechanisms in MDD (Mill and Petronis, 2007; Machado-Vieira et al., 2011; Vialou et al., 2013; Lolak et al., 2014; Lopizzo et al., 2015; Saavedra et al., 2016; Hoffmann et al., 2017; Nagy et al., 2018).

Taken together, the results suggest that galaninergic mechanisms, in several brain regions, are involved in MDD, and

that epigenetic changes mediated by DNA methylation play an important role, in agreement with a candidate gene study (Juhasz et al., 2014).

INVOLVEMENT OF GALANIN IN DEPRESSION AND RESILIENCE – A HYPOTHESIS

The LC in humans is a compact (but less so than in rodents), 'blue' (pigmented) nucleus consisting of a total (both sides) of around 50,000 neurons (German et al., 1988; Baker et al., 1989; Chan-Palay and Asan, 1989; Miller et al., 1999; Szot et al., 2000). As in other mammalian species studied, galanin is expressed in a large proportion of the human noradrenergic neurons (Chan-Palay et al., 1990; Kordower et al., 1992; Miller et al., 1999; Le Maitre et al., 2013), suggesting conservation during evolution. However, there are differences with regard to receptors. Thus the GalR3 receptor seems to be the most prominent receptor in the LC, contrasting the robust expression of GalR1 and GalR2 in the rat LC (O'Donnell et al., 1999; Burazin et al., 2000). Neither GalR1 nor -R2 mRNA was, surprisingly, detected with ISH in human NA neurons, although a GalR1 signal was seen in the LC region and in other regions, suggesting that the probe was functional. However, it cannot be excluded the NA LC neurons contain lower levels of GalR1 than other types of neurons and thus escaped detection. Thus, a distinct species difference seems to exist. Another apparent species difference was the possible lack of galanin expression in the human 5-HT neurons (Le Maitre et al., 2013), as is the case also in the mouse (Larm et al., 2003; Kuteeva et al., 2004; Lein et al., 2007; Fu et al., 2010), versus a robust expression of galanin in rat 5-HT neurons (Melander et al., 1986c; Fuxe et al., 1990; Priestley et al., 1993; Xu and Hokfelt, 1997).

How and when neuropeptides and classic transmitters are released has been explored (see Lundberg and Hokfelt, 1983; Hokfelt, 1991; Lundberg, 1996). Neuropeptides, stored in LDCVs, are mainly released when neurons are firing at a high rate or in bursts, e.g., during stress. This release occurs extrasynaptically, and not only from nerve endings but also from soma/dendrites. Merging this information and the results from MDD patients (Barde et al., 2016) it is possible to generate a hypothesis how depression in humans, likely a (stress-related) subtype of MDD, may develop (**Figure 11**): Under normal circumstances LC neurons fire at low frequencies, releasing NA in cortical regions, acting on post- and pre-synaptic adrenoceptors. Under stress, when LC neurons fire in bursts, also galanin will be released, together with NA, from nerve endings and soma/dendrites of the NA LC neurons, galanin acting on somatic/dendritic GalR3 autoreceptors.

GalR3 is, like GalR1, inhibitory (Smith et al., 1998) and causes hyperpolarization of the LC neurons, the purpose being to act as a 'brake' to prevent overexcitation, to keep the system in balance. This is similar to the proposed function of the 5-HT1A receptor as a "safety valve" of 5-HT neurons (Celada et al., 2013). As a consequence of increased firing and increased galanin release, synthesis of new peptide is initiated, reflected in increased mRNA levels. The fact that in dendrites sites of synthesis and release are close allows for rapid replacement. Thus, if mRNA is translated, increased galanin levels will be available for release from soma and dendrites, a feed-forward process. The additional increase in GalR3 transcript, presumably resulting in increased levels of receptor protein, could represent a robust increase in local galanin signaling. This seems unexpected, because intuitively one would expect downregulation of the receptor, following elevated levels of ligand. However, the inhibition may be strong and long-lasting leading to depletion of NA in the forebrain. The results suggest that mood disorders may be a consequence of a maladaption, an allostatic load (McEwen, 2003).

