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Fighting for recovery on multiple fronts in spinal cord injury

Edited by
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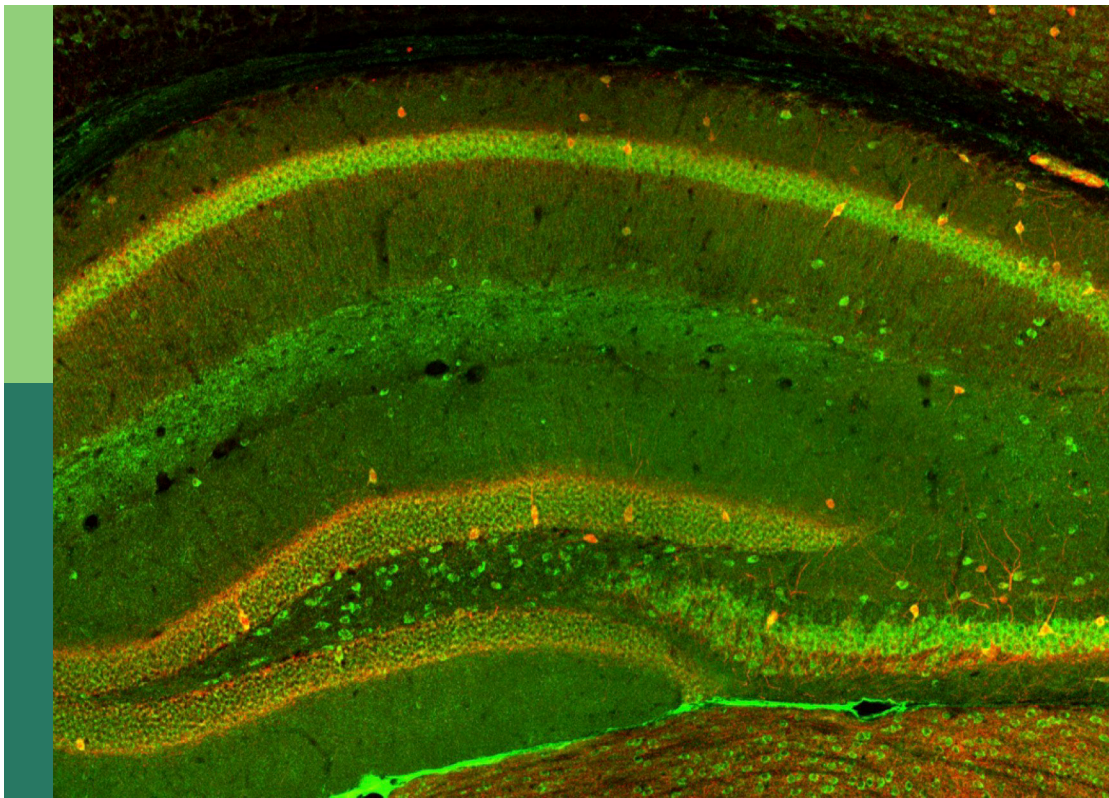

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Fighting for recovery on multiple fronts in spinal cord injury

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Table of contents

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Editorial: Fighting for recovery on multiple fronts in spinal cord injury

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Editorial on the Research Topic
Fighting for recovery on multiple fronts in spinal cord injury

Currently, no approved therapies exist to restore movement, sensation, or autonomic function to the 27 million people world-wide who suffer from a spinal cord injury (SCI). To rectify this, we need to better understand the vast array of processes and pathophysiologies that contribute to this multifaceted disorder. There is significant reason for optimism: the past decade has witnessed a vast increase in knowledge fostered by innovations in research technologies which enable us to understand the nature of the disorder and its underlying mechanisms. Slowly but surely, the promise of this research is being realized through the development and clinical assessment of SCI treatments and assistive technologies to aid the quality of patients' lives. Within this Research Topic, "*Fighting for recovery on multiple fronts in spinal cord injury,*" we have brought together basic and clinical studies to review the current state of the field, discuss the outcomes of clinical trials, and highlight innovative research which together will assist in deciphering the mechanisms of dysfunction, recovery, and treatment after SCI.

The wealth of evidence that treatment effect may be altered due to sex differences has led national funding bodies in the United Kingdom and beyond to require pre-clinical research be conducted on mixed sex populations. Stewart et al. have produced a compelling review suggesting that age may similarly affect an individual's response to SCI treatment. The authors provide a considered and powerful argument utilizing both clinical and basic research, advocating for the use of age as a variable in pre-clinical models. They conclude age may affect SCI treatment outcome and injury pathophysiology, and may need to be accounted for when translating strategies into the clinic. Similarly important for therapeutic potential, Wulf and Tom provide an extensive review of the effect SCI has upon the sympathetic nervous system, concentrating on the impact upon critical organ function. The authors provide persuasive evidence detailing the importance of restoring sympathetic nervous system function to aiding SCI patient health and quality of life. The critical and wide-ranging importance of astrocyte heterogeneity to SCI is appraised by Yu et al. demonstrating potential flaws in current classification systems. They discuss the multiple roles of astrocytes following injury as regulators of inflammation and a component in astrogliosis and subsequently how these glial cells may be targeted as a treatment for SCI recovery. The extracellular matrix is another important element with multiple

roles in both the injured and uninjured spinal cord. Sánchez-Ventura et al. presented a review on the perineuronal nets (PNN), focusing on the potential for targeted modulation and its consequences. There authors highlight several crucial knowledge gaps and discuss insights that can be drawn from development and plasticity in order to find more effective PNN-targeting strategies.

There is an increasing number of clinical trials for SCI providing evidence concerning what impacts patient recovery while also continuously amassing experiences that may guide researchers and clinicians in future practice and treatment translation. Kjell and Svensson share a perspective on the clinical advantages and opportunities of autologous peripheral nerve graft (PNG) therapy for SCI and put PNG therapy in context of our current understanding of SCI recovery and repair. They authors highlight the unique potential of PNGs to repair long tracts and be optimally surgically positioned within the spinal cord, an advantage that may also be exploited in combination with other therapeutic strategies. In clinical trials, primary outcome can be affected and obscured by secondary complications. Franz et al. perform a longitudinal study providing data describing the effect on clinical recovery of the secondary complication heterotopic ossification, common in SCI patients. The heterotopic ossification primarily occurred at the hip joints within 3 months after injury and the authors emphasize that early prevention or treatment needs to be established in order to safeguard functional recovery in patients. Finally, Dietz et al. performed a systematic review of 1,149 SCI clinical trials, and report how trial interventions and outcomes have evolved over time. Among other trends, the authors show that as the numbers of new clinical trials grow each year, studies focused on neuromodulation and rehabilitation dominate the clinical trial landscape. They also identify problems with low reporting of clinical trial results, highlighting a need for better reporting standards for SCI clinical trials.

This Research Topic also includes new original research utilizing experimental SCI models. First, Romanelli et al. utilized human mesenchymal stromal cell-derived extracellular vesicles (EVs) as an anti-inflammatory therapeutic intervention for SCI in rats. The authors found that intralesional, but not systemic, injection of EVs into sites of thoracic contusion improved long-term hindlimb motor functional outcomes and reduced thermal hypersensitivity. Markers of molecular and cellular inflammation were found to be reduced in the EV-treated injured spinal cord tissue, and lesion volume as well as axon tract integrity were improved vs. vehicle-treated animals. Together, these findings suggest a neuroprotective, anti-inflammatory effect of EV treatment. Further, a study by Kondo et al. examines the effects of treadmill training on locomotor recovery in a marmoset SCI model. For this study, the authors developed a new locomotor scoring system, the Marmoset Motor Scale for Locomotion. The authors show that treadmill training improves locomotor recovery in marmosets, with improved frequency of stepping, coordination, and kinematic trajectories vs. untrained animals. Training also induced changes in hindlimb representation within cortical motor maps, with greater cortical area capable of eliciting hindlimb movement. Collectively, these new studies shed new light on mechanisms of two different promising therapeutic interventions for SCI.

In this Research Topic, we highlight that SCI presents great challenges on both microscopic and macroscopic levels and is indeed a challenge on multiple fronts. The contributing authors have highlighted several of the challenges and aspects related to making the trip from "bench to bedside." These contributions exemplify and make it salient that it is only by understanding these challenges that we may find future therapies that are able to improve functional recovery following SCI.

Author contributions

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

Conflict of interest

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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REVIEW
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Reactive Astrocytes in Central Nervous System Injury: Subgroup and Potential Therapy

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Traumatic central nervous system (CNS) injury, which includes both traumatic brain injury (TBI) and spinal cord injury (SCI), is associated with irreversible loss of neurological function and high medical care costs. Currently, no effective treatment exists to improve the prognosis of patients. Astrocytes comprise the largest population of glial cells in the CNS and, with the advancements in the field of neurology, are increasingly recognized as having key functions in both the brain and the spinal cord. When stimulated by disease or injury, astrocytes become activated and undergo a series of changes, including alterations in gene expression, hypertrophy, the loss of inherent functions, and the acquisition of new ones. Studies have shown that astrocytes are highly heterogeneous with respect to their gene expression profiles, and this heterogeneity accounts for their observed context-dependent phenotypic diversity. In the inured CNS, activated astrocytes play a dual role both as regulators of neuroinflammation and in scar formation. Identifying the subpopulations of reactive astrocytes that exert beneficial or harmful effects will aid in deciphering the pathological mechanisms underlying CNS injuries and ultimately provide a theoretical basis for the development of effective strategies for the treatment of associated conditions. Following CNS injury, as the disease progresses, astrocyte phenotypes undergo continuous changes. Although current research methods do not allow a comprehensive and accurate classification of astrocyte subpopulations in complex pathological contexts, they can nonetheless aid in understanding the roles of astrocytes in disease. In this review, after a brief introduction to the pathology of CNS injury, we summarize current knowledge regarding astrocyte activation following CNS injury, including: (a) the regulatory factors involved in this process; (b) the functions of different astrocyte subgroups based on the existing classification of astrocytes; and (c) attempts at astrocyte-targeted therapy.

Keywords: traumatic brain injury, spinal cord injury, reactive astrocytes, scar-forming astrocytes, astrocyte-targeted therapy

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INTRODUCTION

In 1856, Rudolf Virchow described for the first time a type of cell with neuron-supportive functions (Virchow, 1856). Then, in 1895, MV Lenhossék proposed the name astrocyte ("Astrocyten") for this type of neuron-supporting cell (Lenhossék, 1893). Cortical astrocytes originate from radial glia derived from the neuroepithelial cells, radial glial cells originate from the cortical ventricular zone and are characterized by a long basal process that extends from the cortical ventricular zone

to the pial surface (Arellano et al., 2021). During embryonic development, radial glial cells generate intermediate glial progenitors *via* asymmetric division, and these progenitors then migrate, proliferate, and finally transform into astrocytes in nerve tissue. After birth, astrocytes are primarily generated through the direct transformation of radial glial cells in the ventricular zone, the migration and development of postnatal progenitors in the subventricular zone, and the symmetrical division of differentiated astrocytes (Levison and Goldman, 1993; Ge et al., 2012; Verkhratsky and Nedergaard, 2018; Abdeladim et al., 2019). NG2 glial cells comprise another possible source of astrocytes (Nishiyama et al., 2016). Here, astrocytes undergo limited migration along with radial glial processes (Jacobsen and Miller, 2003). Astrocytes of different origins are phenotypically diverse, which is a partial manifestation of the heterogeneity of astrocyte morphology and function (Magavi et al., 2012; Tsai et al., 2012; Molofsky and Deneen, 2015). A combination of heredity, development, and phenotype renders astrocytes a truly opportunistic cell with lifelong adaptive plasticity.

Under physiological conditions, astrocytes perform a variety of functions primarily associated with the maintenance of CNS homeostasis, including the formation and maintenance of the blood–brain barrier (BBB) and blood–spinal cord barrier (BSCB), signal transmission across synapses, the maintenance of neuronal function, and metabolic regulation (Molofsky and Deneen, 2015). In a pathological background, however, astrocytes can become activated. The lifelong adaptive plasticity of these cells and the complexity of the disease background determine the diversity of astrocyte subpopulations after injury (Verkhratsky and Nedergaard, 2018). Following CNS insult, activated astrocytes can sequentially display two different histological phenotypes over time, first becoming reactive astrocytes (RAs), and then scar-forming astrocytes (SAs; Hara et al., 2017). This sequential phenotypic change from the resting state to the activated state is referred to as reactive astrogliosis (Zamanian et al., 2012). However, this histological classification method fails to clearly define RAs and SAs as it is neither objective nor quantitative.

In 2017, Hara et al. (2017) were the first to define several RA- and SA-specific marker genes in the mouse. *Plaur*, *Mmp2*, *Mmp13*, *Axin2*, *Nes*, and *Ctnnb1* were classified as RA marker genes, while SA markers included *Cdh2*, *Sox9*, and chondroitin sulfate proteoglycan (CSPG)-related genes, such as *Xylt1*, *Csgalnact1*, *Chst11*, *Pcan*, *Acan*, and *Slit2*. Nevertheless, RAs and SAs both display high expression levels of several proteins, including GFAP, nestin, β-catenin, N-cadherin, and SOX9. As the disease progresses, there is an overlap of RA subpopulations and RAs interact with Col1 and are converted into SAs *via* the integrin/N-cadherin pathway (Hara et al., 2017; Li X. et al., 2020). This research is of great significance to the understanding of SAs, but due to the lack of further research, the function of SA is not yet clear. Recently, Escartin et al. (2021) redefined RAs as 'astrocytes that undergo molecular, morphological, and functional changes in response to pathological stimuli from surrounding tissue, such as CNS disease, injury, and deleterious experimental manipulation, among others. High GFAP expression levels and cell hypertrophy are considered the minimum criteria for defining RAs (Liddelow et al., 2017).

In addition to the above classification of astrocytes (RAs and SAs), RAs are also divided into different astrocyte subgroups. In 2012, Zamanian et al. undertook a genomic analysis using two mouse injury models (inflammation and cerebral ischemia models) to profile RA phenotypes. The authors found that the RA phenotype was dependent on the type of inducing injury, and identified high Lcn2 and Serpina3n expression levels as strong markers of RA phenotype (Zamanian et al., 2012). In 2017, Liddelow et al. found that neurotoxic RAs, which they named A1 astrocytes, were induced by cytokines (TNF-α, IL-1α, and complement component C1q) secreted by activated microglia, whereas neuroprotective RAs, termed A2 astrocytes, were induced under ischemic and hypoxic conditions. As shown in **Figure 1**. The neurotoxic effect of complement component 3 (C3), a strong marker of A1 astrocytes, has been confirmed in a variety of CNS diseases, especially the interaction between the C3 cleavage fragment, C3a, and its receptor, C3aR, on neurons (Guo et al., 2010; Lian et al., 2015; Li J. et al., 2020; Yadav et al., 2021). However, the A1 and A2 phenotypes were not proposed to be universal or all-encompassing, they were widely misinterpreted as evidence for a binary polarization of reactive astrocytes in either neurotoxic or neuroprotective states, which could be readily identified in any CNS disease, acute or chronic, like the once-popular, but now discarded, Th1–Th2 lymphocyte and M1–M2 microglia polarization theories. Any binary classification method cannot show the diversity of astrocytes across diseases. More importantly, in mouse models of CNS damage, a RA subset was usually a mixture of A1 and A2 or pan-reactive transcripts (Das et al., 2020). So, Escartin et al. (2021) recommend moving beyond the A1–A2 labels and the misuse of their marker genes. In fact, the latest works of the original authors who studied these subtypes no longer use A1/A2. Guttenplan et al. (2021) used the induction conditions of A1 astrocytes but called the induction results neurotoxic reactive astrocytes. Hasel et al. (2021) used the term neuroinflammatory astrocyte, and used the pattern of "Y-zone X-positive astrocytes showing Z phenomenon" to describe the neuroinflammatory astrocyte subgroups he discovered. Based on existing knowledge, this is an ideal way of naming. However, the A1/A2 classification of RAs is still widely used.

In our opinion, under certain conditions, neurotoxic reactive astrocytes, neuroinflammatory astrocytes, and A1 astrocytes are almost the same. *In vitro*, neurotoxic reactive astrocytes and A1 astrocytes are induced in the same way. In the brain of LPS-induced systemic inflammation mouse model, Liddelow et al proposed the concept of A1 astrocytes, and Hasel et al. proposed various neuroinflammatory astrocyte subtypes, A1 astrocytes can be regarded as a subgroup of neuroinflammatory astrocytes. Neurotoxic reactive astrocytes emphasized function, while neuroinflammatory astrocytes emphasized background, both concepts include A1 astrocytes. At present, users of the A1/A2 concept all regard A1 as the representative of neurotoxic astrocytes and A2 as the representative of neuroprotective astrocytes. However,

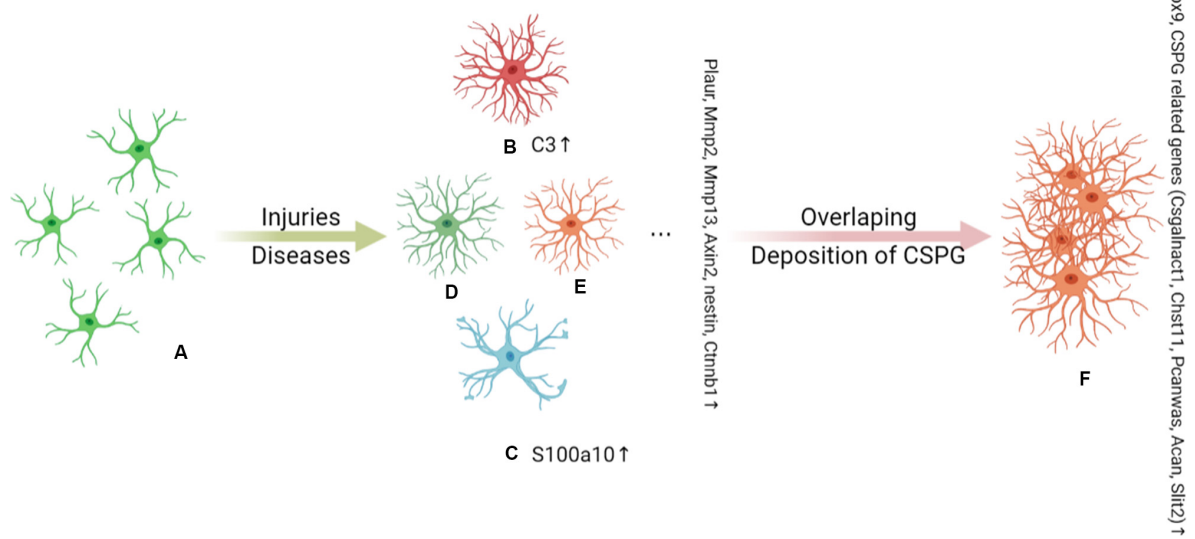

FIGURE 1 | Under the stimulation of injury and disease, **(A)** naive astrocytes are activated into functionally heterogeneous reactive astrocytes (RAs); this heterogeneity is determined by the background of the astrocytes. The *Plaur*, *Mmp2*, *Mmp13*, *Axin2*, *Nes*, and *Ctnnb1* genes are markers of RAs. In an inflammatory background, **(B)** A1 astrocytes are proposed to be a subpopulation of neurotoxic RAs and are marked by C3 expression. **(C)** A2 astrocytes are induced by ischemia and hypoxia and are indicated to play a neuroprotective role in injury and disease. A2 astrocytes can be distinguished by the expression of S100A10. $C3^+$ A1 astrocytes have long dendrites, while $S100a10^+$ A2 astrocytes have hypertrophic cell bodies with few dendrites. There are other as yet unidentified subpopulations of RAs that also play an important role in disease, such as **(D)** and **(E)**. As the disease progresses, there is an overlap of RA subpopulations and chondroitin sulfate proteoglycan (CSPG) deposits, which together induce the conversion of RAs to SAs **(F)**. *Cdh2*, *Sox9*, and CSPG-related genes (*Csgalnact1*, *Chst11*, *Pcan*, *Acan*, and *Slit2*) are markers of scar-forming astrocytes (SAs).

considering their functional heterogeneity, it is likely that not all neurotoxic RAs are A1 astrocytes, and neither are A2 astrocytes. In the background that current knowledge does not allow objective classification of astrocytes, the use of a binary description of reactive astrocytes (A1/A2, neurotoxicity/neuroprotective), seems unavoidable. Recently, Escartin et al. (2021) reached a consensus that the field should move beyond binary descriptors and embrace objective classification based on their increasingly complex functional heterogeneity. And the work by Liddelow and Hasel supports this view (Hasel et al., 2021).

Astrocytes are key factors in secondary neuronal damage and repair inhibition largely due to their dual role in the regulation of neuroinflammation and glial scar formation after CNS injury (Liddelow and Barres, 2017; Adams and Gallo, 2018). This dual role requires the accurate classification of astrocyte subpopulations. In this review, we will focus on the heterogeneity of astrocytes and astrocyte targeted therapy strategies after CNS injuries (TBI and traumatic SCI) to help the development of targeted therapy strategies based on these precise classification of astrocytes.

TRAUMATIC CNS INJURY

Owing to the preventability of most CNS injuries and the complex and expensive medical care they require, TBI and SCI are increasingly recognized as global health priorities. In 2016, approximately 27.08 million new cases of TBI and 0.93 million new cases of SCI were diagnosed. The age-standardized incidence rate was reported to be 369 per 100,000 population for TBI and 13 per 100,000 for SCI (GBD 2016 Traumatic Brain Injury and Spinal Cord Injury Collaborators, 2019). TBI alone caused annual global economic losses of $US400 billion (Maas et al., 2017). From 1990 to 2016, the age-standardized prevalence of TBI increased by 8.4%, whereas that of SCI did not change significantly. However, given the increase in population density, population aging, and the increased use of motor vehicles, the number of people with SCI is expected to increase. TBI has a higher mortality rate (higher acute injury-related mortality), while TSCI is characterized by a higher standardized mortality rate (shorter long-term life expectancy for SCI survivors; Badhiwala et al., 2019). Public health initiatives to prevent injuries, such as the use of bicycle helmets, fall prevention, policy changes affecting the impact of sports, and other public safety measures, are very effective in reducing the morbidity and mortality associated with TBI and SCI (Taylor et al., 2017). The focus of clinical management involves reducing intracranial pressure, medullary cavity pressure, and cerebral edema, as well as systemic supportive treatment (Maas et al., 2021). In most cases, the effects of these interventions on patients are disappointing (Maas et al., 2017). The burden of disability due to CNS injury can also have a devastating effect on the families of patients because it prevents them from engaging in economic activities.

TBI is divided into focal tissue damage and diffuse tissue damage. Focal injuries are caused by direct impact and include scalp injuries, skull fractures, brain contusions, cerebral hemorrhage, and stroke, which form focal TBI lesions that can vary greatly in size (Gaetz, 2004). Diffuse injury is caused by acceleration–deceleration forces, including hypoxia–ischemic injury, meningitis, and vascular injury (Gaetz, 2004). However, tissue damage after TBI is rarely purely focal or diffuse, and a single case usually involves multiple focal and diffuse lesions (Skandsen et al., 2010). TBI-related tissue pathology and its functional consequences are heterogeneous and determined largely by: (a) the mechanical properties of the injury; (b) the degree of injury severity (mild, moderate, or severe); and (c) the anatomical location of the injury (Burda et al., 2016). The spinal cord has a unique anatomical structure and the impact of scars on the function of the spinal cord at later stages of SCI can be devastating. Consequently, greater attention is given to pathological changes occurring over time. Several key time points are worth noting, such as the 3rd day after injury when inflammation peaks.

Traumatic injury in the CNS is characterized by transient mechanical damage and subsequent delayed non-mechanical damage (Burda et al., 2016). Primary injury in the brain is caused by mechanical force, which immediately leads to contusion and bleeding in the affected area. In the spinal cord, injury usually relates to vertebral fracture or dislocation (Oyinbo, 2011). The secondary injury occurs hours, days, months, or even years after the initial injury, and is characterized by the expansion of tissue damage from the center of the disease. According to the research in the rodent model of TBI, secondary injury can be simply divided into two parts. The first is inflammation, which peaks on the 3rd day after injury (Susarla et al., 2014). Under the stimulation of a wide variety of pro-inflammatory factors produced as a result of the primary injury, microglia and astrocytes are activated, peripheral immune cells are recruited, and the inflammation cascade is initiated. These effects are accompanied by the destruction of the neurovascular unit, glutamate accumulation, oxidative stress, axonal damage, and neuronal death (Gyoneva and Ransohoff, 2015). The second part involves scar formation, in which glial scars begin to form on day 7 post-injury (Villapol et al., 2014). The glial scar surrounds the site of injury and limits the spread of a strong inflammatory response (Burda and Sofroniew, 2014); however, glial scars secrete a variety of cytokines and proteoglycans that promote neurotoxicity and inhibit axon regeneration, respectively (Silver and Miller, 2004). The outcome of glial scarring is the development of a fibrotic scar, which creates a physical and chemical barrier to axon regeneration and nerve function recovery after injury (O'Shea et al., 2017).

The role of an astrocyte is determined by its subgroup status and the surrounding environment. This diversity of astrocyte function directly affects the inflammatory response and glial scar formation after injury. After an injury, astrocytes interact with surrounding cells, such as neurons, microglia, and endothelial cells, that together constitute the post-injury microenvironment, which plays a pivotal role in disease development (Abbott et al., 2006; Valori et al., 2019).

Although primary CNS injuries cannot be treated, secondary injuries provide a therapeutic window for the treatment of the resulting diseases (Wang et al., 2014). Accordingly, to identify effective treatment strategies, research attention has increasingly focused on the role of astrocytes in the pathology of CNS damage.

ASTROCYTE ACTIVATION AFTER INJURY

In response to CNS damage, naïve astrocytes are activated and transform into RAs. This transformation involves changes in morphology, increased expression of the intermediate filament proteins GFAP and vimentin, as well as increased proliferation and secretion of inflammatory mediators and growth factors (Karve et al., 2016). After TBI in mouse, astrocytes react within 24 h and reach a peak of approximately 3–7 dpi, showing a continuous reactive state (Susarla et al., 2014). A recent study conducted using a mouse CCI (chronic constriction injury) model reported the occurrence of astrocyte hypertrophy in the lesion site and surrounding area at 3 days post-injury (dpi). At 7 dpi, the morphological changes became long-lasting, and glial scars began to form (Villapol et al., 2014). In this model, reactive gliosis persisted for up to 60 dpi, indicative of a continuous response of astrocytes to brain injury (Villapol et al., 2014). In another study, after sensorimotor cortex aspiration in adult rats, astrocyte activation lasted for 16 weeks (Basiri and Doucette, 2010).

Primary Mechanical Stress

In traumatic CNS injury, mechanical stress can cause neuronal membrane instability and cytoskeleton disintegration (LaPlaca et al., 2009). Astrocytes are activated through plasma membrane stretching. The results of a study using astrocytes cultured on deformable membranes indicated that mechanical strain led to AKT activation in astrocytes *via* the stimulation of P2 receptors and promoted ATP release; this, in turn, activated extracellular signal-regulated protein kinase (ERK; Neary et al., 2005). Additionally, the knockout of the Cav1.2 subunit of L-type voltage-operated calcium channels attenuated the migratory and proliferative abilities of astrocytes, indicating that these channels contribute to astrocyte activation, at least *in vitro* (Cheli et al., 2016). In a mouse model of nerve demyelination, reducing voltage-gated Ca2+ influx in astrocytes during brain demyelination significantly attenuated brain inflammation and astrocyte reactivity (Zamora et al., 2020). Indeed, calcium is required for ERK activation in astrocytes, and inhibiting these Ca2+ channels may be an effective means of preventing astrocyte activation and proliferation. In recent research, Hlavac et al showed rat primary astrocytes exposed to high-rate overpressure were mechanically activated, involving changes in structure and junctional proteins (Hlavac and VandeVord, 2019). Their further study indicated that both extracellular adhesion (*via* FAK activation) and cationic conductance (*via* ion channels) contribute to this progress (Hlavac et al., 2020). Wakida et al. (2020) showed astrocyte phagocytosis was a mechanosensitive response, and astrocytes exposed to fluid shear stress initiated phagocytosis at a faster rate than cells observed under static

conditions. Liu J. et al. (2021) proposed Piezo1(mechanosensing channel) in astrocytes was involved in the mechanical activation of astrocytes caused by mechanical stretching.

Secondary Pathological Process

During the secondary pathological process, the release of intracellular components by the cells injured by primary mechanical stress; activation of microglia and astrocytes at the injured site; production of cytokines and chemokines; and recruitment of peripheral immune cells into CNS, these processes influence each other and produce complex interaction. Peripheral cells released signal factors to recruit extra cells from the periphery and maintain the activation of microglia and astrocytes, leading to excessive activation of astrocytes, which further damaged surrounding tissues and neurons (Gyoneva and Ransohoff, 2015). Additionally, secondary inflammation after CNS injury is the body's reactive inflammation to the injury, which is different from primary neuroinflammation, such as AD, which is caused by the disorder of normal growth and metabolism in cells (Cao et al., 2021).

In the context of post-injury inflammation, the combination of DAMP (HMGB1, Hsp72, HA, ATP) and TLRs drove the complex inflammation network and astrocyte effector events (Struve et al., 2005; Sun et al., 2017; Sun L. et al., 2019; Du et al., 2021; Li et al., 2021b; Michinaga and Koyama, 2021). Cytokines IL-1β, IL-6, TNF-α activated astrocytes by activating the corresponding receptors and downstream signaling pathways (NFκB, MAPK, NO synthase), and led to the secretion of inflammatory substances (HMGB1, NO, ROS) which further promoted the activation cascade of astrocytes (Swanson et al., 2004; Sun et al., 2017; Sun L. et al., 2019; Patil et al., 2021; Qian et al., 2021). Human spinal cord astrocytes induced by IL-1β showed up-regulation of chemokines and axon permissive factors (including FGF2, BDNF, and NGF) expression, and down-regulation of most genes that regulate axon suppression molecules, including ROBO1 and ROBO2 (Teh et al., 2017). After the injury, the EGFR of astrocytes is up-regulated, and mTOR pathway is up-regulated after combining with EGF. The use of EGFR inhibitors effectively reduced reactive astrogliosis (Codeluppi et al., 2009; Li Z. W. et al., 2014). You et al. (2017) proposed that IL-17-JAK/STAT-VEGF axis was involved in the activation of astrocytes after SCI. As a clear target of MIF, the CD74 receptor on the astrocyte membrane binded to MIF, leading to excessive activation of astrocytes, and this process was significantly blocked by c-Jun N-terminal kinase inhibitors (Zhou et al., 2018). But in gecko astrocytes, the combination of MIF and CD74 could not cause obvious inflammation. Du et al. (2021) proved that Vav1 was the key mediator of this phenomenon. In addition, lncRNAPVT1/miR-186–5p/CXCL13/CXCR5 axis and lncRNA H19/miR-1–3p/CCL2 axis were involved in the activation of astrocytes after SCI (Li P. et al., 2020; Zhang P. et al., 2021). MiR-21 regulated the proliferation, secretion, and activation of astrocytes through the PI3K/Akt/mTOR signaling pathway mediated by PTEN, as a positive factor for the recovery of acute SCI (Liu et al., 2018). MiR-17–5p may specifically regulate the proliferation of RAs triggered by LIF through the JAK/STAT3 pathway (Hong et al., 2014). miR-379 (A et al., 2019), miR-124 (Jiang et al., 2020), miR-145 (Wang et al., 2015), and miR-140 (Tu et al., 2017) negatively regulated astrocyte activation and improved the prognosis of the disease. The transcription factors OLIG2 and SP1, as well as FGF, FGFR, and PDGFRβ have all been implicated in glial scar formation (Kang et al., 2014; Koyama, 2014; Pei et al., 2017; **Table 1**). These experimental results obtained in ideal places under different conditions emphasized the heterogeneity of reactive astrocytes at the morphological, functional, biochemical, metabolic, and transcriptome levels. In the complex environment inside the body, they will be covered up.

REACTIVE ASTROCYTES

RAs are astrocytes that undergo molecular, morphological, and functional changes in response to pathological stimuli from surrounding tissue, such as CNS disease, injury, and deleterious experimental manipulation, among others. As mentioned before, the lifelong adaptive plasticity of astrocytes and the complexity of the disease background determine the diversity of astrocyte subpopulations after injury. In animal models of TBI, P2Y (1)R stimulation was shown to reduce the severity of brain edema and cytotoxic swelling (Talley Watts et al., 2013). However, the results of another study suggested that microglia could convert astrocytes into neurons by mediating the downregulation of P2Y (1)R (Shinozaki et al., 2017). Early et al. (2020) proposed that astrocytes exhibited age-related progressive reactive astrocyte response by the models of TBI in mice of different ages. Recently, Hasel et al. (2021) successfully demonstrated the heterogeneity of RAs in the brain of LPS-induced mouse models. They used single-cell sequencing combined with spatial transcriptomics and *in situ* hybridization techniques to show that RAs were transcriptome and spatially heterogeneous under inflammatory conditions; and clarified the highly expressed genes and possible functions of RA subtypes in different anatomical locations (Hasel et al., 2021). Combined, the findings of all these studies have highlighted the high heterogeneity of RAs, which can lead to both neuroprotective and toxic effects after CNS injury (Miller, 2018). Differences in *in vitro* induction conditions; species used in animal models; injury type, degree, and location; and time passed after the injury have all contributed to the contrasting results obtained in different studies. All these make the precise typing of RAs more difficult.

Debris Clearance

The timely removal of dead cells after CNS injury helps limit secondary tissue damage. Phagocytosis is normally carried out by professional phagocytes. However, several electron microscopy-based studies as early as the 1970s showed that astrocytes could swallow small fragments, such as axons or myelin fragments (Ronnevi, 1978). Later, it was discovered that astrocytes were involved in the removal of myelin debris during Wallerian degeneration in the goldfish visual system (Colavincenzo and Levine, 2000). Subsequent studies showed that after CNS injury, astrocytes participate in the removal of axons and myelin fragments, even entire dead cells, thereby protecting injured

TABLE 1 | Molecules and signaling pathways that involved in the activation of astrocytes.

Etiology category	Activation factor
Primary mechanical force	Plasma membrane stretching (Neary et al., 2003, 2005), Cav1.2 voltage-gated Ca^{2+} channels (Cheli et al., 2016; Zamora et al., 2020), high-rate overpressure (Hlavac and VandeVord, 2019; Hlavac et al., 2020), fluid shear stress (Wakida et al., 2020).
Cytokines and growth factors	IL-1β (Teh et al., 2017), IL-6 (Patil et al., 2021), IFN-γ, CNTF, EGF (Li Z. W. et al., 2014), IL-17 (You et al., 2017), TNF-α (Gayen et al., 2020; Patil et al., 2021), LIF (Kerr and Patterson, 2004; Goodus et al., 2016), VEGF (Gao et al., 2015), MIF (Du et al., 2021), FGF (Kang et al., 2014), CTGF (Lu M. et al., 2019).
Chemokines	MCP-1 (Gwak et al., 2012; Joy et al., 2019; Liraz-Zaltsman et al., 2021).
Signal transducers	STAT3, NF-κB, JAK2 (Oliva et al., 2012; You et al., 2017; Li X. et al., 2020), mTOR (Codeluppi et al., 2009), Notch1 (Ribeiro et al., 2021), MAPK (Zhang X. et al., 2021), ERK (Sticozzi et al., 2013; Li et al., 2021a), PKC (Chao et al., 2018), SOX9 (Liu W. et al., 2021).
Receptors	p75NTR (Chen et al., 2020), CB2R (Jing et al., 2020), ET_BR (Koyama, 2021), EGFR (Li Z. W. et al., 2014), TLRs (Kigerl et al., 2014; Rosciszewski et al., 2018), purine receptor (Li et al., 2021b), FGFR (Kang et al., 2014), PDGFRβ (Pei et al., 2017), CD36 (Bao et al., 2012), CD44 (Bourguignon et al., 2007), CD74 (Su et al., 2017).
Chaperone proteins	Sig-1R, Hsp72, PDIs (Michinaga and Koyama, 2021).
Hormones	Neuron-derived estrogen (Lu Y. et al., 2020), noradrenalin (Smith et al., 2005; Bekar et al., 2008).
Oxidative stress molecules	NO (Swanson et al., 2004), ROS (Qian et al., 2021).
Non-coding RNA	lncRNAPVT1/miR-186–5p (Zhang P. et al., 2021), lncRNA H19/miR-1–3p (Li P. et al., 2020), miR-21 (Liu et al., 2018), miR-145 (Wang et al., 2015), miR-140 (Tu et al., 2017), miR-17 (Hong et al., 2014), miR-379 (A et al., 2019), miR-124 (Jiang et al., 2020).
Transcription factor	Olig2, Sp1 (Koyama, 2014).
Protease	uPA (Diaz et al., 2021), USP18 (Liu W. et al., 2021).
Proteins	HMGB1 (Sun et al., 2017; Sun L. et al., 2019), ICAM-1 (Gwak et al., 2012), Galectin-3 (Ribeiro et al., 2021).
Peptides	ET-1 (Goodwin and Grizzle, 1994; Michinaga et al., 2018, 2020a).
Others	HA (Struve et al., 2005), Glutamate (Gwak et al., 2012), ATP, Ca^{2+} (Li et al., 2021b), NG_2 (Huang et al., 2016), Cr (Ma et al., 2017).

neurons from contact-induced cell death (Basiri and Doucette, 2010; Lööv et al., 2012). Morizawa et al. (2017) reported that following brain ischemia, RAs could become phagocytic in a limited spatiotemporal pattern and engulf debris *via* upregulating the phagocytosis-related ABCA1 pathway. Wang et al. showed that astrocytes directly cleared myelin debris through endocytosis after SCI (Wang S. et al., 2020).

Glutamate Excitotoxicity

A sharp increase in extracellular glutamate levels has been detected in both CNS injury models and human patients, and this increase represents the cumulative effect of several pathological events that lead to the overstimulation of glutamate receptors and the occurrence of large cation fluxes (Lima et al., 2021). Glutamate excitotoxicity plays an important role in the development of secondary CNS injury. It can lead to neuronal death, followed by prolonged depolarization and subsequent ion imbalance, ATP depletion, increased intracellular free calcium levels, and, ultimately, more serious tissue damage (Jamjoom et al., 2021).

The glutamate transporters GLAST and GLT-1 are mainly expressed in astrocytes and are downregulated following TBI, which leads to enhanced excitotoxicity (Beitchman et al., 2020). Astrocytic excitatory amino acid transporters (EAATs) can protect against neuronal death induced by microglia-derived glutamate, whereas microglial EAATs exert neither neurotoxic nor neuroprotective effects (Liang et al., 2008). These observations indicate that astrocytic glutamate transporters are key for limiting the development of excitotoxic conditions by reducing the concentration of interstitial glutamate. *In vitro*, oxygen–glucose deprivation/reoxygenation insult can reportedly activate the HMGB1/TLR4 axis and reduce glutamate clearance by inhibiting GLAST expression in primary astrocytes (Lin et al., 2020). Similarly, the downregulation of GLT-1 expression in RAs leads to worse functional and histological outcomes following SCI (Lepore et al., 2011a,b). In addition, during cerebral hemorrhage, astrocytic volume-regulated anion channels release glutamate, further aggravating the damage (Yang J. et al., 2019). Interestingly, Li et al. illustrated that the overexpression of the astrocytic glutamate transporter GLT1 exacerbated phrenic motor neuron degeneration, diaphragm impairment, and forelimb motor dysfunction post cervical contusion SCI, while the transplantation of glial progenitors that overexpress the glutamate transporter GLT1 could overcome the diaphragm dysfunction (Li K. et al., 2014; Li et al., 2015).

Cytotoxic Edema

After CNS injury, the brain and spinal cord tissues undergo edema, leading to intracranial or medullary cavity hypertension, secondary to more serious tissue damage that may lead to fatal brain injury or hernia (Liang et al., 2007). Many studies have shown that the degree of cerebral and spinal cord edema is associated with the severity of trauma and subsequent motor dysfunctions (Miyanji et al., 2007). Cytotoxic edema is characterized by the swelling of all cell types due to excessive water retention. In contrast, astrocytes are the main cause of

brain swelling in brain edema (Liang et al., 2007). AQP-4, expressed in the brain (perivascular and subpial membrane domain) and spinal cord astrocytes, is the most abundant aquaporin in the CNS and represents a major pathway for the entry of excess water into damaged tissue (Nesic et al., 2006; Tait et al., 2008; Saadoun and Papadopoulos, 2010). Astrocytic AQP-4 is primarily responsible for cytotoxic edema after CNS injury (Amiry-Moghaddam et al., 2003).

In animal models of CNS injury, AQP-4 mRNA and protein expression levels are significantly upregulated in activated astrocytes (Finnie et al., 2011; Hemley et al., 2013). Various mechanisms are involved in this process in astrocytes, such as IL-6/NF-κB pathway activation, HMGB1/TLR4/MyD88/NF-κB signaling pathway activation, FOXO3A nuclear translocation, and ERK1/2 phosphorylation (Ito et al., 2006; Kapoor et al., 2013; Sun et al., 2017; Sun L. et al., 2019; Zhang et al., 2019a; Li et al., 2021a). Experiments conducted using AQP-4-deficient mice showed that AQP-4 promotes the formation of cytotoxic edema, whereas the absence of AQP-4 reduces edema severity after acute water intoxication, ischemic stroke, and SCI (Manley et al., 2000; Saadoun et al., 2008). In the rat model of TBI, AQP-4 knockdown reportedly reduces the extent of cytotoxic and post-traumatic brain edema (Lu H. et al., 2020). Kitchen et al. suggested that brain or spinal cord swelling was not only related to the total expression of AQP-4, but also the subcellular translocation of AQP-4 to the BSCB. Their data showed that calmodulin could directly bind to the carboxyl terminus of AQP-4, resulting in specific conformational changes and AQP-4 cell-surface localization. In rat SCI models, trifluoperazine-mediated calmodulin inhibition suppressed AQP-4 localization to the BSCB, led to the ablation of CNS edema, and resulted in accelerated functional recovery relative to that seen in untreated animals (Kitchen et al., 2020). As shown in **Figure 2**. As AQP-4 cell surface localization is controlled by calcium/protein kinase A/calmodulin in astrocytes, targeting calmodulin may also represent a novel treatment method for cytotoxic edema (Kitchen et al., 2015, 2020). In addition to AQP-4, other functional molecules in astrocytes, such as NKCC1, Sur1/Trpm4, AQP-1, and vasopressin are also considered to be initiators of cytotoxic edema formation (Nesic et al., 2008; Jayakumar et al., 2011; Jia et al., 2016; Gerzanich et al., 2019).

BBB/BSCB: Disruption or Recovery

CNS damage can lead to the loss of BBB/BSCB integrity. Astrocytes regulate BBB/BSCB homeostasis through end-feet processes that surround endothelial cells. A series of factors derived from RAs after an injury have opposing effects on the BBB/BSCB (Michinaga and Koyama, 2019; **Table 2**).

Nitric oxide (NO) and excess glutamate derived from RAs after an injury can damage the BBB and the BSCB (Saha and Pahan, 2006; András et al., 2007; Lu L. et al., 2019; Sharma et al., 2019). In animal models of TBI and SCI, the expression of VEGF and MMP-9, both factors that promote BBB permeability, increases in RAs, and inhibiting them reduces BBB/BSCB-related damage after injury (Noble et al., 2002; Gao et al., 2015; You et al., 2017; Michinaga et al., 2018; Liu et al., 2020). Astrocyte-derived ET-1 was shown to induce the upregulation of ICAM-1 and VCAM-1 expression in human brain microvascular endothelial cells and aggravate the destruction of the BBB. ET receptor antagonists such as bosentan, BQ788, and S-0139 can alleviate the loss of BBB integrity in TBI model mice (McCarron et al., 1993; Matsuo et al., 2001; Michinaga et al., 2018, 2020a). Interestingly, studies on mice have highlighted that the APOE E4 variant (APOE4) is a risk factor for poor outcomes in CCI. However, APOE is an important modulator of spontaneous BBB stabilization following TBI (Main et al., 2018; Montagne et al., 2020). Astrocyte-derived neurotrophic factor (MANF) can inhibit inflammation and promote angiogenesis and BBB repair (Li et al., 2018). Astrocyte ablation results in the failure of BSCB repair, local tissue destruction, severe demyelination, and the death of neurons and oligodendrocytes following SCI (Faulkner et al., 2004). After CNS injury, the expression of Shh is increased in astrocytes. The administration of exogenous Shh attenuates BBB destruction, while the application of the Shh inhibitor jervine exerts the opposite effects in mice with TBI (Xing et al., 2020; Michinaga et al., 2021). In the mouse SCI model, Shh/Gli1 signaling is induced in RAs and plays an important role in the permeability of BSCB and locomotor recovery after SCI (Yue et al., 2020). The expression of ANG-1 in astrocytes is decreased after CNS injury, while the administration of recombinant ANG-1 can alleviate the destruction of the BBB/BSCB (Sabirzhanov et al., 2019; Michinaga et al., 2020b). Astrocyte-derived FABP7 enhances BBB integrity through the caveolin-1/MMP signaling pathway after TBI, and displays neuroprotective properties after SCI (Rui et al., 2019; Senbokuya et al., 2019). In addition, astrocyte-derived retinoic acid and IGF-1 have also been shown to participate in BBB/BSCB maintenance and vascular protection (Kong et al., 2015; Bake et al., 2016; Zhou et al., 2016; Li H. et al., 2020). Notably, Shh and MMP-9 can restore or disrupt the BBB or BSCB through multiple mechanisms, and both proteins have the potential to serve as therapeutic targets for CNS injury.