It may be emphasized that the prefrontal cortex has not been included in the discussion, even if significant changes in levels of galanin transcripts and methylation were recorded in this region of depressed subjects (Barde et al., 2016). This is because lack of knowledge about the cellular localization of the galanin system in this brain area. Finally, a similar scenario for an anti-depressive role of a GalR3 antagonist could be sketched for the 5-HT neurons in the DRN, since the galanin and GalR3 transcripts are upregulated both in the male and female DRN region from depressed patients who committed suicide, paralleled by decreased DNA methylation of the GalR3 gene in the female depressed subjects (Barde et al., 2016).

Of note, in the Barde et al. (2016) study the end stage of a mostly long development of the disorder is recorded, where all 'resources' have been mobilized to prevent overexcitation: increased ligand release plus increased receptor availability. Alternatively, the situation may reflect changes beyond patho-physiological regulatory mechanisms, especially when considering the considerable time it takes for depression to arise[7].

Resilience

Even if many humans are exposed to stress of various types and intensity, only comparatively few develop depression, thus displaying resilience to stress (Nestler et al., 2002; Southwick et al., 2005; Han and Nestler, 2017). Resilience appears to represent an active process involving several systems, including not only the mesolimbic dopamine neurons (Han and Nestler, 2017) but also other systems, such as the noradrenergic LC neurons (Charney, 2004; Feder et al., 2009; Krystal and Neumeister, 2009; Sciolino et al., 2015; Valentino and Van Bockstaele, 2015; Isingrini et al., 2016). Specific molecules, e.g., BDNF and neuropeptides like opioids and CRF, have also been implicated (Russo et al., 2012). Of particular interest in the present context is NPY (Kask et al., 2002; Morgan et al., 2002; Heilig, 2004; Krishnan et al., 2007; Zhou et al., 2008; Domschke et al., 2010; Cohen et al., 2012; Sabban et al., 2016; Kautz et al., 2017), a neuropeptide discovered in the Mutt laboratory (Tatemoto et al., 1982). NPY may be involved in the

[7] In the early electrophysiological in vitro experiments on rat LC neurons in slices, a marked and lasting (20 min) desensitization developed after application of galanin (Pieribone et al., 1995). If this is true also for GalR3 in the human LC is not known; nor how such an effect, if present, would contribute to/would be of importance for the results obtained in our study on depressed suicides (Barde et al., 2016), and for the present hypothesis.

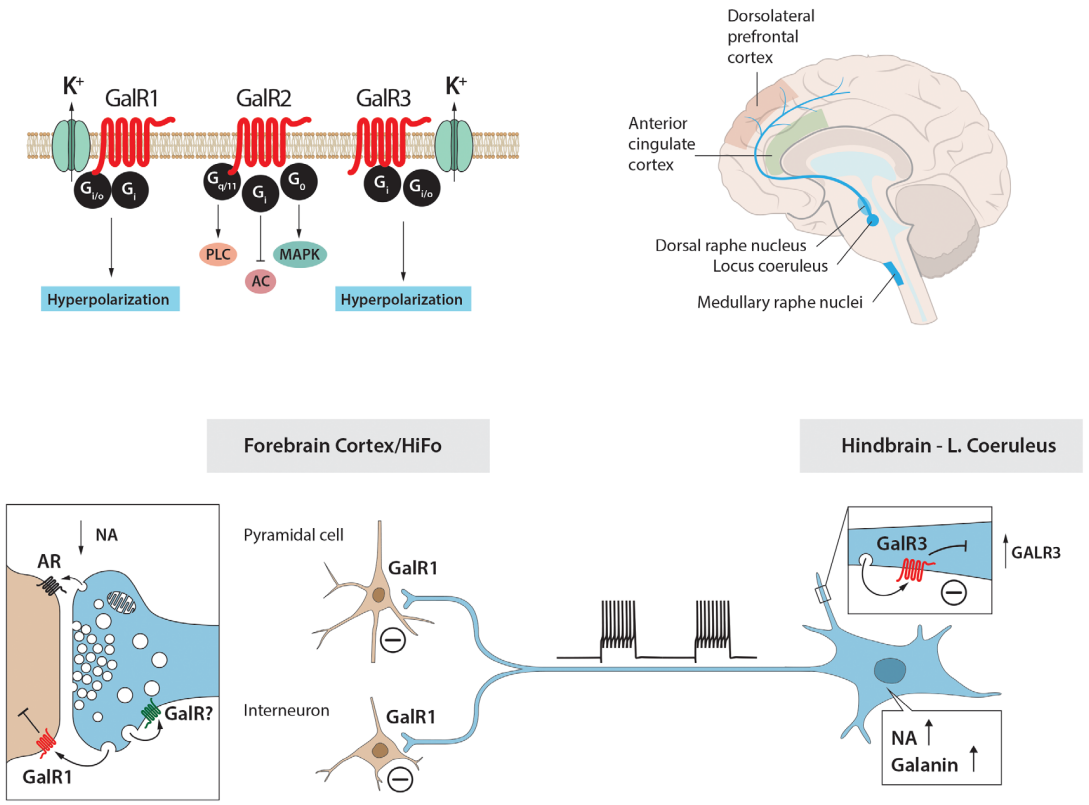

FIGURE 11 | The galanin–locus coeruleus (LC) system in stress and depression: A hypothesis. The hypothesis is built on animal (rat) experiments showing that (i) galanin and GalR1 **(top left)** are present in LC NA neurons; (ii) galanin mRNA levels are increased during stress; (iii) galanin via GalR1 autoreceptors inhibits firing of LC neurons; and (iv) indirect evidence that galanin can be released from soma-dendrites of LC neurons. The second corner stone is results from two studies on human *postmortem* brain with ISH and qPCR. Five regions from *postmortem* brains from depressed subjects who committed suicide and controls were studied and are shown, including LC that projects to anterior cingulate and dorsolateral prefrontal cortices **(top right)**. The results show that also in humans (i) the NA LC neurons express in any case galanin and GalR3 **(top left)**. GalR1 and GalR3 probably have similar transduction mechanisms **(top left)**. Under 'normal' firing only noradrenaline is released in forebrain. A situation after severe stress is depicted in the **lower panel**: LC neurons burst fire (lower panel, **middle**), NA and galanin are released from nerve endings in cortex (**lower panel**, left) and dendrites in the LC (**lower panel**, right), the latter in an attempt to prevent overexcitation (a resilience mechanism). To replace released peptide, galanin transcript levels and synthesis increase, and also GalR3 is upregulated (**lower panel**, right). The increased release, together with elevated galanin and GalR3 levels, result in a too strong inhibition and decreased NA levels in the forebrain (maladaptation) (**lower panel**, left), possibly contributing to depressive symptoms. HiFo, hippocampal formation. Drawing by Mattias Karlén.

control of LC signaling in a similar way as galanin, but this will not be discussed further here, because limited information is available on the expression of NPY and NPY receptors in the brain of normal subjects and subjects who committed suicide.

The present data suggest that the GalR3-mediated 'brake' on the LC neurons is part of the resilience 'machinery' in humans. This is in agreement with animal experiments on rats by Sciolino et al. (2015) who show that exercise increases galanin levels in LC, and that exposure to stress reduces open arm exploration in sedentary rats. But this effect is not seen in exercise rats – and not in rats treated chronically with galanin given intraventricularly (i.c.v.); and it could be blocked by chronic administration of the galanin antagonist M40. Thus, increased galanin levels, presumably in LC, promotes resilience. This is also suggested by earlier animal experiments, as summarized by Sciolino et al. (2015): i.c.v. galanin protects against anxiety under stressful conditions (Bing et al., 1993), but not in the absence of stress (e.g. Holmes et al., 2005); i.c.v. M40 blocks fluoxetine-induced activity in the FST (Lu et al., 2005a); and transgenic mice overexpressing galanin under the dopamine B-hydroxylase promoter (the GalOE/D mouse), i.a. in LC, are protected against stress (Holmes et al., 2002).

The resilience hypothesis is further supported by comparison of the GalOE/D mouse (Steiner et al., 2001) with a mouse overexpressing galanin under the platelet growth factor B (PDGFB) promoter (the GalOE/P mouse) (Holmberg et al., 2005). Analyzing the effect of swim stress with microdialysis, it was shown that NA release in the forebrain is much lower in the GalOE/D than in the GalOE/P mouse (Yoshitake et al., 2004). The histochemical/qPCR analyses revealed that the galanin mRNA levels in the LC are 5 times higher in the GalOE/D than in wild type mice (Steiner et al., 2001). On the other hand, the GalOE/P mouse has a lower galanin expression in the LC and in (noradrenergic) nerve terminals in the forebrain (Kuteeva et al., 2004; Yoshitake et al., 2004). One possible explanation is that

the higher galanin levels in the GalOE/D mouse suppress NA release in the forebrain by autoinhibition of the LC neurons, in agreement with the hypothesis.