Inflammation: Basic Protective Function and the Consequences of Overactivation

Inflammation represents a physiological protective response to injury; however, extreme inflammation, which is inevitable following CNS injury, results in additional tissue damage (Popovich and Jones, 2003; Förstner et al., 2018). RAs promote inflammation after CNS injury by secreting cytokines, chemokines, reactive oxygen species (ROS), NO, and damage-associated molecular patterns, all factors that are involved in the activation of microglia and the recruitment of peripheral immune cells, thereby maintaining and even further aggravating neuroinflammation (Wicher et al., 2017; Linnerbauer et al., 2020). The NF-kB signaling pathway in RAs is a key regulator of inflammation in the CNS (O'Neill and Kaltschmidt, 1997). In animal models of CNS injury, NF-κB is highly activated and the expression of NF-kB-dependent genes is upregulated (Schneider et al., 1999). Inhibiting NF-κB signaling dampens astrocyte responses to brain injury, resulting in neuroprotective effects (Acarin et al., 2001; Brambilla et al., 2005). An *in vitro* study showed that ATP-stimulated human astrocytes

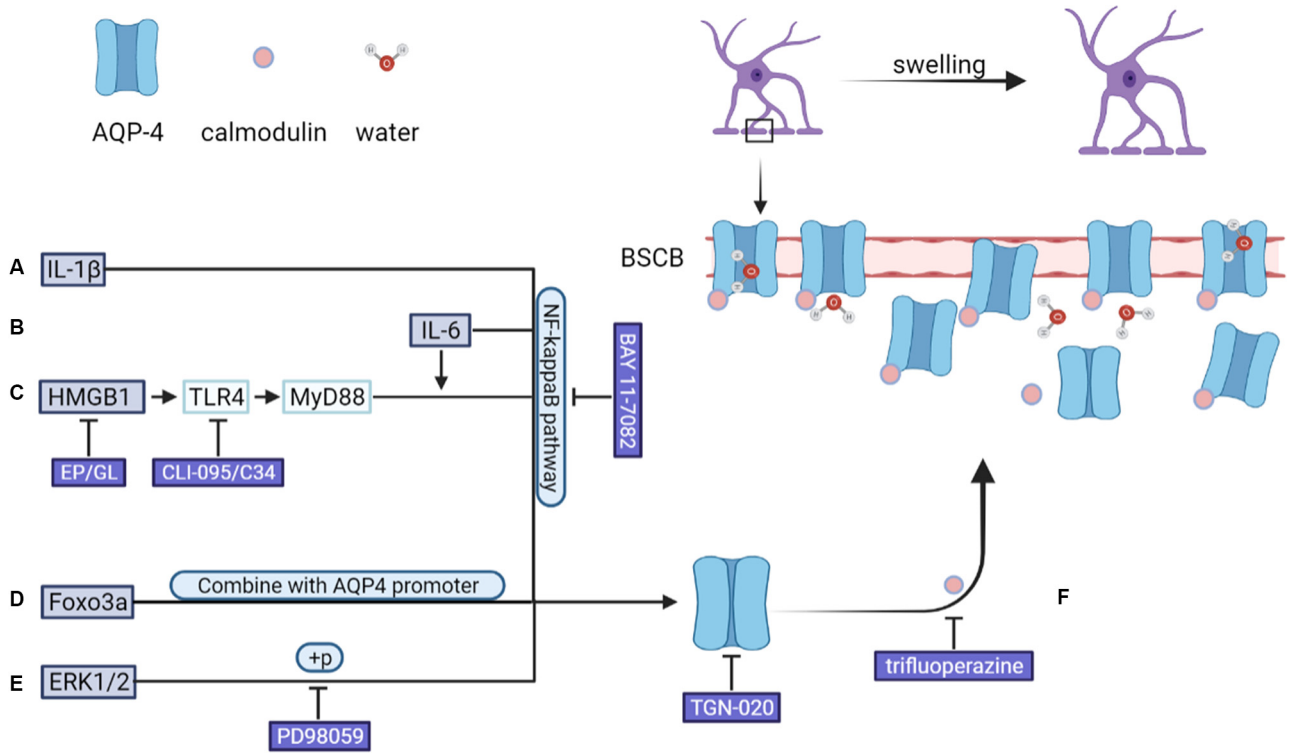

FIGURE 2 | After CNS injury, an increase in the levels of **(A)** IL-1β and **(B)** IL-6 leads to the upregulation of AQP-4 expression through the NF-κB pathway. **(C)** HMGB1 upregulates AQP-4 expression *via* the HMGB1/TLR4/MyD88/NF-κB axis independently of IL-6. **(D)** FOXO3A undergoes nuclear translocation, binds to the *AQP4* promoter, and upregulates AQP-4 expression. **(E)** SCI-induced upregulation on of AQP-4 expression was down-regulated by PD98059 (ERK blocking agent) and TGN-020 (aquaporin-4, AQP4, blocking agent). In addition, **(F)** AQP-4 undergoes a conformational change after binding to calmodulin, after which it localizes to the BSCB, leading to an increase in the amount of water entering astrocytes. ERK, extracellular signal-regulated protein kinase; BSCB, blood–spinal cord barrier.

TABLE 2 | Factors destroy or recover BBB/BSCB.

BBB/BSCB destruction	BBB/BSCB recovery
NO (Sharma et al., 2005, 2019; Saha and Pahan, 2006; Buskila et al., 2007; Gu et al., 2012; Jiang et al., 2014)	MANF (Li et al., 2018)
Excess glutamate (András et al., 2007; Liu et al., 2010; Sulejczak et al., 2016; Lu L. et al., 2019)	Shh (Xia et al., 2013; Xing et al., 2020; Yue et al., 2020; Michinaga et al., 2021)
VEGF (Gao et al., 2015; You et al., 2017)	Ang-1 (Xia et al., 2013; Sabirzhanov et al., 2019; Sun J. D. et al., 2019; Michinaga et al., 2020b)
MMP-9 (Noble et al., 2002; Michinaga et al., 2018; Liu et al., 2020)	fatty acid-binding protein 7 (Rui et al., 2019)
ET-1 (Michinaga et al., 2018, 2020a, 2021)	RA (Mizee et al., 2014; Kong et al., 2015; Zhou et al., 2016)
APOE4 variant (Main et al., 2018; Montagne et al., 2020)	IGF-1 (Bake et al., 2016, 2019; Pitt et al., 2017; Li H. et al., 2020)
	APOE4 (Main et al., 2018)

activated NLRP2 inflammasomes, while the knockdown of NLRP2 significantly reduced the inflammatory response in human astrocytes (Minkiewicz et al., 2013). Many other pro-inflammatory molecules have been associated with astrocyte reactivity, such asS100β, ICAM-1, PrPc, TrkB, D-dopachrome tautomerase, and MIF (Kabadi et al., 2015; Zhang et al., 2019b; Charkviani et al., 2020; Ji et al., 2021; Sulimai et al., 2021). However, using a mouse model of TBI, Myer et al showed that RA ablation aggravated cortical degeneration after moderate CCI, but did not affect cortical degeneration following severe CCI, which suggested that RAs also have a basic protective role in inflammation after injury (Myer et al., 2006). Similar results were obtained with astrocyte ablation after SCI (Faulkner et al., 2004). Long et al. (2020) showed that astrocyte-derived exosomes enriched with miR-873a-5p can inhibit the NF-κB signaling pathway and promote the transformation of protective M2 microglia, thereby inhibiting excessive neuroinflammation. Additionally, Zaheer et al. (2001) showed that activation of the NF-κB signaling pathway resulted in the synthesis of neurotrophic factors (nerve growth factor and brain-derived neurotrophic factor), which is essential for neuronal survival after injury.

RA SUBGROUP WITH NEUROTOXICITY

As early as 2012, Zamanian et al. (2012) discovered a potentially harmful subgroup of RAs. Subsequently, Liddelow et al. (2017) proposed a neurotoxic RA with C3 as a molecular marker and named it A1 astrocytes. A1 astrocytes were induced by cytokines (TNF-α, IL-1α, and complement component C1q) secreted by activated microglia. Although the concept of A1 is not relevant in this field, many previous research results of A1 neurotoxic astrocytes can help subsequent research on the neurotoxic subpopulations of RAs. A1 astrocytes lose many basic functions and gain harmful ones when compared with normal astrocytes. Namely, A1 astrocytes have fewer synapses and a weaker ability for synapse induction; impaired myelin scavenging ability; they can inhibit oligodendrocyte maturation; exhibit stronger neurotoxicity; and kill CNS neurons that have severed axons (Liddelow et al., 2017; Li X. et al., 2020). A1 astrocytes have a significantly different morphology: long dendrites (Zou et al., 2019). This suggests that the morphology of RAs may be changeable. Adding morphological features to the subgroup division can make the typing more specific and accurate. A1 astrocytes are found in a variety of CNS injuries and neurodegenerative diseases but are also present during the normal aging process (Clarke et al., 2018; Yun et al., 2018; Zheng et al., 2021). Alawieh et al. showed that a significant increase in C3 levels after CNS injury triggers continuous microglia degeneration and astrocyte activation, reduces dendrite and synapse density, and ultimately leads to the loss of neurons (Alawieh et al., 2018; Clark et al., 2019). After SCI, mice with C3 deficiency have reduced inflammation and secondary damage and better nerve regeneration and functional recovery after injury compared with that for normal mice (Guo et al., 2010). However, mice with C3aR deficiency show abnormal neurodevelopment that persists into adulthood, and is characterized by locomotive hyperactivity and altered cognitive functions (Pozo-Rodrigálvarez et al., 2021). Wang et al. (2021) proposed a more radical possibility, namely, that A1 astrocytes could directly kill neurons by secreting neurotoxic C3. Several studies have reported that C3 is closely related to the onset of multiple neurodegenerative diseases (Lian et al., 2015; Litvinchuk et al., 2018). These observations suggest that the basic C3 level is necessary for the maintenance of a normal physiological environment in the CNS, whereas excessive C3 availability produces neurotoxic effects after injury. However, it must be acknowledged that the expression of a singular marker "C3" is not a definitive marker that identifies A1 astrocytes. The work of Boisvert et al. (2018) showed that C3 was upregulated on astrocytes in the condition of aging, and did not necessarily, or categorically, indicate A1 astrocytes. Therefore, it is neither accurate nor objective that C3 is used as a singular marker of A1 astrocytes in injury and diseases in humans and other models. Recently, Guttenplan et al. (2021) proposed that saturated lipids contained in APOE and APOJ lipid particles mediated the neurotoxicity of RAs. Astrocytes specifically knock out saturated lipid synthase ELOVL1 to eliminate the formation of long-chain saturated lipids, which reduced astrocyte-mediated toxicity.

In CNS injury, a variety of substances and intracellular signal pathways are involved in the induction and transformation of the functions of RAs (neurotoxicity and neuroprotection; **Table 3**). For instance, the activation of the NF-κB and Notch signal pathways promotes A1 transformation, while exposure to mesenchymal stem cell (MSC)-derived exosomes, which play anti-inflammatory and neuroprotective roles after SCI, suppresses A1 astrocyte numbers by inhibiting the NF-κB signaling pathway (Wang et al., 2018; Liu et al., 2019; Qian et al., 2019). Additionally, activating the FGF2/FGFR1 pathway can reverse the increase in C3 expression levels in astrocytes following ultrasound exposure (Zou et al., 2019). After SCI, the application of electrospun fiber was reported to promote the expression of A1-specific markers, but electrospun fiber-containing TGF elicited the opposite effect (Gottipati et al., 2020). In comparison, in an IL-1β-induced neonatal rat model of white matter injury, astrocytes showed A2 reactivity (Shiow et al., 2017). After TBI, neuron-derived prokineticin 2 and astrocyte-derived estrogen activated STAT3 signaling pathway in astrocytes, leading to the upregulation of A2 astrocytes (Neal et al., 2018; Ma et al., 2020; Wang J. et al., 2020).

TABLE 3 | Neurotoxic astrocyte-related substances and signal pathways.

Effect	Inductive molecule	Signal path	Reference
Reduce neurotoxicity	MSC-exo	NF-κ (-)	Wang et al. (2018) and Liu et al. (2019)
	HSF1	NF-κB (-) MAPKs (-)	Li L. et al. (2021)
	–	Notch (-)	Qian et al. (2019)
	–	FGF2/FGFR1 (+)	Zou et al. (2019)
	TGF-β3	–	Gottipati et al. (2020)
Induce neuroprotection	IL-1β	–	Shiow et al. (2017)
	Astrocyte-Derived Estrogen	JAK-STAT3 (+)	Wang J. et al. (2020)
	PK2	STAT3 (+)	Neal et al. (2018) and Ma et al. (2020)
Reduce neurotoxicity and Induce neuroprotection	miR-21	STAT3 (+)	Su et al. (2019)
	MFG-E8	PI3K-Akt (+) & NF-κB (-)	Xu et al. (2018)
	MSC-EVs	–	Kaminski et al. (2020)
	Wnt-3a	Wnt/β-catenin signaling pathway (+)	Zhang D. et al. (2019)
	Trkβ	–	Miyamoto et al. (2020)

We have previously shown that miR-21, a regulator of the STAT3 pathway, can transform neurotoxic (A1) RAs into an A2 phenotype (Su et al., 2019). MFG-E8, MSC-derived extracellular vesicles (EVs), Wnt-3a, and Trkβ have also been shown to be involved in A1/A2 transformation (Xu et al., 2018; Zhang D. et al., 2019; Kaminski et al., 2020; Miyamoto et al., 2020). Interestingly, FGF2 can inhibit the TGF-β1-induced increase in GFAP expression in astrocytes (Tran et al., 2018). The antagonism between different molecules that induce the same phenotype further underlines the need for the development of a more precise method for typing RAs.

GLIAL SCARS AND SAS

Following CNS injury, naive astrocytes transform into RAs, and then eventually SAs, leading to impaired axon regeneration and functional recovery. This continuous phenotypic change is a manifestation of astrocyte reactivity, which was once considered to be a unidirectional and irreversible process (Hara et al., 2017). Diseases and injuries of the CNS are usually accompanied by a certain degree of scar formation, although scar formation differs according to disease and injury (Smith et al., 2015). Glial scars are mainly involved in the repair process after CNS injury. After SCI, damage repair efficiency is low and the resulting pathological changes cannot be overcome. Consequently, here, we focus on astrocyte-mediated scar formation after SCI (Bradbury and Burnside, 2019). SCI lesions exhibit three compartments: a non-neural (stromal) lesion core, astrocyte scar borders, and spared but reactive neural tissue. SAs participate in the formation of astrocyte scar borders (Sofroniew, 2018). The scarring process begins on day 7 post-injury and involves the misalignment of activated astrocytes and the deposition of inhibitory CSPGs. SAs can be identified from 14 dpi (Hara et al., 2017).

Various mediators are involved in glial scar formation, including TGF-β1/2, IFN-γ, FGF, MMP-9, fibrinogen, and STAT3 (Moon and Fawcett, 2001; Herrmann et al., 2008; Hsu et al., 2008; Schachtrup et al., 2010). The glial scar represents a physical barrier that enwraps damaged tissues and restricts the migration of inflammatory cells from the non-neural lesion core to the CNS parenchyma (Voskuhl et al., 2009; Sofroniew, 2015). Glial scars fill the interstitial spaces and induce the formation of new capillaries (Rolls et al., 2009). RA ablation impairs glial scar formation, leading to extensive infiltration of inflammatory cells and loss of neurons (Gu et al., 2019). Importantly, however, RA ablation also exerts an unwelcome inhibitory effect on axon regeneration (Anderson et al., 2016). CSPGs deposited in glial scars inhibit oligodendrocyte precursor cell differentiation and remyelination, the two most important processes underlying axon regeneration. CSPG inhibition or inactivation effectively improves motor function (Bradbury et al., 2002; Silver and Miller, 2004; Siebert et al., 2011; Lang et al., 2015; Tran et al., 2018). Wallerian degeneration of damaged axon protrusions leads to continuous extracellular deposition of axons and myelin debris. Myelin-related molecules (MAG, Nogo, OMGP), in conjunction with CSPGs, inhibit neuronal regeneration and neural plasticity (Sofroniew, 2018). However, the deletion of CSPG-related genes or CSPG receptor blockade only enhances synaptic remodeling and cannot directly overcome the protective effects of the astrocyte scar and lesion cores of non-neural tissue to produce meaningful spontaneous axonal regeneration (Hossain-Ibrahim et al., 2007; García-Alías et al., 2009). A combination of TGF-β1/2 antibodies reduced CNS scar formation in an adult rat model of brain injury; however, this was not accompanied by an increase in axon regeneration (Moon and Fawcett, 2001). GFAP$^{-/-}$vim$^{-/-}$ mice show normal scar formation after TBI or SCI, but the scar density is low and accompanied by bleeding (Pekny et al., 1999). Three genetically targeted loss-of-function interventions—preventing astrocyte scar formation, attenuating scar-forming astrocytes, and ablating chronic astrocytic scars—all failed to promote spontaneous axon regrowth. However, exogenous administration of axon-specific growth factors, coupled with growth-activating priming injuries, stimulated axon regeneration, which was reversed by glial scar ablation (Anderson et al., 2016).

Glial scars transform into fibrous scars 14 dpi, and SAs are produced at the same time. SAs are known to originate from the interaction between RAs and type I collagen *via* the integrin/N-cadherin pathway. Antibodies targeting collagen-binding integrin and N-cadherin neutralizing antibodies both inhibited this process (Hara et al., 2017). Immunofluorescence analysis identified the presence of SOX9-positive nuclei in astrocytes of a wild-type brain scar 30 days after the cortical puncture. In contrast, SOX9 expression was strictly limited to the cytoplasm in the DBN−/− brain. DBN may also participate in the transformation of RAs into SAs (Schiweck et al., 2021). Inhibiting the RA/SA conversion may represent an ideal treatment for CNS injury. For this, the restrictive effect of RAs on inflammation should not be affected, only the formation of the glial scar boundary should be inhibited so as to alleviate the inhibitory effect of the surrounding environment on axon regeneration.

In summary, the dual role of the glial scar in axon regeneration may result from the low inherent regeneration potential of neurons. The growth-activating effect of the glial scar cannot bridge the gap between the neuronal regeneration potential and the physical hindrance represented by glial scars; when a glial scar is ablated, neurons cannot regenerate axons on their own without the growth-activating effect of the glial scar. Han et al. (2020) proposed to increase the intrinsic regenerative power of neurons by restoring cellular energy, and successfully promoted the germination and regeneration of axons after SCI by enhancing mitochondrial transport and energy metabolism. Therefore, in the case of preserving glial scars, enhancing the regeneration potential of neurons may also be a feasible treatment option.

STRATEGIES FOR ASTROCYTE-TARGETED THERAPY

Based on the dual role of astrocytes in CNS injury, multiple attempts have been undertaken to enhance the beneficial effects of astrocytes or reduce their harmful effects. Here, we

TABLE 4 | Diverse astrocyte targeted therapy strategies.

Target	Treatment	Model	Mechanism	Curative effect	Reference
Inhibit excessive activation of astrocytes	MP	*In vivo* *In vitro*	Down-regulate astrocyte activation and inhibit CSPG expression	Improve neuron repair and promote neurite outgrowth after excitotoxic injury	Liu et al. (2008)
	Melatonin PPR	*In vivo* *In vivo*	Inhibit astrocyte activation Down-regulate TNF-α, IL-1β, reduce GFAP+ astrocyte cells	Reduce neuronal apoptosis Reduce the degree of cerebral edema and seizures	Babaee et al. (2015) Song Y. et al. (2020)
	TBHQ	*In vivo*	Reduce the production of M1 microglia and inflammatory cytokines, significantly reduce the excessive activation of astrocytes	Reduce neuronal death and lesion volume, improve motor function and cognitive deficits	Zhang et al. (2020)
	AS-IV	*In vitro*	AS-IV reduces the activation of the CXCR4/JNK pathway and ultimately up-regulates the Keap1-Nrf2 signaling	Prevent OGD/R-induced astrocyte apoptosis	Yang J. et al. (2021)
	Simvastatin	*In vivo* *In vitro*	Simvastatin manipulates the caveolin-1 expression in lipid rafts in the astrocyte cell membrane, reduces EGFR phosphorylation, and finally reduces IL-1 production and astrocyte activation	Protect neurons	Li et al. (2009) and Wu et al. (2010)
	ONO-2506	*In vivo*	Inhibit the production of S100B by astrocytes to inhibit the activation of astrocytes	Reduce neuropathic pain after SCI	Ishiguro et al. (2019)
	Edaravone	*In vivo*	Reduce astrocyte proliferation in a rat model of propofol-induced brain injury through the BDNF/TrkB pathway.	Reduce inflammation	Yang Y. et al. (2021)
Reduce Edema	Functionalized Phenylbenzamides	*In vivo* *In vitro*	Reduce AQP-4-mediated water Permeability	Reduce brain edema and improve prognosis	Farr et al. (2019)
	TGN-020	*In vivo*	Inhibit the expression of AQP-4, GFAP, PCNA	Reduce spinal cord edema and promote axon regeneration	Li et al. (2019)
	Atorvastatin	*In vivo*	Inhibit p38MAPK-dependent pathway to down-regulate the expression of AQP4	Reduce ischemic brain edema	Cheng et al. (2018)
	Goreisan	*In vivo*	Decrease AQP-4expression level	Reduce brain water content, alleviate motor deficits	Nakano et al. (2018)
	Trifluoperazine	*In vivo* *In vitro*	Prevent calmodulin from directly binding to the carboxyl terminus of AQP-4, which inhibit AQP-4 localization BSCB	Relieve CNS edema and accelerate functional recovery	Kitchen et al. (2020)
	Bosentan	*In vivo* *In vitro*	Decrease the expression levels of MMP-9, VEGF-A, and Ang-1 in the brain after injury	Reduce BBB dysfunction and cerebral edema	Michinaga et al. (2020a)
	BQ788	*In vivo*	Reduce GFAP-positive astrocytes and their products: VEGF-A and MMP9	Promote the recovery of BBB function and reduce cerebral edema	Michinaga et al. (2018)

(Continued)

TABLE 4 | Continued

Target	Treatment	Model	Mechanism	Curative effect	Reference
	Ulinastatin	*In vivo*	Decrease the activation of ET-1 and inhibit the expression of pro-inflammatory VEGF and MMP-9	Reduce brain edema after TBI	Liu T. et al. (2021)
	EP/GL	*In vivo*	Inhibit the activation of astrocytes, reduce the expression of AQP4, and inhibit the activation of the TLR4/NF-κB signaling pathway	Improve motor function and reduce early spinal cord edema	Sun et al. (2017) and Sun L. et al. (2019)
Astrocyte reprogramming	OCT4, NANOG	*In vitro*		Astrocytes are reprogrammed into the generation of cells expressing neural stem/precursor markers	Corti et al. (2012)
	SOX2	*In vivo*		Resident astrocytes are reprogrammed into proliferating neuroblasts	Niu et al. (2013)
	Zfp521	*In vivo* *In vitro*		Astrocytes are reprogrammed into iNSCs or neurons	Su et al. (2014) and Zarei-Kheirabadi et al. (2019a,b)
	Transcription factors PAX6, NGN2 and ASCL1	*In vitro*		Reprogramming of astrocytes into neurons	Heins et al. (2002) and Berninger et al. (2007)
	Combination of transcription factors Brn-2a, MyT1L, and ASCL1	*In vivo*		Reprogramming of astrocytes into neurons	Torper et al. (2013)
	Transcription factors NeuroD1	*In vivo*		Reprogramming of astrocytes into neurons	Puls et al. (2020)
Reduce the toxicity of RAs and protect neurons	Drug-Loaded Nano-Structured Gel	*In vivo* *In vitro*	Down-regulate A1 astrocytes, reduce iNOS and Lcn2	Improve early exercise ability of injury and protect neurons	Vismara et al. (2020)
	Ponesimod	*In vivo* *In vitro*	Reduce A1 astrocyte polarization by activating the STAT3 signaling pathway	Prevent neuronal death from early brain injury after subarachnoid hemorrhage	Zhang L. et al. (2021)
	Epidermal Growth Factor Hydrogels	*In vitro*	Down-regulate negative A1-like genes (Fbln5 and Rt1-S3) and up-regulate potentially beneficial A2-like genes (Clcf1, Tgm1, and Ptgs2)	Enhance neuroprotection and neuroplasticity	Chan et al. (2019)
	RTMS	*In vivo* *In vitro*	Reduce the production of inflammatory mediators, promote HIF-1α signaling, transform A2 astrocytes into A1 astrocytes	Reduce neuronal apoptosis, promote blood vessel repair, and improve cognitive function.	Zong et al. (2020)
	Physical exercise	*In vivo*	Down-regulate the expression of IL-1α, C1q, and TNF, up-regulate the release of TGFβ, and promote the conversion of A1astrocytes to A2 astrocytes	Promote white matter repair and cognitive improvement	Jiang et al. (2021)
	RvD1	*In vivo* *In vitro*	Induces higher levels of mitochondrial autophagy in astrocytes to protect the mitochondrial morphology and membrane potential of the astrocytes	Reduce cognitive impairment and brain edema, improve the neuron survival rate after TBI	Ren et al. (2020)

(Continued)

TABLE 4 | Continued

Target	Treatment	Model	Mechanism	Curative effect	Reference
	Baicalin	*In vivo* *In vitro*	Inactivate SDH to inhibit ROS production and reduce the loss of GS protein in astrocytes after injury	Reduce excitotoxicity and protect neurons	Song X. et al. (2020)
	LEC	*In vivo*	Reduce lipid peroxidation of astrocytes and increase their glutamate uptake	Reduce excitotoxicity and protect neurons and oligodendrocytes	Lima et al. (2021)
	Agathisflavone	*In vitro*	Increase the expression of neurotrophic factors, reduce the expression of GFAP and hypertrophy of astrocytes	Protect neurons and promote neurite growth	de Amorim et al. (2020)
	Ganglioside GM1	*In vivo* *In vitro*	GM stimulates the expression of genes related to glucose metabolism and enhances glycolysis in astrocytes	Protect neurons	Finsterwald et al. (2021)
Others	Sodium houttuyfonate	*In vivo* *In vitro*	Reduce NLRP3 inflammasome activation, TLR4 activity, phosphorylation of ERK and NF-κB	Reduce inflammation and promote angiogenesis	Yao et al. (2021)
	Ferrostatin-1	*In vitro*	Suppress the ROS levels and activate the Nrf2/HO-1 signaling pathway	Alleviate astrocytes inflammation and ferroptosis	Li S. et al. (2021)

mainly review the existing attempts at astrocyte-targeted therapy (**Table 4**).