The behavioral analyses of these two mouse lines reveal that the GalOE/P mouse displays an increased time of immobility in the FST, that is a depression-like behavior (Kuteeva et al., 2005). In contrast, there are no differences in immobility time on tail suspension between GalOE/D and wild type mice (Holmes et al., 2005). One interpretation is that the GalOE/D mouse is resilient to stress thanks to increased inhibitory galanin signaling in the LC, again, in agreement with the hypothesis.

DRUG TREATMENT VIA NEUROPEPTIDE RECEPTORS

The fact that neuropeptides routinely signal via GPCRs is promising from the perspective of drug development, since >30% of all prescription drugs act via such receptors (Luttrell et al., 2015; Hauser et al., 2017; Santos et al., 2017). Thus, >200 neuropeptide receptors are potential drug targets.

Principles for Peptidergic Co-signaling

Peptide signaling in the rodent, and possibly primate, brain likely always means co-transmission with one or more small molecule transmitters – and other peptides. How could this affect treatment of patients? Are there any problems, or even advantages? Here some thoughts.

To fully block signaling at least two antagonists may be required. For example, a substance P antagonist is potentially a pain killer, since this excitatory peptide is released from nociceptors. However, the clinical tests failed for the reasons discussed (Hill, 2000; Herbert and Holzer, 2002). An obvious explanation would be that at least two additional excitatory transmitters are co-released with substance P from the same nerve endings in the dorsal horn: glutamate and CGRP, which are co-stored together with substance P in the same LDCVs (Merighi, 2002) (**Figure 1E**). So, blockade of one (the NK1) receptor may not be sufficient to achieve analgesia.

There could be advantages with peptide transmitters: First, neuropeptides are 'weak' messengers. Thus, an antagonist will not have the potentially detrimental effects of blocking 'strong' and functionally essential fast transmitters, like those for GABA and glutamate. Although glutamate antagonists have many obvious indications for treating disease, it has been difficult to develop clinically efficacious and safe medicines. For example, glutamate is the major transmitter in nociceptors, and glutamate ligands have been strong candidates for pain treatment (Neugebauer, 2007), but glutamate antagonists like ketamine have yet to emerge as a clinically safe and widely used treatment alternative for pain (Bell et al., 2017). This said, and important in the context of the present review (MDD), the introduction of ketamine and ketamine analogs causing rapid antidepressant effects in subjects with treatment resistant depression has been, to say the least, an exciting advance (Zarate et al., 2006; Abdallah et al., 2015; Lener et al., 2017).

Furthermore, if neuropeptides are only released when neurons are firing at high frequency or firing in bursts, then antagonists would only have an effect under these circumstances, that is only affecting an activated system. For example, galanin is present in >20 nuclei in the rat brain. However, stress may primarily activate LC and 5-HT neurons, which in rats may be the only neurons releasing galanin. And an antagonist will consequently antagonize only the effects of galanin released from these two systems. The remaining galanin systems are silent and will therefore not be affected by the antagonist – so likely only few side effects. In contrast, a NA reuptake inhibitor will affect *all* NA neurons, resulting in increased extracellular amine levels in virtually all brain regions, probably leading to side effects. The same is of course true for SSRIs and serotonin, as well as for SNRIs and serotonin plus NA.

Treatments via Peptidergic Mechanisms Are Effective

The discovery by the pharmaceutical company Merck of small non-peptide molecules passing the blood-brain-barrier (Uslaner et al., 2013) and acting as antagonists at orexin/hypocretin receptors (de Lecea et al., 1998; Sakurai et al., 1998) has resulted in a new medicine: Suvorex/Belsomra, approved by the federal drug administration (US FDA) for treatment of insomnia (Coleman et al., 2012; Yang, 2014): in less than 20 years from bench to patient! The small molecule co-transmitter in these orexin/hypocretin neurons is glutamate (Rosin et al., 2003), and these neurons also express the opioid peptide dynorphin (Chou et al., 2001). Furthermore, monoclonal antibodies[8] to calcitonin gene-related peptide (CGRP) (Aimovig, erenumab), a peptide also present in nociceptors (Rosenfeld et al., 1983), are now approved by FDA and EMA for treatment of migraine (Silberstein et al., 2017; Edvinsson et al., 2018). In fact, antibodies to the CGRP *receptor* and CGRP *antagonists* are also efficacious in treatment of migraine (Silberstein et al., 2017; Edvinsson et al., 2018). Here some 35 years passed from the discovery to the clinic. Moreover, the NK1 antagonist (Aprepitant) mentioned in relation to depression is now used for treatment of chemotherapy-induced emesis (Pendergrass et al., 2004), a serendipitous finding

Drugs Acting on Galaninergic Signaling

It has been difficult to generate small molecules that pass the blood-brain-barrier and act on central galanin receptors. Bartfai and associates made significant contributions, starting with chimeric peptide ligands (Bartfai et al., 1992). For several years, these were important tools in the galanin field, although they did not penetrate into the brain/spinal cord from the periphery. GalR3 antagonists (SNAP 39899 and related compounds) were then the first molecule acting on the brain after peripheral administration (Swanson et al., 2005; Barr et al., 2006; Konkel et al., 2006a,b). An allosteric modulator, a GalR2 agonist passing the blood-brain-barrier, was also reported (Lu

[8]Monoclonal antibodies were 'invented' more than 40 years ago (Kohler and Milstein, 1975; Milstein, 1990), and have during the last decades become indispensable tools for treatment of an array of diseases.

et al., 2010), followed by further GalR2 ligands (Saar et al., 2013a,b). Several overviews of the field have been published (Mitsukawa et al., 2008; Hoyer and Bartfai, 2012; Webling et al., 2012; Freimann et al., 2015).

Based on the discussion above it appears that a GalR3 antagonist is a promising candidate for treatment of depression. Experiments in rats, suggest that the GalR1 receptor in LC also is a target for treatment of addiction (Picciotto, 2008; Genders et al., 2018a). However, in humans the correct receptor may be GalR3. In fact, in two genetic studies on alcoholism, both the galanin gene (Belfer et al., 2006) and, interestingly, the GalR3 gene, but not the other two receptor genes, have been implicated (Belfer et al., 2007). Of note, a GalR3 knockout mouse exhibits an alcohol-preferring phenotype (Genders et al., 2018b).

Why would a GalR3 antagonist be an advantageous choice over reuptake blockers? Analysis of regions of the *postmortem* MDD brains and controls (**Table 1**) reveals upregulation of galanin and GalR3 not only in the LC but also in the DRN (Barde et al., 2016). These changes are likely associated with higher levels of released galanin and of available receptors. Thus, a GalR3 antagonist could disinhibit blockade of *two* monoamine systems critical in mood disorders and restore both NA and 5-HT forebrain levels, relieving depressed mood. Since no changes are seen in the ACC, and since galanin and GalR3 are *down*regulated in the male DLPFC, these systems are likely 'silent'. Thus, treatment with a GalR3 antagonist may overall have a high degree of selectivity with less side effects.

LIMITATIONS AND FUTURE PERSPECTIVES

The key message of the present review is that the neuropeptide galanin and its subtype 3 (GalR3), both coexisting in noradrenergic LC neurons, are involved in MDD as part of the resilience machinery and GalR3 as a target for treatment. The hypothesis is based on solid and reproduced animal experiments from several laboratories. However, the translation to humans represents a major 'jump,' only involving one single (large) experiment on postmortem brains and a supporting candidate gene study. Thus, the hypothesis needs confirmation, preferably by other laboratories and methods. GWAS reports are negative, and no support based on imaging is published. A key experiment would be to label a GalR3 antagonist and carry out both *in vitro* autoradiography and positron emission tomography to analyze GalR3 binding sites/receptors. Moreover, the results are mainly based on transcript analysis, and it will be necessary to show translation into receptor protein, both in the rodent and human brain. The final proof would be to test a GalR3 antagonist in the clinical setting, but that would require generation of new, non-toxic molecules. Therefore, the retraction of major pharmaceutical companies from the neuroscience field represents a major disappointment. Further aspects on 'limitations' can be found in Barde et al. (2016).