Inhibit Excessive Activation of Astrocytes

In the inflammatory phase after CNS injury, excessive activation of astrocytes aggravates the inflammatory cascade and has a negative impact on the prognosis of the disease (Johnson et al., 2013). Methylprednisolone (MP) is a typical representative of an RA-targeting molecule that has already been used in the clinic. MP can reduce astrocyte activation and downregulate the expression of CSPG, thereby promoting the growth of neurites after injury (Liu et al., 2008). Melatonin can exert similar effects (Babaee et al., 2015). PPR, TBHQ, AS-IV, and simvastatin can all reduce the production of inflammatory mediators and inhibit excessive astrocyte activation, thereby protecting neurons and improving prognosis (Li et al., 2009; Wu et al., 2010; Song Y. et al., 2020; Zhang et al., 2020; Yang J. et al., 2021). ONO-2506 can also attenuate astrocyte activation, thus minimizing secondary damage and relieving neuropathic pain after SCI (Ishiguro et al., 2019). As a variety of free radical scavengers, edaravone alleviated astrocyte proliferation and inflammation in a rat model of propofol-induced brain injury (Yang Y. et al., 2021). The selective inhibitor of D-dopachrome tautomerase, a close homolog of MIF protein, effectively attenuated the inflammatory activation of astrocytes after SCI and improves motor function, which helps to develop the application of anti-inflammatory drugs in CNS injuries (Ji et al., 2021). In fact, anti-inflammatory drugs have been used in the clinical treatment of CNS injuries for a long time.

Reduce Edema

AQP-4 is the best-characterized astrocyte-related molecule. Functionalized phenylbenzamide, TGN-020, atorvastatin, and goreisan all target AQP-4, improving post-injury edema and prognosis (Cheng et al., 2018; Nakano et al., 2018; Farr et al., 2019; Li et al., 2019). Using a rat model of SCI, Kitchen et al administered trifluoperazine to inhibit the direct binding of calmodulin to the carboxyl terminus of AQP-4, which inhibited its localization to the BSCB. This effect relieved CNS edema and accelerated functional recovery relative to untreated animals (Kitchen et al., 2020; **Figure 2**). However, in a review by Nesic et al. (2010), the authors proposed that the therapeutic effect of AQP-4 depends not only on the time interval after SCI or the animal model but also on the balance between the protective effect of increased AQP-4 levels on hypoxia and the harmful effects associated with sustained astrocyte swelling. ET-1 has also received widespread attention as a putative therapeutic target. Both bosentan (an ET_A/ET_B antagonist) and BQ788 (an ET_B antagonist) effectively attenuated BBB disruption and cerebral edema in both patients and mice with TBI, whereas the ET_A antagonists ambrisentan and FR139317 elicited no effect (Michinaga et al., 2018, 2020a; Liu T. et al., 2021). This suggests that the deleterious effect of ET-I following CNS injury mainly depends on ET_BR. Additionally, EP/GL inhibited the

activation of astrocytes, reduced the expression of AQP4 and early spinal cord edema (Sun et al., 2017; Sun L. et al., 2019).

Reduce the Toxicity of RAs and Protect Neurons

A drug-loaded nano-structured gel and ponesimod were shown to improve motor performance in the early stages after injury and protect neurons by suppressing the activation of the neurotoxic phenotype of RAs (Vismara et al., 2020; Zhang L. et al., 2021). Epidermal growth factor-containing hydrogels can reportedly alter astrocyte behavior, i.e., they downregulate the expression of deleterious neurotoxicity-related genes (*Fbln5* and *Rt1-S3*) while upregulating that of potentially beneficial neuroprotective phenotype-associated genes (*Clcf1*, *Tgm1*, and *Ptgs2*), thereby indirectly enhancing neuroprotection and neuroplasticity (Chan et al., 2019). RTMS, HSF1, and physical exercise also lead to the conversion of the neurotoxic phenotype into the neuroprotective phenotype, which promotes functional recovery after injury (Zong et al., 2020; Jiang et al., 2021; Li L. et al., 2021). Mitochondria may also play a role in A1 polarization. Incubation with cobalt chloride (CoCl2) converted astrocytes from an A2 to an A1 state, concomitant with a reduction in mitochondrial migration. Trkβ agonists can convert A1 astrocytes to an A2 phenotype *via* reducing mitochondria migration (Miyamoto et al., 2020). Mitochondrial transplantation after CNS injury decreases the release of inflammatory factors such as IL-1β and TNF-α and significantly suppresses astrocyte and microglia activation, thus protecting neurons and promoting functional recovery (Zhang Z. et al., 2019). Resolvin D1 protected mitochondrial morphology and membrane potential in astrocytes, removed damaged mitochondria and thereby enhanced the survival of neurons (Ren et al., 2020). This prompts us to pay attention to the impact of the energy status of RAs on their function in the context of disease. A better understanding of the changes occurring in mitochondrial morphology and function after CNS insult may yield novel strategies for the treatment of CNS injuries. Baicalin and LEC were shown to stabilize astrocytes after injury and increase their glutamate uptake, effects that can reduce excitotoxicity and protect both neurons and oligodendrocytes (Song X. et al., 2020; Lima et al., 2021). Agathisflavone and ganglioside GM1 promoted the neuroprotective effect of astrocytes (de Amorim et al., 2020; Finsterwald et al., 2021).

Astrocyte Reprogramming

Astrocytes retain limited neural stem cell potential and can be reprogrammed into a stem cell-like state to replenish neurons lost after injury (Kriegstein and Alvarez-Buylla, 2009; Verkhratsky and Nedergaard, 2018). The transcription factors OCT4, SOX2, NANOG, and zinc-finger nuclear protein Zfp521 can individually reprogram mature astrocytes into neural stem cells (Corti et al., 2012; Niu et al., 2013; Su et al., 2014; Yang H. et al., 2019; Zarei-Kheirabadi et al., 2019b). The transcription factors PAX6, NGN2, and ASCL1, participate in the transformation of astrocytes into neurons *in vitro* (Heins et al., 2002; Berninger et al., 2007), similar to that seen with the combination of three nerve conversion factors (ASCL1, Brn-2a, and MyT1L) *in vivo* (Torper et al., 2013). Noristani et al. (2016) showed that more than 10% of autologous astrocytes were transdifferentiated and expressed classic neural stem cell markers after SCI. Decreased Notch signaling due to stroke was shown to be necessary for astrocyte neurogenesis (Magnusson et al., 2014). The transcription factors NeuroD1, SOX2, and ZFP521 can all be used to reprogram astrocytes into neurons or neural stem cells after SCI (Zarei-Kheirabadi et al., 2019a; Puls et al., 2020).

Others

Sodium houttuyfonate effectively inhibited the activation of microglia cells while promoting the activation of astrocytes and angiogenesis (Yao et al., 2021). Ferrostatin-1 alleviated astrocytes inflammation and ferroptosis by suppressing the ROS levels and activating the Nrf2/HO-1 signaling pathway (Li S. et al., 2021). Additionally, many other molecules, such as USP18 (Liu W. et al., 2021), p-ERK1/2 (Li et al., 2021a), CREB (Pardo et al., 2016), HSPA12B (Xia et al., 2016), CCR5 (Joy et al., 2019), also represent potential therapeutic targets that merit further investigation.

Although attention has bright prospects, the difficulty in obtaining human CNS tissue and the substantial differences between rodents and human astrocytes (Zhang et al., 2016) represent unavoidable obstacles to the identification or development of strategies for the treatment of CNS injury, that is, how to translate research results from animal studies to humans. Although astrocytes induced by human pluripotent stem cells provide a possible cell model, these astrocytes differ from astrocytes under normal physiological conditions, at least partially. How to transfer research results from animal models to human patients will likely also be the focus of research attention in the future.

CONCLUSIONS

The importance of astrocytes in CNS disease and injury is widely recognized; however, our understanding of astrocyte functions is still in its infancy. The continuous development and breakthrough of instruments and technologies provide conditions for accurate typing of astrocytes. The combination of single-cell and spatial transcriptome sequencing shows promise as a means of determining astrocyte heterogeneity after injury. Through the sequencing of several key times after injury, the time and space distribution of each astrocyte subpopulation can be determined. For example, astrocyte subpopulation D appears on the 7th day after SCI, mainly distributed in the core of injury. Further investigations to determine the temporal and spatial specificity of different astrocyte subpopulations with their specific genetic markers, thereby revealing their respective roles in injury, will provide a more precise indication to allow the targeting of specific astrocyte subpopulations for the treatment of CNS injuries. Such as the study of Hasel et al. (2021), in the mouse inflammation model, they divided astrocyte subgroups according

to the difference between transcriptome and anatomical location and found that Cluster 8 is widely present in inflamed brains, but few in normal brains. In subsequent studies, treatment attempts can be made against Cluster 8 to inhibit the production of Cluster 8, or convert Cluster 8 into a neuroprotective or even neutral RAs subgroup to reduce inflammation. Although they have been proposed to express unique marker genes, little is known regarding the process involved in the transformation between RAs and SAs given that research attention has primarily focused on inflammation and glial scar formation after injury. In the absence of theoretical support, there is no way to talk about the treatment of targeted SA. As detailed in this review, clarifying how SAs are generated may provide ideal treatment and management options for CNS injuries. Based on the precise type of astrocytes, targeting harmful RA subgroups in the early stage of injury to reduce neuronal death and tissue destruction, and changing the extracellular matrix and reducing scar formation through the regulation of SA in the later stage to weaken the external inhibitory factors of nerve regeneration. This kind of treatment is worth looking forward to.

AUTHOR CONTRIBUTIONS

BN designed the research and revised the manuscript. YZ found some articles. GY wrote the article. All authors contributed to the article and approved the submitted version.

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Enhancing Functional Recovery Through Intralesional Application of Extracellular Vesicles in a Rat Model of Traumatic Spinal Cord Injury

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Local inflammation plays a pivotal role in the process of secondary damage after spinal cord injury. We recently reported that acute intravenous application of extracellular vesicles (EVs) secreted by human umbilical cord mesenchymal stromal cells dampens the induction of inflammatory processes following traumatic spinal cord injury. However, systemic application of EVs is associated with delayed delivery to the site of injury and the necessity for high doses to reach therapeutic levels locally. To resolve these two constraints, we injected EVs directly at the lesion site acutely after spinal cord injury. We report here that intralesional application of EVs resulted in a more robust improvement of motor recovery, assessed with the BBB score and sub-score, as compared to the intravenous delivery. Moreover, the intralesional application was more potent in reducing inflammation and scarring after spinal cord injury than intravenous administration. Hence, the development of EV-based therapy for spinal cord injury should aim at an early application of vesicles close to the lesion.

Keywords: exosomes, inflammation, motor function, locomotion, neuroregeneration, traumatic spinal cord injury, scarring, neuroimaging

INTRODUCTION

Traumatic spinal cord injury (tSCI) is a complex clinical condition considered to be one of the most debilitating neurological disorders in industrialized societies. The initial tissue disruption following injury triggers an inflammatory response aggravating the spinal cord lesion (David and Kroner, 2015; Ahuja et al., 2017; Couillard-Despres et al., 2017).

It is well documented that the upregulation of pro-inflammatory cytokines during the early phase of secondary damage leads to further neuronal death and motosensory losses (Pineau and Lacroix, 2007; Bastien and Lacroix, 2014; Anwar et al., 2016).

The principal sources of pro-inflammatory cytokines at the lesion site are the microglia and the "classically-activated" macrophages (Pineau and Lacroix, 2007; Zhou et al., 2014; Honjoh et al., 2019). The former are the resident immune cells of the central nervous tissue, which upregulate the secretion of pro-inflammatory cytokines, such as interleukin-1α and β (IL-1α and IL-1β), interleukin 6 (IL-6) and tumor necrosis factor-α (TNF-α), very rapidly after tSCI (Hayashi et al., 2000; Pan et al., 2002; Yang et al., 2004; Pineau and Lacroix, 2007). These cytokines are responsible for the recruitment of circulating leukocytes to the lesion site and for fostering their activation. Activated microglia and blood-derived macrophages maintain a deleterious inflammatory state leading, among other issues, to apoptosis of oligodendrocytes, activation of astrogliosis, and formation of a fibroglial scar (Fitch et al., 1999; Lu et al., 2000; Beattie et al., 2002; Gao et al., 2013; Ren and Young, 2013; Garcia et al., 2016). The Janus-faced nature of the fibroglial scar resulted in discrepant findings showing beneficial and deleterious properties. However, the scar is believed to impede or even block regenerative processes (Karimi-Abdolrezaee and Billakanti, 2012; Anderson et al., 2016; Bellver-Landete et al., 2019; Bradbury and Burnside, 2019).

Hence, the over-expression of pro-inflammatory cytokines initiates and sustains the relentless inflammatory response, which exacerbates tissue injury and hinders functional recovery (Popovich et al., 1997; Kigerl et al., 2009; Prüss et al., 2011; Ahuja et al., 2017). Studies on tSCI patients have shown a correlation during the acute stage post-injury between lower concentrations of pro-inflammatory cytokines (e.g., TNF-α and IL-1β) in the blood and CSF, and a favorable neurological outcome (Biglari et al., 2015; Kwon et al., 2017). This observation underscores the importance of controlling the inflammatory processes as early as possible after the initial trauma. Yet, although many "single-molecule" therapies have been explored for the treatment of tSCI, effective treatments improving functional recovery remain controversial in pre-clinical studies and represent an unmet medical need.

Using a rat tSCI contusion model, we recently demonstrated that an early intravenous application of human umbilical cord mesenchymal stromal cells (hUC-MSCs) reduced inflammation and astrogliosis at the site of injury (Romanelli et al., 2019). Our observation supports previous reports (Chen et al., 2008; Quertainmont et al., 2012; DePaul et al., 2015; Badner et al., 2016), which showed that the intravenous administration of MSCs decreased the inflammatory response after tSCI in rats. These reports also showed that MSCs application elevated serum levels of anti-inflammatory cytokine IL-10 and decreased levels of pro-inflammatory TNF-α. However, the unforeseen need for MSCs in an acute intervention following tSCI precludes the timely use of patients' own MSCs, which would first need to be expanded *ex vivo*. Therefore, acute MSC-based therapies for tSCI are bound to be performed with allogenic material. In addition, the transplantation of proliferative whole cells constitutes a considerable risk for complications and tumorigenicity (Barkholt et al., 2013).

Accumulating evidence suggests that the biological and therapeutic effects of MSCs are contained in secreted factors acting over long distances, rather than resulting from direct cell-cell interactions within the lesioned tissues (Caplan and Dennis, 2006; Ruppert et al., 2018; Zhou et al., 2019). Among the various factors secreted by MSCs, extracellular vesicles (EVs) represent promising candidates to mediate the biological activities associated with MSCs and could provide a breakthrough for the development of novel therapies (Gimona et al., 2017; Campanella et al., 2019; Rohde et al., 2019). Through their capacity to transfer bio-active molecules, such as DNA, messenger RNAs (mRNAs), microRNAs (miRNAs), proteins, and lipids (Colombo et al., 2014), EVs are increasingly being recognized as important components of intercellular communication for numerous physiological and pathological processes. Moreover, EVs can reach distant targets and readily cross barriers, such as the blood-brain barrier (Saint-Pol et al., 2020).

We reported recently that an intravenous administration of hUC-MSCs-derived extracellular vesicles (named EVs hereafter) following tSCI reduced the inflammatory and scarring processes more efficiently than the application of their parental cells (Romanelli et al., 2019). In line with our findings, other studies have reported that the administration of MSC-derived EVs attenuated inflammation and improved functional recovery after tSCI (Huang et al., 2017; Ruppert et al., 2018; Liu et al., 2020; Noori et al., 2020). The possibility that a higher local concentration and a faster contact with the lesioned tissue further potentiate the beneficial activities of EVs following tSCI remained to be examined. In this study, we compared the long-term functional and structural outcomes obtained following acute intralesional or systemic application of EVs secreted by hUC-MSCs in a rat spinal contusion model.

MATERIALS AND METHODS

Purification of EVs

Single donor-derived human umbilical cord multipotent stromal cells (hUC-MSC at passage number 4) were expanded in alpha-modified minimum essential medium (α-MEM, Sigma, Darmstadt, Germany) supplemented with 10% v/v pooled human platelet lysate (pHPL) and dipeptiven (5.5 mg/ml, Fresenius-Kabi, Graz, Austria). The growth medium was fibrin-depleted by centrifugation at 2,500× *g* for 20 min and further clarified by filtration through a 0.22 μm Stericup filter (Merck, Darmstadt, Germany). At 70% confluence, cells were washed twice with phosphate-buffered saline (PBS, Sigma) and the culture medium was replaced with pHPL-EV-depleted medium, which was prepared as described earlier (Pachler et al., 2017). After 24 h, EVs were isolated from 2.5 L of conditioned medium (CM) derived from 336 × 10^6 hUC-MSCs by tangential flow filtration (TFF) and ultracentrifugation. Briefly, cell debris was removed from CM by centrifugation at 2,500× *g* for 20 min at 18°C (Centrifuge Model 5810 R, Eppendorf, Hamburg,

Germany). The supernatant was filtered through a 0.22 μm Stericup filter (Merck) and the clarified CM was reduced to 60 ml by TFF using a 100 kDa molecular mass cut-off column (Spectrum Labs-Repligen, Breda, The Netherlands). Concentrated CM was further centrifuged at 120,000× *g* for 180 min at 18°C in a Sorvall model WX-80 ultracentrifuge using a fixed-angle rotor model Fiberlite F37L-8x100 to pellet hUC-MSC-EVs. The resulting pellets were washed gently once with PBS and subsequently resuspended in Ringer's Lactate (Fresenius-Kabi) in an appropriate volume to achieve a dose of 20–40 × 10^6 cells secretome equivalent/ml. The resulting EV suspension was clarified by centrifugation at 3,000× *g* for 10 min at 4°C (Centrifuge Model 5810 R, Eppendorf) and sterile filtered through a 0.22 μm Stericup filter (Merck). Dilution was made from 2 × 10^{12} particle/ml stock and aliquots were stored at −80°C until use.

Animal Groups

Experiments were performed in conformity with the Directive 2010/63/EU of the European Parliament and of the council of 22 September 2010 on the protection of animals used for scientific purposes and were approved by the Federal Ministry of the Republic of Austria for Education, Science and Research (BMBWF-66.019/0036-V/3b/2018).

Female F344-rats of 10–12 weeks of age (140–190 g body weight) were purchased from Charles River Laboratories (Sulzfeld, Germany) and kept for at least 4 weeks in the animal facility to acclimatize to the handling by the experimenters. Prior to surgery, rats were randomly divided into three treatment groups each comprising 26 rats that would receive acutely after contusion *via* intra-parenchymal (i.pa.) injection either: (a) 2 μl of Ringer-lactate (i.pa. vehicle) or (b) 2 μl of Ringer-lactate containing 1.5 × 10^9 extracellular vesicles (i.pa. EVs) into the SCI lesion site, or (c) 100 μl of Ringer-lactate solution containing 1.5 × 10^9 extracellular vesicles intravenously (i.v. EVs) *via* tail vein injection. In addition, a fourth group (sham) was composed of sham-operated rats that only underwent laminectomy ($n = 26$). Every treatment group was additionally divided into three time points of analysis; i.e., 24 h ($n = 6$), 14 days ($n = 10$) and 56 days ($n = 10$). Nine rats were excluded from the analyses due to inadequate contusion, because they died during surgery or due to post-surgery complications requiring euthanasia (Sham: $n = 1$, i.pa. vehicle: $n = 3$; i.pa. EVs: $n = 1$; i.v. EVs: $n = 4$). Experimenters were blinded to the content of injections and treatment groups until the end of the data acquisition and analysis.

Surgeries

The operative narcosis was obtained by an intra-muscular injection of a cocktail of medetomidine hydrochloride (Narcostart 150 μg/kg body weight), Midazolam (Midazolam Accord 2 mg/kg body weight), and Fentanyl (Fentanyl-Janssen 10 μg/kg body weight). Body temperature was maintained at 37°C *via* a rectal probe-coupled heating pad. O_2 saturation and pulse were monitored using a pulse-oximeter (Emka Technologies, Paris, France). A dorsal laminectomy was performed at thoracic level 8 (Th8) leaving the exposed underlying dura mater intact. The neighboring vertebrae (Th7 and Th9) were fixed on the foramina intervertebralia using two Adson forceps. Using an impactor (Infinite Horizon, Precision System and Instrumentation PSI, Fairfax Station, VA, USA), a contusion of 200 kdyn was applied on the spinal cord at Th8 level. Force applied and displacement of tissue were recorded, and only rats with contusion force of 200 kdyn and approximately 1,000 μm of displacements were included in the study.

Immediately after contusion, 2 μl of either Ringer-lactate or Ringer-lactate containing EVs were slowly injected into the spinal cord lesion site at midline with a depth of 0.9 mm using a pulled-glass micropipette mounted on a stereotaxic apparatus (Stoelting, Wood Dale, IL, USA). The third group of rats received an intravenous application of EVs *via* tail vein injection. The rats belonging to the sham group underwent only a laminectomy. Post-operative analgesia was provided directly after surgery and daily for 5 days with meloxicam [1 mg/kg body weight subcutaneous (s.c.)]. On the first two days post-surgery, rats additionally received buprenorphine (0.03 mg/kg body weight s.c.) twice per day. To prevent the occurrence of infection, enrofloxacin (10 mg/kg body weight) was administered s.c. on the day of surgery and daily thereafter, until the 5th day post-surgery. The bladder was manually voided 2–3 times per day. Rats with tSCI were housed on special soft bedding (Arbocell Comfort White bedding, Rettenmaier Austria GmbH, Vienna, Austria). Food and water were freely accessible at a lowered height in the cages.

Motor and Sensory Tests

At least 2 weeks of training prior to the surgery were used to familiarize the rats with the different tests employed. Additionally, the last training pre-surgery served to establish a baseline for comparison of performance measured after surgery and following treatment.

BBB Score

The motor function of hindlimbs was assessed using the non-linear BBB-score scale (Basso et al., 1995), which ranges from (0) total paralysis to (21) normal locomotion. Accordingly, rats moving freely within a 1-m diameter arena were scored for 4 min by two observers blinded to group belonging. Measurements were performed on days 1, 4, 7, 11, 14 and then once per week until the 8th week post-injury. Scores of left and right hindlimbs did not differ significantly, and their means have been used for each time point. BBB sub-scores (ranging from 0 to 13) were also calculated to quantify recovery based on toe clearance, paw position, trunk stability and tail use, independently of forelimb–hindlimb coordination (Popovich et al., 2012).

Horizontal Ladder Walk Test

Video recordings were acquired for each rat as they traveled across a horizontal ladder (1 m length, 3 mm rung diameter, 5–20 mm rung irregular spacing) from a neutral cage to reach their home cage with littermates. Total left and right hindlimb correct steps and foot-faults (missteps) were assessed by one observer. The test was performed prior to the surgery, to establish a baseline score, and at 4 and 8 weeks after tSCI. For each time

point, the rats walked three times across a ladder with different rung patterns for each repetition. The percentage of missteps was calculated for each rat and averages were used to compare groups.

CatWalk

Gait analyses of voluntary locomotion were performed using the CatWalk XT system (Noldus Information Technology, Wageningen, Netherlands). Every session consisted of six valid runs defined as transit across the recording window without pause. Sessions were performed two times prior to surgery and thereafter at weeks 3, 5, and 7. Each paw was documented individually. In addition to the evaluation of single gait parameters, we compared the treatment groups using a p(LDA) we recently described (Timotius et al., 2021), which is a linear discriminant analysis providing a weighted combination of the nine most SCI-affected Catwalk parameters. Importantly, two rats of the vehicle group had to be excluded from the analysis for the 3 weeks post-injury time point since their level of locomotor recovery, leading to insufficient stepping ability, was too low to be analyzed using the CatWalk system.

Plantar Test Hargreave's Method

Rats were first placed in the recording chambers for 20 min of acclimation (Plantar test device from Ugo Basile, Gemonio, Italy). An infrared source (65 Watt) was focused on the plantar surface of the hind paws and the "time to withdrawal" from the heat stimulus was recorded. A cut-off at 15 s was set to avoid burn injury. The plantar test was performed prior to the surgery, as the baseline, and then at 4, 6, and 8 weeks post-injury. Four measurements for each hind paw were recorded at each time point. Scores of left and right hind paws did not differ significantly, and their means have been used for each time point.

Bladder Function Assessment

Following tSCI, the urinary bladder of rats needed to be emptied manually up to three times per day until a reflex voiding appeared. This progressive functional recovery was monitored during the 8 weeks of recovery based on the average volume of urine that needed to be manually voided during the daily care. The "bladder functionality score" was defined using the bladder size assessed by palpation: Large 1 point, Medium/Large 2 points, Medium 3 points, Small/Medium 4 points, Small 5 points, Very Small 6 points, and Empty 7 points. A daily mean score was calculated for each rat.

Histology

On day 14 or day 56 after injury (14 dpi or 56 dpi), rats were deeply anesthetized by intraperitoneal injection of ketamine (273 mg/kg body weight), xylazine (7.1 mg/kg body weight), and acepromazine (0.625 mg/kg body weight) and transcardially perfused with 0.9% NaCl containing 10 unit/ml heparin (Sigma), followed by 0.1 M phosphate-buffered 4% paraformaldehyde, pH 7.4. Following perfusion, spinal cords were dissected and further post-fixed for 1 h at room temperature in the same paraformaldehyde solution. Tissues were then washed three times and stored in PBS. Prior to histological analysis, four spinal cords from each experimental group were randomly selected and processed for MRI and μCT imaging.

Magnetic Resonance Imaging (MRI)

Magnetic resonance imaging was performed on a Bruker BioSpec 7 T system (BioSpec 70/20 USR with Paravision 6.0.1. software version, Bruker BioSpin, Ettlingen Germany) in a Tx/Rx configuration, using an 86 mm transmit volume coil (MT0381, Bruker Biospin, Germany) for transmitting (Tx) and a 2-element mouse brain surface coil (MT0042, Bruker Biospin, Germany) for receiving (Rx). Spinal cords ($n = 4$ for each group) were placed in a standard 15 ml Falcon tube and held close to the coil with a custom-made holder. To remove the background signal, scanned samples were immersed in Fomblin (Solvay, Brussels, Belgium).

The scan protocol consisted of high-resolution anatomical scans (FOV 24.0/4.0/5.2 mm, 80 μm isotropic voxels, producing an image size of 300 × 50 × 60 pixels) and a diffusion tensor measurement scan (FOV 24.0/4.2/5.1 mm, 150 μm isotropic voxels, image size 240 × 28 × 34 pixels). Sagittal slice orientation, corresponding to the smallest sample dimension, was selected in order to minimize scan time. Two anatomical scans were acquired for volumetric analysis: T2-weighted (T2), and T1-weighted with Inversion Recovery (T1-IR). A diffusion-tensor scan (DTI) was performed with a DTI-EPI sequence. Optimal field homogeneity for the DTI-EPI scan was achieved by additional localized shimming using MAPSHIM algorithm.

The timing of the inversion recovery pulse in T1-IR sequence was selected based on prior pilot scans. The optimal timing partially suppresses gray and white matter and completely attenuates scar tissue, while preserving signal from liquids, thus enabling to individuate cysts within the injury site. Cysts were segmented automatically with an in-house developed macro and subsequently corrected by an experienced evaluator. Damaged tissue was delineated manually on consecutive sections based on signal alterations detected on either T2 or T1-IR images. The remaining (non-lesioned) tissue was determined automatically with a simple macro routine, which subtracted the "damaged" ROIs from the whole non-zero signal in every slice. The volumetric analyses included tissue located between 5 mm rostral and caudal from the lesion epicenter. All segmentation was performed in FIJI/ImageJ v.1.53t (Schindelin et al., 2012). One spinal cord from the i.pa. EVs group was excluded from *ex vivo* volumetric analysis due to distortion in the acquisition.

Diffusion tensor data was reconstructed using diffusion tensor imaging (DTI) algorithm in DSI Studio[1]. Prior to reconstruction, images were inspected for distortions and imaging artifacts, after which one sample from the i.pa. EV-treated group was exempt from further analysis. Where needed, eddy current and motion artifact correction were applied using DSI Studio built-in modules. The B-table was checked by an automatic quality control routine to ensure its accuracy (Schilling et al., 2019). The tracking index for generating tracts was fractional anisotropy (FA), and seeds with any orientation and position within the seed voxel were taken as starting points of tracts. The tracking threshold was set to 0.35, differential tracking threshold 0.1, angular threshold 30°, step size 0.06 mm, and minimal fiber length 1 mm.

[1] http://dsi-studio.labsolver.org

The number of tracts was measured along the spinal cord samples over a range of equidistant regions of interest placed every 0.75 mm and extending 4.50 mm on either side of the impact epicenter. The epicenter was determined from a high-resolution T2 image as the thinnest portion of the sample in the sagittal plane. For every region, the number of tracts was measured twice, interchanging the "Seed" and "ROI" attributes (i.e., tract start/tract end) in the reconstruction algorithm. The final number of tracts between the two ROIs was calculated as the arithmetic mean and assigned to the midpoint between the two ROIs. To minimize the effect of distortions due to magnetic field inhomogeneity (air bubbles or poor shimming) on measurement results, the number of tracts counted between neighboring points were compared to that measured between second neighbor points. Regions displaying strong deviation between two measurements were excluded from the analysis.

Micro-Computed Tomography (μCT)

μCT Imaging

Spinal cords (n = 4 for each group) were contrasted prior to scanning with incubation in Accupaque-350 (GE Healthcare, Munich, Germany) diluted 1:2 in PBS for 48 h. The spinal cords were then placed on 2 mm thick and 4 mm wide extruded polystyrene boards (XPS) and fixed using parafilm. The mounted spinal cords were placed upright and evenly spaced around the inner wall of a cylindrical sample holder with 19 mm diameter and held in place with pieces of XPS boards. Up to four spinal cord samples were scanned at the same time. Scans were performed at 70 kVp with 85 μA and a 0.5 mm Al Filter. A total of 3,400 projections/180° were integrated 4 times for 650 ms and averaged. The scans were reconstructed to an isotropic resolution of 6 μm. Approximately 21 mm of spinal length was scanned with the lesion epicenter positioned in the center of the scan.

The scans were converted to DICOM slices and evaluated using Fiji (ImageJ v1.53a; Schindelin et al., 2012). The spinal cords were rotated and aligned on the Z-Axis of the image using the rotate function of TransformJ (version 2016/01/09). The images were cropped to the XY extent of the spinal cord and in the Z direction to at least 5 mm above and below the lesion epicenter. Due to damage during tissue manipulation, a rat of the sham group was excluded.

Classification of Spinal Tissue

The outer borders of the spinal cord and borders of the damaged tissue were drawn using the lasso and polygon tools in ImageJ. These selections were interpolated along the Z-Axis using the Interpolate Regions of Interest (ROIs) function of the ROI Manager. The interpolated ROI was then manually validated slice by slice. Classification of damaged tissue was validated and corrected in the orthogonal views if necessary.

Classification of Cyst

Inside the damaged tissue ROI, cysts were defined with a threshold adjusted for each image, based on the intensity of the staining solution. Each scan was filtered using a 3D Gauss filter with a sigma of 4 (24 μm) followed by 3D unsharp masking (sigma 4, strength 0.6). The thresholding resulted in a binary mask which was converted to selections and added to the ROI manager.

Volumetry

The classifications of the spinal cord, damaged tissue, and cyst were combined into a single grayscale classification image with different intensity levels for background, the spinal tissue, damaged tissue, and cyst. Measurements were performed on this classification image and one transversal segment was measured in 10 slice segments (total 60 μm). The average cross-section area of the spinal cord, damaged tissue, and cyst was measured for each segment. For comparison between samples, the spinal cords were aligned according to the lesion epicenter.

Immunohistology

A segment of 15 mm centered on the lesion was selected and transferred into 0.1 M phosphate-buffered 30% sucrose solution for 72 h (n = 5–6 per group and time point). Then, samples were embedded in OCT embedding compound (Tissue-Tek, Sakura, Umkirch, Germany) and frozen in 2-methylbutane over liquid nitrogen. Using a cryostat (Leica CM1950), coronal sections of 15 μm were collected in 10 series (each containing every 10th section) on Superfrost Plus microscope slides (Thermo Scientific, Vienna, Austria).

For immunohistological analyses, sections were washed with PBS + 0.1% Tween-20 (Sigma-Aldrich). The blocking solution was composed of PBS containing 1% bovine serum albumin (Sigma-Aldrich), 0.2% fish skin gelatin (Sigma-Aldrich), and 0.1% Tween-20. The primary antibodies: guinea-pig anti-GFAP (1:500; Progen, Heidelberg, Germany), goat anti-Iba1 (1:300; Abcam, Cambridge, UK), rabbit anti-collagen I (1:100; Abcam), goat anti-ChAT (1:100; Novus Biologicals, Abingdon, UK), and rabbit anti-NG2 chondroitin sulfate proteoglycan (1:200; Merck/Millipore) were diluted in blocking solution and applied overnight at 4°C. The secondary antibodies: Alexa Fluor 568 donkey anti-rabbit (1:1,000; Invitrogen, Vienna, Austria), Alexa Fluor 647 donkey anti-guinea pig (1:1,000; Dianova, Hamburg, Germany), and Alexa Fluor 568 donkey anti-goat (1:1,000, Molecular Probes, Vienna, Austria) were applied overnight at 4°C. Nuclei were stained using 4'6-diamidino-2-phenylindole (DAPI; 0.5 μg/ml, Sigma-Aldrich). Finally, sections were mounted with fluorescent mounting medium (ProLong Gold, Thermo Fisher Scientific) and were examined using a confocal fluorescence microscope (Zeiss LSM710) or a slide scanner (Olympus VS120).

Histological Analyses

Spared Tissue

Immunodetection GFAP was used to distinguish intact neural tissue from the necrotic and scarred tissue, and therewith calculated the volume of remaining spared spinal cord tissue, taking the sham rats as reference. The volume of spinal spared tissue was calculated within a segment ranging from 3,150 μm caudal to 3,150 μm rostral of the lesion epicenter based on one section every 1,050 μm. Using the Fiji ImageJ software (Schindelin et al., 2012), the area of intact neural tissue was manually delineated on the micrographs. The total spared volume corresponded to the sum of the volumes extrapolated

from the intact areas measured on each section and the distance between the sections (1,050 μm).

Microglia/Macrophages Cell Density

The intensity of the inflammatory response was determined based on the density of Iba1-expressing cells in the gray matter of the ventral horn (volume of interest: 425 μm × 425 μm × 15 μm), by counting all Iba1$^+$ cells with a nucleus located in the volume of interest. The densities were calculated on the sections located at 2,100 μm and 3,150 μm, both rostral and caudal from the injury epicenter, and pooled together (four sections in total per rat). The tissue destruction at positions closer to the epicenter did not allow reliable quantification in the ventral horn. Additionally, corresponding positions along the rostral-caudal axis of the spinal cord of sham group rats were analyzed.

Reactive Gliosis and Scarring

GFAP, NG2, and collagen I expression were quantified according to the area stained by immunodetection on the tissue sections. Immunofluorescence images were acquired with fixed parameters and binarized as previously described (Romanelli et al., 2019). For the analysis of reactive gliosis, the quantification of GFAP expression was performed in one ventral horn (area of interest: 425 μm × 425 μm) on sections located at 2,100 μm and 3,150 μm, both rostral and caudal from the injury epicenter, and pooled together (four sections in total per rat). The ventral horn could not be reliably defined in regions closer to the epicenter, which was therefore not included in the analysis. Scarring was investigated based on the deposition of NG2 chondroitin sulfate proteoglycan and collagen I and quantified according to the area stained by immunodetection on the tissue sections positioned at the epicenter as well as 1,050 μm, 2,100 μm, and 3,150 μm rostral and caudal from the epicenter (seven sections in total per rat). Additionally, corresponding positions of sham group rats were analyzed along the rostral-caudal axis of the spinal cord. The area covered by the staining was determined using the Fiji ImageJ software (Schindelin et al., 2012).

Number of Choline Acetyltransferase-Expressing Motor Neurons

The number of cells expressing choline acetyltransferase (ChAT) was determined on sections located 2,100 μm, and 3,150 μm, both rostral and caudal from the injury epicenter of each rat (four sections per rat). The number of ChAT$^+$ cells was counted in both ventral horns and results were pooled together. Additionally, corresponding positions along the rostral-caudal axis of the spinal cord of sham group rats were analyzed.

Cytokine Expression in the Spinal Cord and Serum After tSCI

One day after injury or laminectomy, rats (n = 6 per group) were deeply anesthetized by intraperitoneal injection of ketamine (273 mg/kg body weight), xylazine (7.1 mg/kg body weight), and acepromazine (0.625 mg/kg body weight), and a maximal volume of cardiac blood was collected. Following decapitation, the spinal cords were rapidly dissected for RNA extraction.

Total RNA of each spinal cord was isolated from a 1 cm segment centered on the lesion site, or the corresponding position in the sham group, using the TRIzol reagent (Sigma-Aldrich) according to the manufacturer's protocol. RNA concentrations were determined with a NanoVue plus (GE Healthcare, UK). RNAs were reverse transcribed into first-strand cDNA using the iScript TM Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories, Vienna, Austria) according to the manufacturer's protocol. Quantitative gene expression analyses were performed using TaqMan RT-PCR technology. Technical duplicates containing 10 ng of reverse transcribed RNA were amplified with the GoTAQ Probe qPCR Master Mix (Promega) using a two-step cycling protocol (95°C for 15 s, 60°C for 60 s; 50 cycles, Bio-Rad CFX 96 Cycler). The following validated exon-spanning gene expression assays were employed: pPIA Rn.PT.39a.22214830 and TBP Rn.PT.39a.22214837 from Integrated DNA Technologies (IDT, Leuven, Belgium); arginase 1 Rn00691090; IL-6 Rn01410330; IL-18 Rn01422083; caspase 1 Rn00562724; TNF-α Rn99999017; NLRP1a Rn01467482 and NLRP3 Rn04244620 from ThermoFisher; and IL-1β NM_031512.2 from Sino Biological (Eschborn, Germany).

The relative expression levels of the target genes were normalized on two validated reference genes, Peptidylprolyl isomerase A (PPIA) and TATA-binding protein (TBP). C_q values were analyzed using QBasePlus v. 3.2 (Biogazelle NV, Zwijnaarde, Belgium). Expression of target genes in control and treatment conditions were normalized to represent the relative expression in terms of "fold changes".

Following cardiac blood collection, the serum was isolated by centrifugation and stored at −80°C until analyses. The MSD V-Plex Proinflammatory Panel 2 Rat Kit (Rockville, Maryland, USA) was used to measure serum concentrations of IL-1β, IL-4, IL-6, IL-10, IL-13, TNF-α, IFN-γ, and KC/GRO according to the manufacturer's instructions. Samples were run in duplicates. Plates were quantified with a MESO QuickPlex SQ 120 (Meso Scale Discovery), and data were analyzed using DISCOVERY WORKBENCH Data Analysis software (Meso Scale Discovery).

Statistics

Statistical analyses were performed using the Prism 8 software (GraphPad, San Diego, CA, USA). One-way ANOVA and two-way ANOVA tests were performed, followed by a Tukey *post hoc* test. Repeated measures one-way ANOVA or two-way ANOVA was used where indicated. Baseline scores pre-tSCI were not included in group analyses of treatment effect over time. The measurements performed on the three SCI-groups were compared using one-way ANOVA, followed by Tukey *post hoc* test, to detect the effects of EV treatments following SCI (significances marked with asterisks on the graphs). Moreover, when data were also acquired for the sham group, the SCI and sham groups were compared with an additional one-way ANOVA, followed by Tukey *post hoc* test, to detect effect related to SCI (significances marked with pound signs on the graphs). Statistical significance was assumed for $p < 0.05$. Data are shown as mean ± standard deviation.

RESULTS

Using a moderate to severe contusion tSCI rat model (200 kdyn, Infinite Horizon Impactor) previously described in Romanelli et al. (2019), we compared the effects of EVs applied intravenously (i.v.) or injected intra-parenchymal (i.pa.) directly in the lesion site. The intra-parenchymal application allows for the rapid delivery of EVs at the lesion in high concentrations. Here, we analyzed the impact of EV application immediately after tSCI and in particular the impact of these two administration routes on the functional and structural outcomes.

Acute Intralesional Application of EVs Improves Functional Outcomes After tSCI

Recovery of locomotor function after tSCI was monitored according to the widely used locomotor Basso-Beattie-Bresnahan (BBB) scale (maximal score 21; Basso et al., 1995). On the first day post-tSCI, hindlimbs of rats were nearly paralyzed (BBB score 0.5 ± 0.7; **Figure 1A**). Over the following days, the mobility of joints and the walking pattern progressively recovered in all tSCI groups (**Figure 1A**). Strikingly, from the 2nd week post-injury onward, the recovery of locomotion in i.pa. EV-treated rats was significantly more robust compared to the recovery following vehicle application. At 56 days post-injury, the BBB score reached 15.2 ± 1.9 in i.pa. EV-treated rats, which corresponds to a consistent forelimb–hindlimb coordination with predominant parallel paw placement at the initial floor contact (**Figure 1A**; **Table 1**). In contrast, the BBB scores of vehicle and i.v. EV-treated rats only reached 11.6 ± 0.5 and 12.7 ± 1.7 respectively, which corresponds to consistent weight-supported plantar steps with occasional forelimb–hindlimb coordination (**Figure 1A**; **Table 1**).

Furthermore, we analyzed the BBB sub-score, which combines various parameters of locomotion, independently of forelimb–hindlimb coordination (maximal score 13). Starting at 28 days post-injury, the BBB sub-score was significantly higher in rats that received i.pa. EV application, as compared to the vehicle-treated rats (**Figure 1B**; **Table 1**). Importantly, at 56 days post-injury, the BBB sub-scores of i.pa. and i.v. EV-treated rats (6.1 ± 2.7 and 4.9 ± 1.8, respectively) were significantly higher than the sub-score of vehicle-treated rats (1.6 ± 2.1, $p < 0.001$), and no significant difference was detected between the EV-treated groups at this time point.