CONCLUDING REMARKS

The discovery of new drugs for treatment of mental illness has often been the result of serendipity (Celada et al., 2013; Millan et al., 2015a). The present review suggests that results from experimental animal studies can generate hypotheses that can be further validated by examining *postmortem* brains from relevant patient groups, perhaps leading to new pharmacological treatment strategies.

In animal studies the neuropeptide galanin has shown consistent changes in expression in response to a variety of stimuli, including stress. In agreement, results on the four galanin system genes (Juhasz et al., 2014), together with a recent study conducted with *postmortem* brains from depressed suicides (Barde et al., 2016), suggest involvement of galaninergic mechanisms in depression. On the basis of these studies, it is hypothesized that galanin may, via inhibitory GalR3 autoreceptors, act as a 'brake' to prevent overexcitation of LC neurons, representing a *resilience* mechanism to protect against depression. Galanin is, however, only *one* factor in a comprehensive network of built-in safeguards against overexcitation of LC neurons, reflecting the functional importance of a strict control of noradrenergic LC neurons which project to virtually all parts of the central nervous system.

It is now some 45 years since fluoxetine was generated, and 35 years since the first monoamine (serotonin)-reuptake inhibitor (Zimelidine) was launched (Spector et al., 2018). Since then SSRIs, NRIs and SNRIs have been the most widely used drugs for treatment of depression. Here we speculate that an antagonist at GalR3 receptors in noradrenergic LC neurons could lead to enhanced NA release in the forebrain – and recovery from disease. Since GalR3 is also likely present and upregulated in 5-HT neurons, it is possible that the same GalR3 antagonist also could normalize 5-HT release in the forebrain as well. Such a GalR3 antagonist has been developed (Swanson et al., 2005). However, the GalR3 antagonist SNAP 37889 has shown *in vitro* toxicity (Koller et al., 2016), and clinical trials have been terminated due to safety concerns.

If a GalR3 antagonist without toxic side effects will be developed and if ever tested in the clinic, the question might arise: Why would this drug be an advantage over a combined reuptake inhibitor, like Venlafaxine? Since the GalR3 antagonist works by a different mechanism it may, hypothetically, avoid some of the well-known side effects of reuptake inhibitors by a restricted site of action, versus the reuptake inhibitors increasing monoamine levels at *all* sites in the brain. It may be further speculated that the well-known delay of onset may be avoided, since the postulated disinhibition of the NA and 5-HT neurons via GalR3 antagonism should be a fast effect, perhaps without the complex 'compensatory' changes occurring after treatment with SSRIs and related to the 5-HT1A receptors (Celada et al., 2013). A third consideration is the consistently higher relevance of GalR3 gene variants compared to those of the serotonin transporter in stress-related depression, which might serve as a basis of personalized treatment. To what extent treatment resistant subjects will be helped is another issue that needs to be addressed. Finally, the use of agents acting at multiple

sites, e.g., blocking monoamine re-uptake plus the NK1 receptor, may represent a way forward (Millan, 2009). In this respect, perhaps a drug combining blockade of a galanin receptor with another receptor/mechanism could represent an interesting alternative?

AUTHOR CONTRIBUTIONS

TH and SB wrote and revised the manuscript. GB, GJ, and NM wrote key sections of the manuscript. All authors contributed to manuscript revision, and read and approved the submitted versions.

FUNDING

The primary research from the host laboratory reviewed in this article was supported by the Swedish Research Council (04X-2887), the Marianne and Marcus Wallenberg Foundation, the European Community (NewMood, LSHM-CT-2004-503474; 2004-2008), Karolinska Institutet, AFA, the Swedish Brain Foundation, an Unrestricted Bristol-Myers-Squibb Neuroscience grant, NARSAD and the Torsten Söderberg Foundation.

ACKNOWLEDGMENTS

We thank Dr. Eric Kandel, Columbia University, New York, for critically reading and correcting the original draft of the manuscript, and Dr. Gilad Silberberg for valuable comments. We are particularly grateful to Reviewer AG for valuable suggestions and corrections of the final version. We express thanks to Drs. Marian DiFiglia (**Figure 1D**), Adalberto Merighi (**Figure 1E**), Dr. Mingdong Zhang and Ms. Joanne Bakker for allowing reproduction of photos (**Figure 5**).

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Conflict of Interest Statement: TH has shares in Bioarctic, Stockholm, Sweden and Novo Nordisk, Copenhagen, Denmark.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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