The CatWalk XT system was further used to analyze limb coordination during voluntary locomotion. Assessment of the step cycle regularity after tSCI confirmed the strongly impaired coordination 3 weeks after injury (**Figure 1C**; **Table 1**). As expected, sham rats showed a regularity index consistently close to 100%. In the weeks following tSCI, a progressive, but incomplete recovery of interlimb coordination, as well as a decrease of the inter-individual variability, were observed in all injured groups (**Figure 1C**; **Table 1**). Application of EVs, either i.pa. or i.v., did not significantly accelerate the recovery of step cycle regularity, as compared to the vehicle treatment (**Figure 1C**). At 7 weeks post-injury, the three tSCI groups still presented a regularity index significantly lower than the sham group. In addition, we calculated a p(LDA) representing a weighted combination of the nine CatWalk parameters most affected by contusion SCI (Timotius et al., 2021). As expected, the p(LDA) value decreased significantly after contusion and progressively recovered over time for all treatment groups. Furthermore, 5 weeks after injury, the p(LDA) of i.pa. EVs treated rats was greater in comparison to the vehicle-treated rats, although the difference did not reach significance (i.pa.vehicle: 0.045 ± 0.018; i.pa. EVs: 0.064 ± 0.013; $p = 0.06$; **Figure 1D**; **Table 1**).

In addition, we assessed skilled walking with the horizontal ladder walk test. The baseline performance of rats was good and we observed less than 3% of step cycles containing missteps (**Figure 1E**; **Table 1**). In contrast, 4 and 8 weeks after tSCI, the percentage of step cycles containing missteps was significantly larger in all three injured groups, as compared to the sham group (**Figure 1E**; **Table 1**). After 8 weeks ofs tSCI, the rats treated with EVs performed slightly better than vehicle-treated rats. However, this difference was not statistically significant.

The disturbance of sensory function following tSCI was examined using the plantar test Hargreaves' method. The shorter hind paws' withdrawal latencies to thermal stimuli measured 4 weeks following tSCI revealed the emergence of thermal hypersensitivity in all lesioned groups (**Figure 1F**; **Table 1**). The withdrawal latencies of both groups treated with EVs normalized during the 8 weeks of recovery and were eventually comparable to the latency measured in the sham group (**Figure 1F**; **Table 1**). In contrast, 8 weeks after tSCI, rats that had received the vehicle treatment still withdrew their hind paws significantly faster upon thermal stimulus as compared to the sham group ($p < 0.001$). It is worth noting that 6 weeks post-injury, the rats treated with an i.v. EV application already reacted with withdrawal latencies comparable to the sham group, whereas the latencies measured in the i.pa. EV and vehicle-treated groups were still significantly shorter ($p < 0.001$; **Figure 1F**; **Table 1**).

Finally, in addition to the paralysis and partial functional recovery of hindlimb muscles, urinary bladder dysfunction is also observed in this rat model of tSCI. Loss of bladder control and functionality is a devastating problem for a large proportion of SCI patients. We assessed, by palpation, the recovery of bladder voiding capacity over time, based on a scoring system describing the accumulation of urine in the disabled bladder (score ranged from 1 point: very large bladder, to 7 points: empty bladder). The reappearance of a voiding capacity through compensatory reflexes was detected from day 5 after tSCI onward (**Supplementary Figure 1**). From the 3rd week post-injury until the end of the observation period, the bladder volumes palpated in all groups of injured rats were similar to those of sham rats with intact bladder functionality.

Acute Intralesional Application of EVs After tSCI Quenches the Inflammatory Response

We examined, 24 h post-injury, the capacity of acute EV applications to install a milder inflammatory environment within the lesioned spinal cord. As we have observed previously (Romanelli et al., 2019), tSCI provokes a strong upregulation of the expression of the pro-inflammatory cytokines IL-1β and

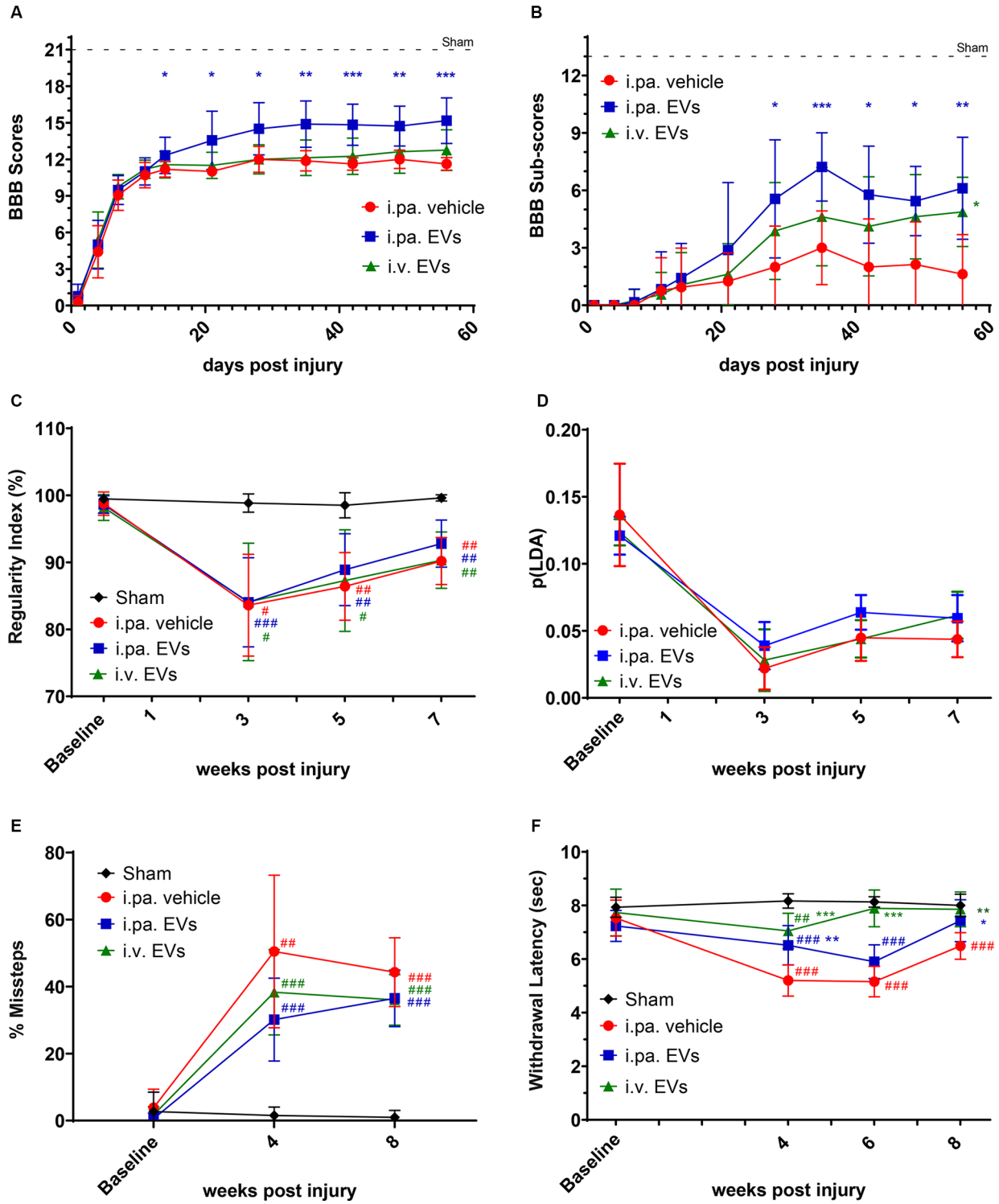

FIGURE 1 | Acute intralesional application of EVs improved functional recovery after tSCI. The locomotor function of rats was monitored by **(A)** BBB scores and **(B)** sub-score in the tSCI groups. **(C)** Inter-limb coordination was assessed by Catwalk regularity index. **(D)** The p(LDA) was calculated to combine the nine most SCI-affected Catwalk parameters. **(E)** Skilled walking was tested with the horizontal ladder walk test and the percentage of step cycles containing missteps was monitored over time. **(F)** Thermal sensitivity was assessed according to the withdrawal latencies to a thermal stimulus using the plantar test Hargreaves' method. Statistical differences to sham group using two-way ANOVA and Tukey *post hoc* test: $^{(\#)}p < 0.05$, $^{(\#\#)}p < 0.01$, $^{(\#\#\#)}p < 0.001$, statistical differences to i.pa vehicle group using two-way ANOVA and Tukey *post hoc* test on tSCI groups (without sham): $^{(*)}p < 0.05$, $^{(**)}p < 0.01$, $^{(***)}p < 0.001$. EVs, extracellular vesicles; tSCI, traumatic spinal cord injury; BBB, Basso-Beattie-Bresnahan.

TABLE 1 | Summary of functional tests.

	Sham	i.pa. vehicle	i.pa. EVs	i.v. EVs
BBB scores				
1 day post-injury		0.3 ± 0.5 *n* = 17	0.8 ± 1.0 *n* = 19 n.s.	0.3 ± 0.5 *n* = 16 n.s.
4 days post-injury		4.4 ± 2.1 *n* = 17	5.0 ± 2.0 *n* = 19 n.s.	5.4 ± 2.3 *n* = 16 n.s.
7 days post-injury		9.1 ± 1.2 *n* = 17	9.5 ± 1.2 *n* = 19 n.s.	9.7 ± 1.0 *n* = 16 n.s.
11 days post-injury		10.7 ± 1.0 *n* = 17	11.0 ± 1.1 *n* = 19 n.s.	11.2 ± 0.7 *n* = 16 n.s.
14 days post-injury		11.2 ± 0.6 *n* = 17	12.3 ± 1.5 *n* = 19*	11.6 ± 1.1 *n* = 16 n.s.
21 days post-injury		11.0 ± 0.0 *n* = 8	13.6 ± 2.4 *n* = 9*	11.5 ± 1.1 *n* = 8 n.s.
28 days post-injury		12.0 ± 1.1 *n* = 8	14.5 ± 2.2 *n* = 9*	12.0 ± 1.2 *n* = 8 n.s.
35 days post-injury		11.9 ± 0.8 *n* = 8	14.9 ± 1.9 *n* = 9**	12.1 ± 1.5 *n* = 8 n.s.
42 days post-injury		11.6 ± 0.5 *n* = 8	14.8 ± 1.7 *n* = 9***	12.2 ± 1.5 *n* = 8 n.s.
49 days post-injury		12.0 ± 0.8 *n* = 8	14.7 ± 1.6 *n* = 9**	12.6 ± 1.8 *n* = 8 n.s.
56 days post-injury		11.6 ± 0.5 *n* = 8	15.2 ± 1.9 *n* = 9***	12.7 ± 1.7 *n* = 8 n.s.
BBB sub-scores				
1 day post-injury		0.0 ± 0.0 *n* = 17	0.0 ± 0.0 *n* = 19 n.s.	0.0 ± 0.0 *n* = 16 n.s.
4 days post-injury		0.0 ± 0.0 *n* = 17	0.0 ± 0.0 *n* = 19 n.s.	0.0 ± 0.0 *n* = 16 n.s.
7 days post-injury		0.0 ± 0.0 *n* = 17	0.1 ± 0.7 *n* = 19 n.s.	0.2 ± 0.7 *n* = 16 n.s.
11 days post-injury		0.8 ± 1.7 *n* = 17	0.8 ± 1.9 *n* = 19 n.s.	0.6 ± 1.1 *n* = 16 n.s.
14 days post-injury		0.9 ± 2.0 *n* = 17	1.4 ± 1.8 *n* = 19 n.s.	1.1 ± 1.7 *n* = 16 n.s.
21 days post-injury		1.2 ± 1.5 *n* = 8	2.9 ± 3.5 *n* = 9 n.s.	1.6 ± 1.6 *n* = 8 n.s.
28 days post-injury		2.0 ± 2.1 *n* = 8	5.6 ± 3.1 *n* = 9*	3.9 ± 2.5 *n* = 8 n.s.
35 days post-injury		3.0 ± 1.9 *n* = 8	7.2 ± 1.8 *n* = 9***	4.6 ± 2.6 *n* = 8 n.s.
42 days post-injury		2.0 ± 2.5 *n* = 8	5.8 ± 2.5 *n* = 9*	4.1 ± 2.6 *n* = 8 n.s.
49 days post-injury		2.1 ± 2.2 *n* = 8	5.4 ± 1.8 *n* = 9*	4.6 ± 2.2 *n* = 8 n.s.
56 days post-injury		1.6 ± 2.1 *n* = 8	6.1 ± 2.7 *n* = 9**	4.9 ± 1.8 *n* = 8*
% Regularity index (CatWalk)				
Baseline	99.5 ± 0.6 *n* = 9	99.1 ± 1.6 *n* = 8 n.s.	98.6 ± 1.3 *n* = 9 n.s., n.s.	98.4 ± 1.9 *n* = 8 n.s., n.s.
21 days post-injury	98.9 ± 1.4 *n* = 9	83.6 ± 7.6 *n* = 6$^{\#}$	84.0 ± 6.6 *n* = 9 n.s.,$^{\#\#\#}$	84.6 ± 8.3 *n* = 8 n.s.,$^{\#\#}$
35 days post-injury	98.5 ± 1.9 *n* = 9	77.8 ± 16.8 *n* = 8$^{\#}$	88.9 ± 5.4 *n* = 9 n.s.,$^{\#\#}$	86.6 ± 7.3 *n* = 8 n.s.,$^{\#\#}$
49 days post-injury	99.6 ± 0.5 *n* = 9	86.3 ± 8.5 *n* = 8$^{\#}$	92.8 ± 3.5 *n* = 9 n.s.,$^{\#\#}$	89.5 ± 4.6 *n* = 8 n.s.,$^{\#\#}$
p(LDA) (Catwalk)				
Baseline		0.135 ± 0.041 *n* = 8	0.121 ± 0.014 *n* = 9	0.124 ± 0.010 *n* = 8
21 days post-injury		0.027 ± 0.013 *n* = 6	0.039 ± 0.018 *n* = 9	0.028 ± 0.023 *n* = 8
35 days post-injury		0.045 ± 0.018 *n* = 8	0.064 ± 0.013 *n* = 9	0.044 ± 0.014 *n* = 8
49 days post-injury		0.045 ± 0.012 *n* = 8	0.059 ± 0.017 *n* = 9	0.062 ± 0.018 *n* = 8
Missteps (Ladder Walk)				
Baseline	2.7 ± 5.8 *n* = 10	3.8 ± 5.5 *n* = 8 n.s.	0.6 ± 1.7 *n* = 9 n.s., n.s.	1.7 ± 2.4 *n* = 8 n.s., n.s.
28 days post-injury	1.5 ± 2.5 *n* = 10	50.5 ± 22.8 *n* = 8$^{\#\#}$	30.1 ± 12.4 *n* = 9 n.s.,$^{\#\#\#}$	38.3 ± 12.7 *n* = 8 n.s.,$^{\#\#\#}$
56 days post-injury	1.0 ± 2.1 *n* = 10	44.3 ± 10.3 *n* = 8$^{\#\#\#}$	36.5 ± 8.4 *n* = 9 n.s.,$^{\#\#\#}$	36.0 ± 7.5 *n* = 8 n.s.,$^{\#\#\#}$
Withdraw Lat. (s) (Hargreaves Test)				
Baseline	7.9 ± 0.4 *n* = 10	7.5 ± 0.7 *n* = 8 n.s.	7.2 ± 0.6 *n* = 9 n.s.,$^{\#}$	7.7 ± 0.9 *n* = 8 n.s., n.s.
28 days post-injury	8.2 ± 0.3 *n* = 10	5.2 ± 0.6 *n* = 8$^{\#\#\#}$	6.5 ± 0.7 *n* = 9**,$^{\#\#\#}$	7.1 ± 0.7 *n* = 8 ***,$^{\#\#}$
42 days post-injury	8.1 ± 0.2 *n* = 10	5.1 ± 0.6 *n* = 8$^{\#\#\#}$	5.9 ± 0.6 *n* = 9 n.s.,$^{\#\#\#}$	7.9 ± 0.7 *n* = 8 ***, n.s.
56 days post-injury	8.0 ± 0.4 *n* = 10	6.5 ± 0.5 *n* = 8$^{\#\#\#}$	7.4 ± 0.8 *n* = 9*, n.s.	7.9 ± 0.6 *n*=8**, n.s.

Summary of measurements addressing motor and sensory recovery. The results are displayed as means ± standard deviations. Statistically significant differences to the sham group are marked with $^{(\#)}p < 0.05$, $^{(\#\#)}p < 0.01$, and $^{(\#\#\#)}p < 0.001$. Statistically significant differences to the vehicle are marked with $^{(*)}p < 0.05$, $^{(**)}p < 0.01$, and $^{(***)}p < 0.001$. Non-significant differences are marked with n.s..

IL-6 at the lesion site at this time point. Compared to vehicle-treated rats, the i.pa. application of EVs significantly reduced the induction of IL-1β and IL-6 expression by more than 90% ($p < 0.001$; **Figure 2A**). A similar reduction in IL-1β expression was also detected following the i.v. application of EVs. However, IL-6 expression was less efficiently suppressed by i.v. application of EVs and showed a reduction of approximately 77% (**Figure 2A**). In contrast, the expression of arginase 1, TNF-α, NLRP1a, NLRP3, Caspase 1, and IL-18 was neither significantly regulated by tSCI at this time point, nor were their expressions modulated by the application of EVs (**Figure 2A**).

Furthermore, we investigated the level of circulating inflammatory cytokines in the serum at 24 h post-injury. At this early time point, we could not detect significant differences in the serum concentrations of IL-1β, IL-4, IL-6, IL-10, IL-13, TNF-α, IFN-γ, and KC/GRO between the injured rats treated with vehicle or EVs (data not shown). Hence, at the beginning of the phase of secondary damages, the application of EVs mainly addressed the local inflammatory processes at the lesion site.

To investigate in more detail the impact of EVs on the course of the local inflammatory response, we calculated the density of Iba1-expressing microglia/macrophages in the ventral horns of the lesioned spinal cord during the sub-acute phase (14 days post-injury) and at the beginning of the chronic phase (56 days post-injury; **Figures 2B–F**; **Table 2**). Fourteen days after contusion, a significantly higher density

of Iba1$^+$ cells was detected in the gray matter of the lesioned spinal cord (vehicle group $2.54 \times 10^4 \pm 0.17 \times 10^4$ Iba1$^+$ cells/mm^3), as compared to the situation observed in sham rats ($0.88 \times 10^4 \pm 0.13 \times 10^4$ Iba1$^+$ cells/mm^3, $p < 0.001$). As shown in **Figure 2B**, 14 days after tSCI, only the i.pa. EVs significantly dampened (-15%) the accumulation of Iba1$^+$ cells ($2.16 \times 10^4 \pm 0.07 \times 10^4$ Iba1$^+$ cells/mm^3, $p < 0.05$), as compared to the vehicle-treated rats. In contrast, no significant difference was observed at this time point between the i.v. EVs ($2.31 \times 10^4 \pm 0.28 \times 10^4$ Iba1$^+$ cells/mm^3) and vehicle-treated rats (**Figures 2B,D–F**; **Table 2**). The number of Iba1$^+$ cells detected on day 56 post-injury decreased in the vehicle-treated rats by roughly one-third as compared to 14 days post-injury (**Figure 2C**; **Table 2**). At this late time point, no treatment-associated differences were observed between the tSCI groups. Nevertheless, all lesioned groups presented significantly higher densities of Iba1-expressing cells than the sham group ($p < 0.001$; **Figures 2C–F**; **Table 2**).

Acute Intralesional Application of EVs Reduces Scarring After tSCI

During the sub-acute phase post-tSCI, reactive astrocytes proliferate and build a glial scar at the lesion site that acts as a physical barrier. Furthermore, astrocytes residing in the intact parenchyma proximal to the lesion become hypertrophic and upregulate the expression of the glial fibrillary acidic protein (GFAP; Silver and Miller, 2004; Renault-Mihara et al., 2008; Bradbury and Burnside, 2019). The immunodetection of GFAP can therefore be used to monitor ongoing reactive gliosis (**Figures 3A,B,G,H**; **Table 2**). At 14 days post-tSCI, examination in the perilesional area revealed that the percentage of area covered with GFAP had more than doubled in tSCI rats that received the vehicle treatment, as compared to the sham group ($p < 0.001$; **Figure 3G**; **Table 2**). In contrast, EV treatment following tSCI significantly lowered the accumulation of GFAP by approximately 35%, compared to the vehicle treatment ($p < 0.01$; **Figure 3G**; **Table 2**). The accumulation of GFAP observed at 56 days post-injury was similar to the respective values at 14 days post-injury for all groups. At this late time point, rats that had received EVs after tSCI exhibited significantly less astrogliosis-associated GFAP accumulation compared to vehicle-treated rats (**Figure 3H**).

Following tSCI, non-cellular components, such as CSPGs (Lemons et al., 1999) and collagen (Tobin et al., 1980), accumulate in the fibroglial scar and create an inhibitory environment for axonal sprouting (Hermanns et al., 2001; Ohtake and Li, 2015). We assessed the impact of early EV applications on the accumulation of collagen I and NG2-CSPGs by immunodetection (**Figures 3C–F,I–L**). Analysis at 14 days post-injury demonstrated that both administration routes of EVs reduced the deposition of NG2-CSPGs and collagen I compared to rats treated with the vehicle (**Figures 3I,K**; **Table 2**). However, only the i.pa. application of EVs reached statistical significance (collagen I $p < 0.01$; NG2 $p < 0.01$). The accumulation of collagen I and NG2-CSPGs detected at 56 days post-injury was comparable to the 14 days time point, but no significant difference was detected between the treatment groups (**Figures 3J,L**; **Table 2**).

Impact of Acute Application of EVs on Structural Integrity After tSCI

Examination of the spinal cords at 14 days and 56 days post-injury revealed that the lesion epicenter was characterized by a large necrotic cavity surrounded by a thin rim of spared tissue (**Figures 4A–F**). Taking the sham rats as a reference, we calculated that vehicle-treated rats lost approximately 40% of the spinal tissue within a region comprised of 3,150 μm rostral and 3,150 μm caudal to the lesion epicenter 14 days post-tSCI (**Figure 4G**; **Table 2**). At this time point, the volumes of intact parenchyma detected in rats that received EVs were not significantly different from the vehicle-treated rats (**Figure 4G**; **Table 2**). In all three tSCI groups, the amount of remaining intact tissue further decreased until 56 days post-injury (**Figures 4G,H**; **Table 2**). At this later time point, a significantly larger preservation of tissue was detected in the rats that received i.pa. EV application, compared to the vehicle-treated group ($p < 0.05$; **Figure 4H**).

To gain more insight into the morphology of the lesion site at 56 days post-injury, *ex vivo* magnetic resonance imaging (MRI) on the spinal cord was performed before processing the samples for histological analyses. First, T1-IR, T2, and DTI-EPI MRI sequences were acquired (**Figures 5A,H**), followed by contrast agent-enhanced μCT imaging (**Figures 5B,C**). MRI and μCT provided a detailed 3D representation of the damaged tissue, revealed the complex cyst morphology, and accurately pinpointed the position of the lesion within the spinal cord (**Figures 5C,D**). The combination of T1-IR and T2 MRI acquisition sequences was the most sensitive for the detection of altered tissue and allowed differentiating, for example, regions with intensive scarring and those with degeneration of axonal tracts (**Figure 5A**). MRI and micro-computed tomography (μCT)-based volumetric analyses conducted from 5 mm rostral to 5 mm caudal to the lesion epicenter addressed the total cyst volume (**Figure 5E**), the total volume of damaged tissue (necrosis, scar, edema, etc.; **Figure 5F**) and total volume of remaining tissue that appeared to be intact (**Figure 5G**). Comparison between the various groups did not reveal significant differences between tSCI rats that received EV treatments or vehicle.

Ex vivo diffusion tensor imaging (DTI) is a powerful technique to evaluate tissue integrity in the spinal segments surrounding the lesion site. At 56 days post-injury, we examined the integrity of longitudinal fiber tracts using DTI in a 9 mm segment of the spinal cord centered on the lesion. As can be seen in **Figure 5H**, the number of longitudinal tracts decreased progressively towards the lesion epicenter, reflecting the progressive disappearance of intact axonal bundles. The severity of the lesion precluded a reliable measurement directly at or across the lesion epicenter. Nevertheless, DTI measurements revealed significantly more longitudinal tracts rostral and caudal to the lesion in the injured rats that received the EVs, as

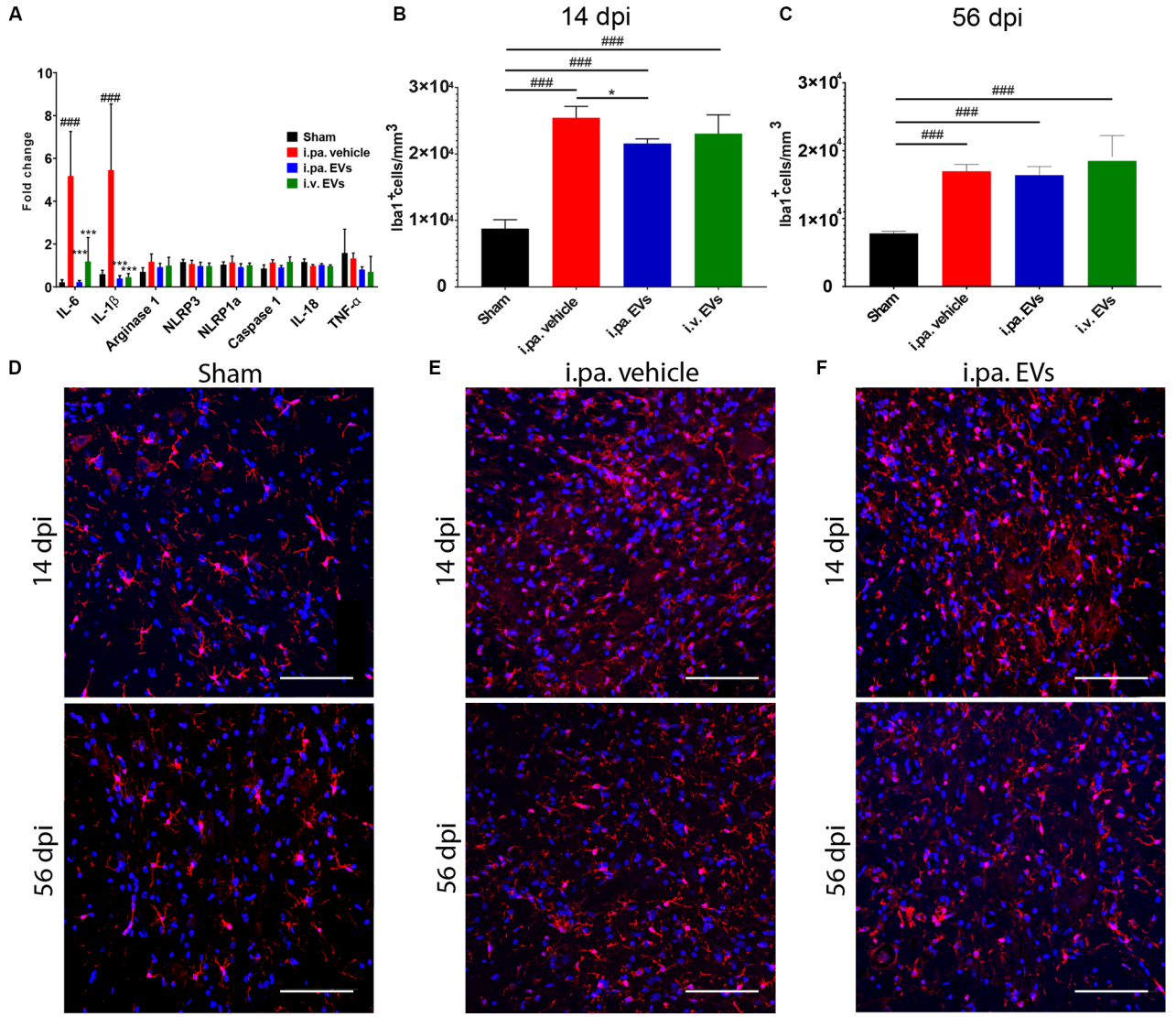

FIGURE 2 | Acute intralesional application of EVs quenched the inflammatory response after tSCI. **(A)** Impact of EV treatment on the expression level of pro-inflammatory genes at the lesion site after tSCI. The density of Iba1-expressing cells was quantified at 14 days **(B)** and 56 days **(C)** after tSCI in the ventral horn. Representative immunodetection of Iba1 in the spinal cord of rats from **(D)** the sham group, **(E)** from the i.pa. vehicle-treated tSCI group and **(F)** from the i.pa. EVs-treated SCI-group (2 mm rostral from the epicenter). Immunodetection of Iba1-expressing microglia (red) was performed 14 days and 56 days post-tSCI. Nuclear counterstain was obtained with DAPI (blue). Scale bar = 50 μm. Statistical differences to sham group using one-way ANOVA and Tukey *post hoc* test: $^{(\#\#\#)}p < 0.001$, statistical differences to i.pa. vehicle group using one-way ANOVA and Tukey *post hoc* test on tSCI groups (without sham): $^{(*)}p < 0.05$, $^{(***)}p < 0.001$. Days post-injury: dpi.

compared to the vehicle ($p < 0.001$). Although more fibers were detected following i.pa. application of EVs, as compared to i.v. administration, this difference did not reach statistical significance.

Finally, we assessed whether the number of motor neurons surviving in the ventral horns of the spinal cord correlated with the better motor function recovery observed following i.pa. application of EVs. Immunodetection of choline acetyltransferase (ChAT) expression at 14 days and 56 days post-injury revealed that the number of motor neurons was significantly decreased (approx. 35%) near the lesion epicenter as compared to the sham group. However, no significant differences were detected following the early application of EVs after the injury as compared to the vehicle treatment (**Table 2**).

DISCUSSION

To the best of our knowledge, this constitutes the first report showing that an intralesional application of EVs successfully improves long-term structural and functional outcomes in a rat model of tSCI. We have recently published that the

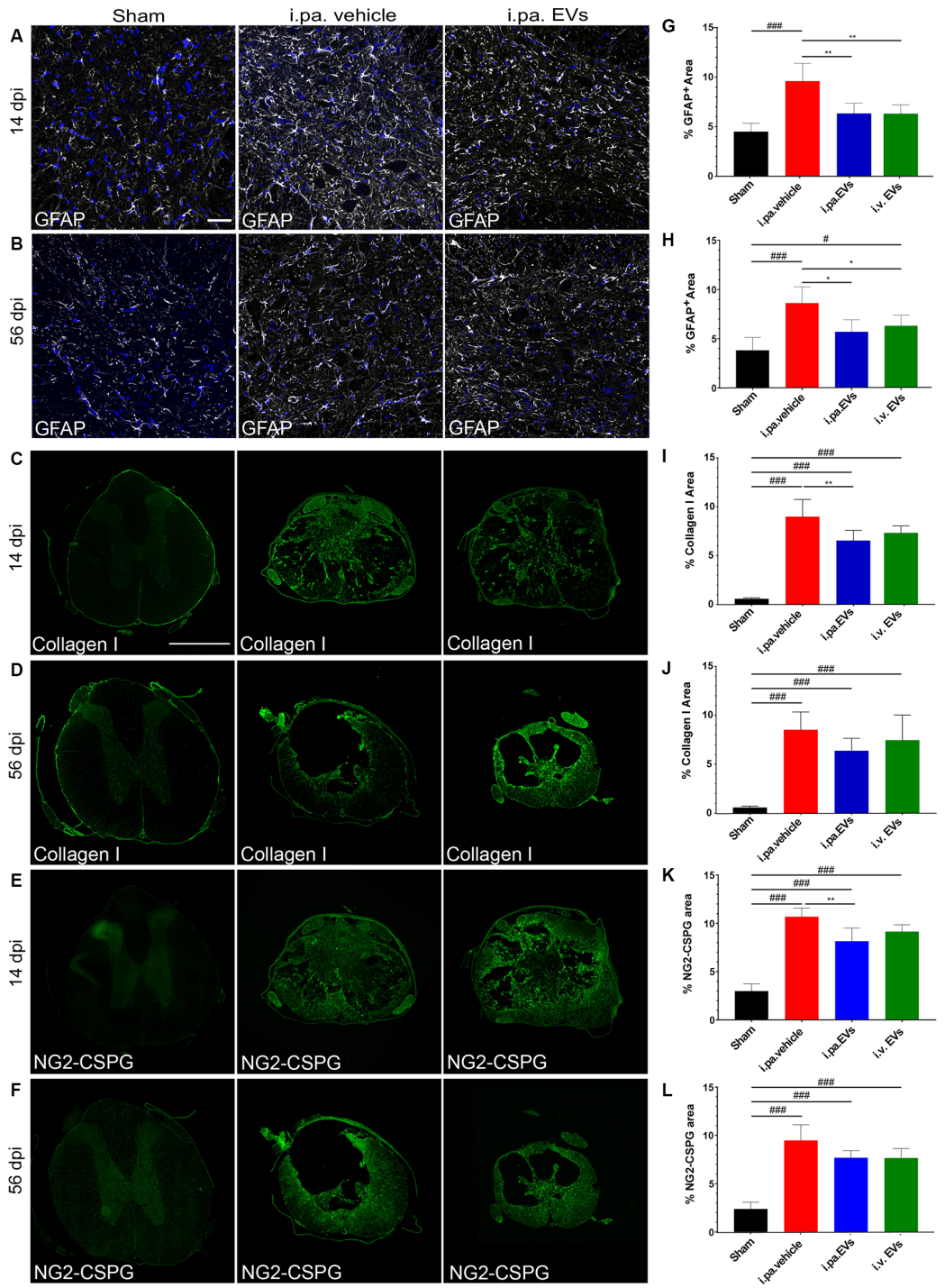

FIGURE 3 | Acute intralesional application of EVs reduced astrogliosis and scarring after tSCI. Immunodetection of GFAP **(A,B)**, collagen I **(C,D)**, and NG2 **(E,F)** in the spinal cord of a sham rat and following tSCI. **(A,B)** Immunodetection of GFAP-expressing astrocytes (white) in the ventral horn of sham rats, i.pa. vehicle-treated tSCI rats and i.pa. EVs-treated tSCI rats at 14 days **(A)** and 56 days **(B)** post-injury (3 mm rostral from lesion epicenter); Nuclear counterstain with DAPI (blue). Scale bar **(A)** = 50 μm. **(C,D)** Immunodetection of collagen I (green) in the spinal cord of sham rats, i.pa. vehicle-treated and i.pa. EVs-treated rats at 14 days **(C)** and 56 days **(D)** post-tSCI at the lesion epicenter. **(E,F)** Immunodetection of NG-2 (green) in the spinal cord of sham rats i.pa. vehicle-treated and i.pa. EVs-treated rats at 14 days **(E)** and 56 days **(F)** post-tSCI at the lesion epicenter. Scale bar **(E)** = 1,000 μm. **(G,H)** Percentage of section area covered by GFAP at 14 days **(G)** and 56 days **(H)** post-tSCI. **(I,J)** Percentage of section area covered by collagen I at 14 days **(I)** and 56 days **(J)** post-tSCI. **(K,L)** Percentage of section area covered NG2 at 14 days **(K)** and 56 days **(L)** post-tSCI. Statistical differences to sham group using one-way ANOVA and Tukey *post hoc* test: $^{(\#)}p < 0.05$, $^{(\#\#\#)}p < 0.001$, statistical differences to i.pa. vehicle group using one-way ANOVA and Tukey *post hoc* test on tSCI groups (without sham): $^{(*)}p < 0.05$, $^{(**)}p < 0.01$. Days post-injury: dpi.

TABLE 2 | Summary of histological measurements.

	Sham	i.pa. vehicle	i.pa. EVs	i.v. EVs
Iba1$^+$ cells/mm^3				
14 days post-injury	8,780 ± 1323 $n = 5$	25,441 ± 1,712 $n = 5^{\#\#\#}$	21,566 ± 715 $n = 6^{*,\#\#\#}$	23,051 ± 2,837 $n = 5$ n.s.,$^{\#\#\#}$
56 days post-injury	7,809 ± 333 $n = 6$	16,947 ± 1,028 $n = 4^{\#\#\#}$	16,380 ± 1,272 $n = 5$ n.s.,$^{\#\#\#}$	18,483 ± 3,110 $n = 5$ n.s.,$^{\#\#\#}$
% GFAP+ area				
14 days post-injury	4.5 ± 0.8 $n = 5$	9.6 ± 1.8 $n = 5^{\#\#\#}$	6.3 ± 1.0 $n = 6^{**}$, n.s.	6.3 ± 0.9 $n = 5^{**}$, n.s.
56 days post-injury	3.8 ± 1.3 $n = 6$	8.6 ± 1.6 $n = 4^{\#\#\#}$	5.7 ± 1.2 $n = 5^{*}$, n.s.	6.3 ± 1.1 $n = 6^{*,\#}$
% collagen I area				
14 days post-injury	0.6 ± 0.1 $n = 5$	9.0 ± 1.8 $n = 5^{\#\#\#}$	6.5 ± 1.0 $n = 6^{**,\#\#\#}$	7.3 ± 0.7 $n = 5$ n.s.,$^{\#\#\#}$
56 days post-injury	0.6 ± 0.1 $n = 6$	8.5 ± 1.8 $n = 4^{\#\#\#}$	6.4 ± 1.3 $n = 5$ n.s.,$^{\#\#\#}$	6.4 ± 0.5 $n = 5$ n.s.,$^{\#\#\#}$
% CSPG-NG2 area				
14 days post-injury	3.0 ± 0.8 $n = 5$	10.7 ± 0.9 $n = 5^{\#\#\#}$	8.1 ± 1.3 $n = 6^{**,\#\#\#}$	9.1 ± 0.7 $n = 5$ n.s.,$^{\#\#\#}$
56 days post-injury	2.4 ± 0.7 $n = 6$	9.5 ± 1.6 $n = 4^{\#\#\#}$	7.7 ± 0.7 $n = 5$ n.s.,$^{\#\#\#}$	7.6 ± 1.0 $n = 6$ n.s.,$^{\#\#\#}$
Spared tissue volume (mm^3)				
14 days post-injury	38.3 ± 3.1 $n = 6$	23.5 ± 3.9 $n = 5^{\#\#\#}$	28.1 ± 3.1 $n = 6$ n.s.,$^{\#\#\#}$	27.9 ± 3.9 $n = 5$ n.s.,$^{\#\#\#}$
56 days post-injury	41.0 ± 2.9 $n = 6$	17.8 ± 2.4 $n = 4^{\#\#\#}$	22.0 ± 1.9 $n = 5^{*,\#\#\#}$	19.0 ± 2.3 $n = 6$ n.s.,$^{\#\#\#}$
Number ChAT+ cells				
14 days post-injury	60.6 ± 6.4 $n = 5$	47.2 ± 6.7 $n = 5^{\#}$	47.7 ± 8.5 $n = 6$ n.s.,$^{\#}$	36.5 ± 5.4 $n = 4$ n.s.,$^{\#\#\#}$
56 days post-injury	51.0 ± 3.2 $n = 6$	35.2 ± 12.7 $n = 4^{\#}$	31.7 ± 6.5 $n = 4$ n.s.,$^{\#\#}$	36.0 ± 8.0 $n = 6$ n.s.,$^{\#}$

The results are displayed as means ± standard deviations. Statistically significant differences to the sham group are marked with $^{(\#)}p < 0.05$, $^{(\#\#)}p < 0.01$, and $^{(\#\#\#)}p < 0.001$. Statistically significant to differences to the vehicle are marked with $^{(*)}p < 0.05$ and $^{(**)}p < 0.01$. Non-significant differences are marked with n.s.

potency of intravenous application of the MSC-EVs to diminish inflammation and scarring surpassed the benefits of whole-cell MSC injection in a rat model of tSCI (Romanelli et al., 2019). Here, we demonstrate that an acute application of EVs directly into the lesion site was significantly more potent to improve long-term functional outcomes. From the BBB scores and sub-scores, we propose that the high intralesional concentration of EVs that was obtained by the direct intra-parenchymal application boosts the locomotor recovery after tSCI. Intriguingly, the recovery of normal thermoception after SCI was quicker in rats treated with i.v. EVs than in rats treated with i.pa. EVs. It may be speculated that following intravenous application, EVs have greater access to the soma of neurons located in the dorsal root ganglions which transmit pain and thermal sensations.

The size and position, as well as the complex system of cysts, could be clearly depicted in the spinal cords *en bloc* using contrast agent-enhanced μCT and MRI in rats entering the chronic phase post-tSCI. In contrast, the histological analysis on cryosections was more susceptible to tissue deformation. It should be noted that μCT and MRI revealed the presence of loose undefined material in several cysts that was not detected in the histological analyses. This material, presumably necrotic tissue, may have been too unstable to withstand cryosectioning and histological processing. Using μCT and MRI-based volumetric measurements, we could not detect the slightly larger volume of intact tissue measured by the histological analysis following i.pa. application of EVs. Whether μCT and MRI are less precise for volumetric analyses than histology or whether the small number of spinal cords analyzed causes this discrepancy remains to be clarified.

The preservation and recovery of spinal neuronal network organization is paramount for its functionality. Taken that as little as 7–10% of the neuronal connections across the lesion site may be enough to obtain significant functionality (Eidelberg et al., 1977; Blight, 1983; Fehlings and Tator, 1995; Kakulas, 1999), even small improvements of connectivity could be relevant. We took advantage of the anisotropic water diffusivity in myelinated axonal bundles to perform diffusion tensor imaging (DTI). Due to the small size of the rat spinal cord and the respiratory motion artifacts, we performed our analyses *ex vivo*. The *ex vivo* measurement on the other side probably decreased the detection sensitivity to anisotropy. Nevertheless, at 8 weeks post-tSCI, we detected significantly more fiber tracts along the rostro-caudal axis in rats that had received EV treatments, as compared to the vehicle-treated rats. We hypothesize that the higher fractional anisotropy resulted from a better structural preservation of the white matter tracts. However, in the absence of measurements at earlier time points, we cannot rule out that regenerative processes also contributed to the higher number of fibers detected.

Acute intralesional application of EVs significantly decreased the local expression level of IL-1β and IL-6. Reduction of inflammation during the acute phase post-SCI is thought to improve the long-term functional outcome (Hausmann, 2003; Arnold and Hagg, 2011; Couillard-Despres et al., 2017; Orr and Gensel, 2018). We recently demonstrated that EVs can directly interact with microglia *in vitro* to dampen their inflammatory phenotype (Romanelli et al., 2019; Warnecke et al., 2020). Hence, EVs applied immediately after tSCI likely target resident microglia at the lesion site to reduce the induction of the local inflammatory response. This modulation was still detectable at 14 days post-injury and resulted in significantly reduced inflammation and scarring. Hence, in addition to the reduction in the density of Iba1-expressing cells, EV applications diminished the reactive astrogliosis by roughly 35% at 14 days post-injury. Interestingly, only the intra-parenchymal EV application could significantly reduce the deposition of collagen I and NG2 at the lesion site, which indicates a stronger anti-scarring activity. In contrast

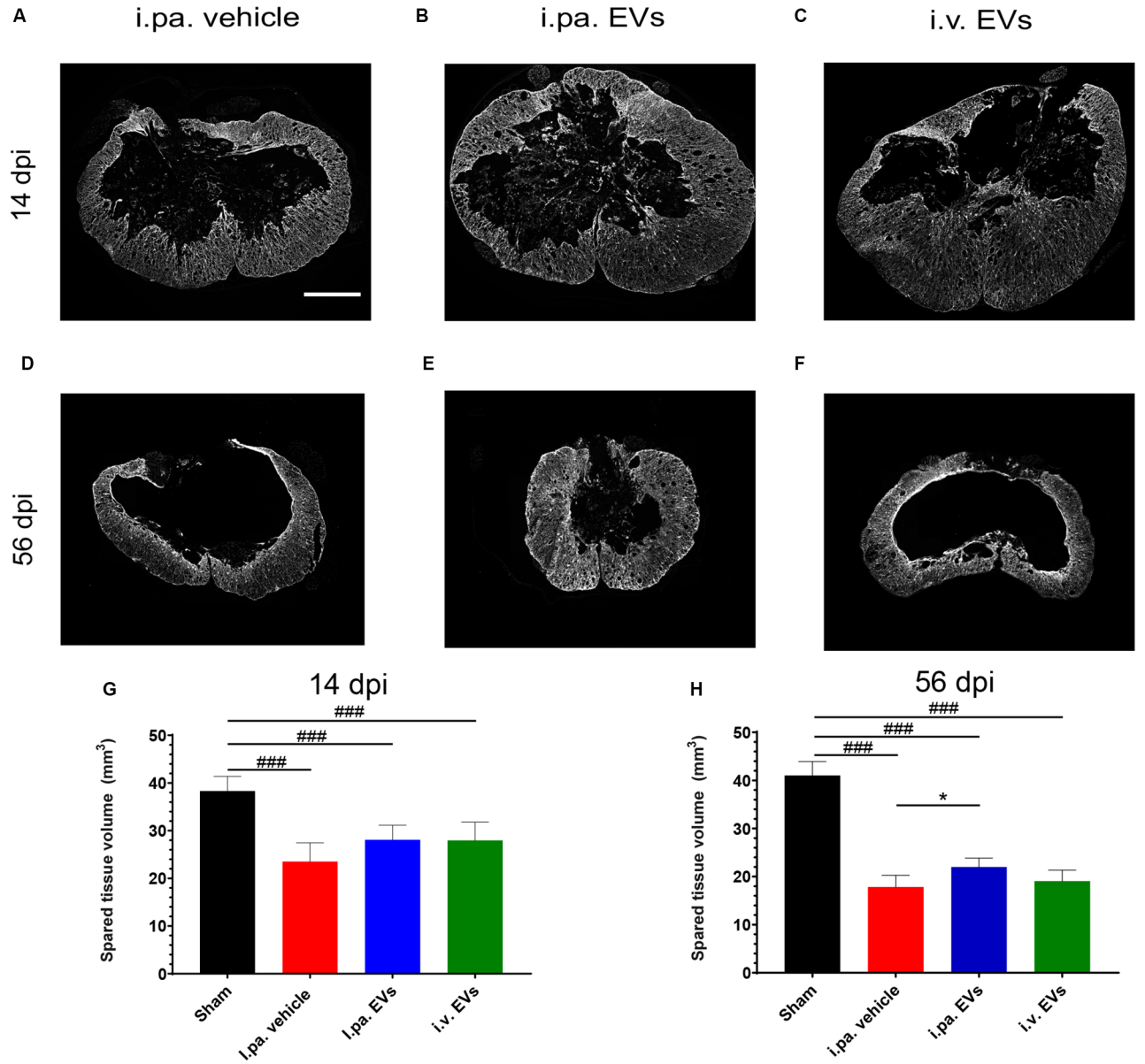

FIGURE 4 | Early intralesional application of EVs spared more parenchyma after tSCI. **(A–F)** Representative micrographs of GFAP immunodetection on spinal cord sections at lesion epicenter at 14 days **(A–C)** and at 56 days post-tSCI **(D–F)**. Scale bar = 500 μm. The total volume of healthy-appearing parenchyma was calculated from 3 mm rostral to 3 mm caudal to the lesion epicenter, or the equivalent position in the sham rats at 14 days **(G)** and 56 days post-tSCI **(H)**. Statistical differences to sham group using one-way ANOVA and Tukey *post hoc* test: $^{(\#\#\#)}p < 0.001$, statistical differences to i.pa. vehicle group using one-way ANOVA and Tukey *post hoc* test on tSCI groups (without sham): $^{(*)}p < 0.05$. Days post-injury: dpi.

to the pathophysiology of tSCI in rodents, astrogliosis and scarring in human tSCI evolve over a longer time span and become prominent at intermediate and chronic stages (Puckett et al., 1997; Hagg and Oudega, 2006). Hence, successful application of EVs against scarring in humans may rely on repeated application, covering a longer time window after tSCI.

The beneficial impact of intravenous application of EVs has been reported using EVs secreted by MSCs of various origins (Huang et al., 2017; Ruppert et al., 2018; Sun et al., 2018; Liu et al., 2019). Although the modes of action of MSC-EVs following tSCI are still to be elucidated, the control of inflammatory processes, reduction of scar formation, and the improvement of vasculature integrity appear to be paramount. Knowledge over the active components of UC-MSC-EVs remains still partial, but the immunomodulatory effects of EVs appear to be relevant to the therapeutic impact. Adenosine signaling may contribute to the immunomodulatory activity of EVs *via* the conversion of AMP to adenosine by the GPI-anchored 5′-ecto-nucleotidase CD73 present on enriched UC-MSC EVs

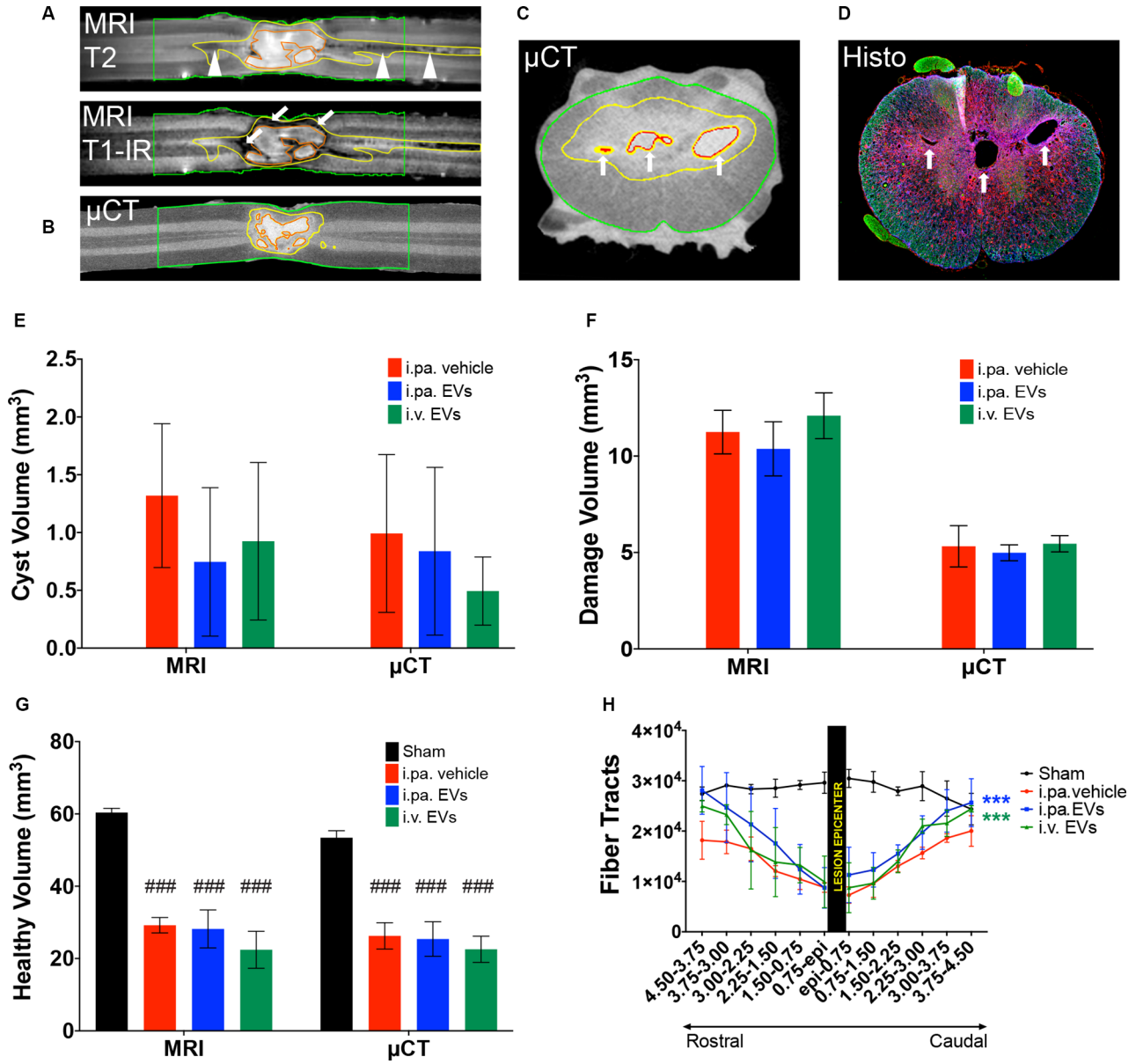

FIGURE 5 | Acute application of EVs improved structural outcomes after tSCI. Representative magnetic resonance (MRI) and micro-computed tomography (μCT) images of vehicle-treated rats at 56 days post-tSCI. Corresponding horizontal sections obtained with **(A)** T1-IR and T2 MRI sequences or **(B)** contrast agent-enhanced μCT. Arrows point to dense scar tissue in **(A)**, arrowheads point to degenerating tracks in **(A)**. Corresponding cross-sections obtained with **(C)** contrast agent-enhanced μCT and **(D)** immunohistology (GFAP in blue, NF-H in green and Iba1 in red). Arrows point to cysts in **(C)** and **(D)**. **(A)** to **(C)**: red lines: cysts; yellow lines: damaged tissue and green lines: sample borders. Quantification of the **(E)** volume of cysts, **(F)** volume of damaged tissue including cysts, and **(G)** volume of intact-appearing tissue. **(E–G)** Statistical differences to sham group using one-way ANOVA and Tukey *post hoc* test on all groups: $^{(\#\#\#)}p < 0.001$. **(H)** Longitudinal fiber tracts rostral and caudal to the lesion epicenter detected by DTI. Statistical differences to vehicle group using two-way ANOVA and Tukey *post hoc* test on tSCI groups (without sham): $^{(***)}p < 0.001$.

(Priglinger et al., 2021). A recent report by Zhai et al. (2021) demonstrated that the use of EVs produced by virus-transformed hUC-MSC engineered to increase the levels of CD73 alleviated inflammation after SCI in a mouse model, and regulated macrophage polarization *in vitro*. Along the same line, Xu et al. (2018) demonstrated that CD73-deficient mice displayed overwhelming immune responses and poor locomotor recovery after SCI. From these data, the authors concluded that CD73 had a protective effect on secondary damage and that the potential mechanism underlying this effect was the restoration of tissue homeostasis *via* extracellular adenosine signaling.

Recently, an intra-cisternal application of bone marrow derived MSCs was also reported to improve the structural

and functional outcomes following tSCI in a rat model (Romero-Ramírez et al., 2020). The biodistribution of the MSC-secreted factors, such as EVs, following intra-cisternal application remains unclear as tSCI provokes swelling of the spinal cord that could hinder cerebrospinal fluid circulation and the distribution of secreted factors. Indeed, the mild impact of MSCs intra-cisternal application reported suggests that these factors may not have reached the lesion epicenter in high concentrations and/or not rapidly enough after tSCI to be as effective as the intralesional application of EVs described in our study.

In conclusion, we have demonstrated that the acute intralesional application of EVs *via* direct intra-parenchymal injection is more effective than the intravenous route to address the early processes of secondary damage in a rat model of tSCI. Furthermore, the intralesional treatment significantly improved the long-term functional outcomes. Even if more invasive in nature than the intravenous application, local injection of biologicals in the spinal parenchyma surrounding the lesion site has been shown to be safe when performed in a slow and gentle fashion in patients with SCI (Levi et al., 2018). Decades of research have indicated that treatment of spinal cord injury will require a manifold approach with different types of intervention for the different phases post-injury. Our observation demonstrates that EVs are particularly potent to address the processes of inflammation and scarring when applied very early and close to the site of injury. Moreover, an improvement of the lesion microenvironment and the additional sparing of parenchyma obtained with EV treatment constitute valuable assets for follow-up interventions aiming for endogenous axonal regeneration or involving cell therapies.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Federal Ministry of the Republic of Austria for Education, Science and Research (BMBWF-66.019/0036-V/3b/2018).

AUTHOR CONTRIBUTIONS

PR, LB, PH, SŠ, BB, ER, MG, DH, MD, and SC-D designed the project. PR, LB, PH, SŠ, DJ, CK, PZ, TS, MG, DH, and MD performed experimental work and acquired data. PR, LB, DH, MD, and SC-D wrote the manuscript. Manuscript corrections were done by all authors. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIALS

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fncel.2021.795008/full#supplementary-material

SUPPLEMENTARY FIGURE 1 | Bladder functionality after tSCI. Time course of the bladder functionality score after tSCI for vehicle-treated, i.pa. EVs-treated as well as i.v. EVs-treated rats. Urinary bladder voiding function was assessed according to filling volumes detected by palpation (1 = very large; 7 = empty bladder).

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Impact of Heterotopic Ossification on Functional Recovery in Acute Spinal Cord Injury

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Objective: In spinal cord injury (SCI), heterotopic ossification is a frequent secondary complication, commonly associated with limited range of motion of affected joints, which could lead to secondary disability in activities of daily living. Additionally, heterotopic ossifications might challenge the effect of regeneration-promoting therapies on neurological and functional recovery. This study evaluated the impact of heterotopic ossification on clinical recovery within the first year after SCI.

Methods: The study was conducted as a monocentric longitudinal paired cohort study. Recruitment was based on consecutive sampling in the framework of the European Multicenter about Spinal Cord Injury (EMSCI). Recovery profiles were determined using standardized neurological and functional clinical assessments within the 1st year following SCI. All study participants underwent at least two comprehensive standardized neurological and functional clinical examinations according to the International Standards for Neurological Classification of SCI and the Spinal Cord Independence Measure, respectively. Data regarding the diagnosis and treatment of heterotopic ossification were obtained by reviewing the patient medical records. The most similar "digital twin" from the entire EMSCI database were matched in terms of age, acute neurological and functional status to each individual with SCI, and heterotopic ossification.

Results: Out of 25 participants diagnosed with heterotopic ossification, 13 individuals were enrolled and matched to control individuals. Most individuals presented with motor complete injury (75%). Ossifications were most frequently located at the hip joints (92%) and mainly occurred within the first 3 months after SCI. Individuals with heterotopic ossification achieved around 40% less functional improvement over time compared to their matched counterparts, whereas neurological recovery was not altered in individuals with SCI and heterotopic ossification.

Conclusion: Heterotopic ossification—a common complication of SCI—unfavorably affects functional recovery, which in the end is most relevant for the best possible degree of independence in activities of daily living. Upon presentation with heterotopic ossification, neurological improvement achieved through potential restorative therapies

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Abbreviations: AIS, ASIA Impairment Scale; AP, alkaline phosphatase; ASIA, American Spinal Injury Association; CRP, C-reactive protein; EMSCI, European Multicenter Study about Spinal Cord Injury; HO, heterotopic ossification; ISNCSCI, International Standards for Neurological Classification of Spinal Cord Injury; LEMS, lower extremity motor score; SCI, Spinal Cord Injury; SCIM, Spinal Cord Independence Measure; SL, sensory level; TLT, total light touch score; TPP, total pin-prick score; UEMS, upper extremity motor score.

might not translate into clinically meaningful functional improvement. Diagnostic algorithms and effective early prevention/treatment options for heterotopic ossification need to be established to ensure the best possible functional outcome.

Clinical Trial Registration: NCT01571531 (https://clinicaltrials.gov).

Keywords: spinal cord injury, functional recovery, heterotopic ossification, complication, ADL, activities of daily living, independence, SCI

INTRODUCTION

The extent of neurological impairment after spinal cord injury (SCI) represents the most powerful predictor of spontaneous sensorimotor and autonomic improvements and subsequently functional recovery. Patients with initially motor complete SCI (AIS-A or AIS-B) display very limited sensorimotor improvements with the inability to restore for example standing and walking function, whereas patients with initially motor incomplete SCI (AIS-C or AIS-D) typically show substantial neurological and functional recovery (Kirshblum et al., 2021a). The aim of restorative therapeutic strategies such as stem cell transplantation or pro-regenerative drug administration is to expedite sensorimotor and autonomous improvement beyond natural recovery. While neurological recovery mainly depends on the integrity of its neural underpinning within the spinal cord, functional recovery referring to mobility, self-care, bowel, bladder, and respiratory function, requires timely and proper administration of rehabilitative interventions (Richard-Denis et al., 2018). A number of secondary complications arising after SCI, e.g., infections, spasticity, pain conditions, pressure injuries, and heterotopic ossification (HO), might negatively affect functional recovery, which is commonly assessed in a standardized fashion with the Spinal Cord Independence Measure (SCIM; Catz et al., 1997; Catz and Itzkovich, 2007; Itzkovich et al., 2007). For example, the presentation with advanced pressure injuries in patients with acute SCI has been shown to impair functional outcome (SCIM score; Donhauser et al., 2020).

Heterotopic ossification (HO) is a complication affecting soft tissues, which can occur after total arthroplasty, traumatic brain injury, or SCI (Déjérine and Ceillier, 1918; Garland, 1991a; Taly et al., 1999). In SCI, it mostly emerges during the first months after SCI below the level of injury (Garland, 1991a; van Kuijk et al., 2002). Most frequent localizations of HO in SCI are the proximal joints, particularly the hip joints (Garland, 1991a; Ranganathan et al., 2015). Around one-fifth of individuals with SCI have been described to present with HO (van Kuijk et al., 2002; Sakellariou et al., 2012; Ranganathan et al., 2015). HO occurs more frequently at younger ages, in men, after cervical or high thoracic traumatic SCI, and typically in more pronounced injury severities (Wittenberg et al., 1992). HO is discussed to favor secondary complications such as urinary tract infections, pneumonias, pressure injuries, and thromboembolic events (Wittenberg et al., 1992; Sakellariou et al., 2012). HO can severely affect the range of motion of affected joints and thus has a great potential to significantly affect the functional outcome, quality of life, and overall healthcare burden (Dryden et al., 2005; Cipriano et al., 2009; Craven et al., 2012). Potentially effective restorative and/or rehabilitative treatments could be challenged by HO to an extent that the gain of neurological recovery by the intervention will not translate into clinically meaningful function (Franz et al., 2012; Lu et al., 2012; Kucher et al., 2018).

While algorithms for early detection and diagnosis of HO are steadily improved (Brooker et al., 1973; Freed et al., 1982; Bressler et al., 1987; Pistarini et al., 1995; Wick et al., 2005; Rosteius et al., 2017) and different (prophylactic) treatment options are being evaluated (Banovac and Gonzalez, 1997; Meiners et al., 1997; Aubut et al., 2011; Genet et al., 2011; Museler et al., 2017), a detailed analysis of HO-associated consequences for the clinical outcome has yet to be determined (Garland, 1991a,b). Thus, this study is intended to clarify to which extent HO affects neurological and functional capabilities over the first year after injury.

MATERIAL AND METHODS

Study Design, Setting and Participants

This longitudinal paired cohort study was conducted in the framework of the "European Multicenter Study about Spinal Cord Injury" (EMSCI) at the Spinal Cord Injury Center Heidelberg, Germany (Curt et al., 2004). The study protocol was approved by the ethics committee of the Heidelberg University Hospital (S-188/2003) and registered at ClinicalTrials.gov (Register-no. NCT01571531). The present study is reported according to the guidelines entitled "Strengthening the Reporting of Observational studies in Epidemiology" (STROBE; Vandenbroucke et al., 2007; von Elm et al., 2007). Before enrolment, informed consent was obtained from all study participants. All participants of EMSCI enrolled from July 2002 to January 2020 were considered for data analyses.

EMSCI aims to include all eligible patients with acute traumatic or single event ischemic SCI according to consecutive sampling. Exclusion criteria comprise nontraumatic cause of SCI (except for single event ischemic incidences), impaired capabilities of cooperation or giving informed consent, peripheral nerve lesions above the level of the spinal lesion, medical history of polyneuropathy, and additional traumatic brain injury. Individuals with SCI who were assigned to the cohort of participants with HO were only assessed at the SCI Center at Heidelberg University Hospital. Participants assigned to the paired control group were identified within the whole EMSCI network. Within EMSCI, initial assessments must be performed within the first 6 weeks after injury. All study participants undergo recurrent comprehensive

clinical examinations in defined time windows (up to day 40, between day 70 and 98, from day 150 to day 186, and from day 300 to day 546 after SCI) within the first year after injury. These comprise neurological examinations according to the "International Standards for Neurological Classification of Spinal Cord Injury" (ISNCSCI; Kirshblum et al., 2011; American Spinal Injury Association, 2019) and functional tests, such as the Spinal Cord Independence Measure (SCIM; Catz et al., 1997; Catz and Itzkovich, 2007). Data were collected by expertly trained examiners to ensure high quality standards (Curt et al., 2004; Schuld et al., 2013; Franz et al., 2022). Data collection and management were coordinated by means of the in-house established EMSCI database (Rupp et al., 2005).

During the study period, all EMSCI study participants from the SCI Center at Heidelberg University Hospital were included in this retrospective data analysis of HO. If at least ISNCSCI and SCIM assessments were completely conducted at the early stage (0–40 days after injury) as well as the late stage (150–546 days after injury) of SCI as previously published (Prang et al., 2021).

Diagnosis of Heterotopic Ossification

Heterotopic ossification as a secondary diagnosis (according to ICD-10) during the first year after SCI led to an assignment of individuals to the HO cohort. Besides relevant clinical symptoms such as redness, swelling, restricted range of motion (Citak et al., 2016), elevated serum levels of alkaline phosphatase (AP), and acute-phase-marker C-reactive protein (CRP; Singh et al., 2003; Estrores et al., 2004), diagnosis of HO was confirmed by plain X-ray, computed tomography, ultrasound, MRI, and 3-phase-bone technetium-99 m scintigraphy (**Figure 1**; Brooker et al., 1973; Freed et al., 1982; Bressler et al., 1987; Pistarini et al., 1995; Wick et al., 2005). The extent of HO in each participant was classified according to the Brooker stages based on available imaging results (Brooker et al., 1973).

Clinical Examination and Quantification of Neurological Outcome

At each exam stage, the neurological assessment was done according to ISNCSCI (Rupp et al., 2021a). This examination includes a standardized motor examination of five upper and lower extremity key muscles and the assessment of two sensory modalities at 28 key sensory points on each side of the body. Based on this, the sensory, motor, and neurological levels of injury, as well as the severity of the SCI graded by the ASIA Impairment scale (AIS), are determined (American Spinal Injury Association, 2015).

Sum scores were calculated for each examination step and side of the body: "upper extremity motor score" (UEMS, maximum = 5 key muscles × 5 max. motor score × body sides = 50 points), "lower extremity motor score" (LEMS, maximum = 5 key muscles × 5 max. motor score × body sides = 50 points), "total light touch score" (TLT, maximum = 28 dermatomes × 2 max. sensory score × 2 sides of the body = 112 points), "total pin-prick score" (TPP, maximum = 28 dermatomes × 2 max. sensory score × 2 sides of the body = 56 × 2 = 112).

For more detailed information on the scoring, scaling and classification process according to ISNCSCI please see Rupp et al. (2021a).

Quantification of Functional Outcome and Independence

The functional capabilities of participants were assessed based on the SCIM (Catz et al., 2007; Itzkovich et al., 2007). The SCIM is a measure of caregiver and assistive device independence in individuals with SCI. It covers the most important aspects of daily living. In EMSCI, an outdated version (SCIM II) was used up until 2007, followed by the current version (SCIM III) from 2007 (Catz et al., 1997; Catz and Itzkovich, 2007). SCIM II and III are compatible on the sub-total level. Sub-total scores are calculated for three different general domains of daily living: (1) "Self Care" (maximum score of 20), (2) "Respiration and Sphincter Management" (maximum score of 40), and (3) "Mobility" (maximum score of 40). Finally, the sum of all scores is defined as the total sum score (maximum score of 100, meaning complete independence).

Matching Procedure

For evaluation of adequate matching partners, the whole EMSCI database was systematically queried applying an iterative approach with margins of matching criteria as narrow as possible being applied. The following matching criteria were applied only if corresponding EMSCI time frames were available in the early stage (either 0–14 or 15–40 days after injury) *and* in the late stage (either 150–186 or 300–546 days after injury): (1) complete ISNCSCI and SCIM assessment within 40 days after injury, (2) identical AIS, (3) neurological level of injury (NLI) ±1 spinal segment, (4) maximum difference of UEMS and LEMS of ±7 points each, (5) divergence of maximum ±4 points in SCIM total scores. In the case of multiple competing matching partners, the one being most akin to the HO-participant was selected. In this process, the lowest possible difference in initial SCIM scores was the determining factor between potential matching partners, followed by the best possible similarity in initial motor function (UEMS/LEMS). In case of persisting ambiguity, the next step was to ensure that the age difference was as small as possible without a fixed cut-off. As the final step in the decision-making process, the gender of the potential matching partners was considered. As the diagnosis of HO is not routinely documented in EMSCI, it is conceivable that a few individuals in the control group were accidentally affected by HO.

Statistical Analysis

Processing (McKinney, 2010), statistics (Virtanen et al., 2020), and presentation (Hunter, 2007) of data were performed using Python Data Science Stack. Due to non-parametric distribution of data, one-sided Wilcoxon signed rank test for matched samples (zero method: "zplit") was used to determine potential differences in neurological and functional recovery. A threshold of $p < 0.05$ was defined for statistical significance.

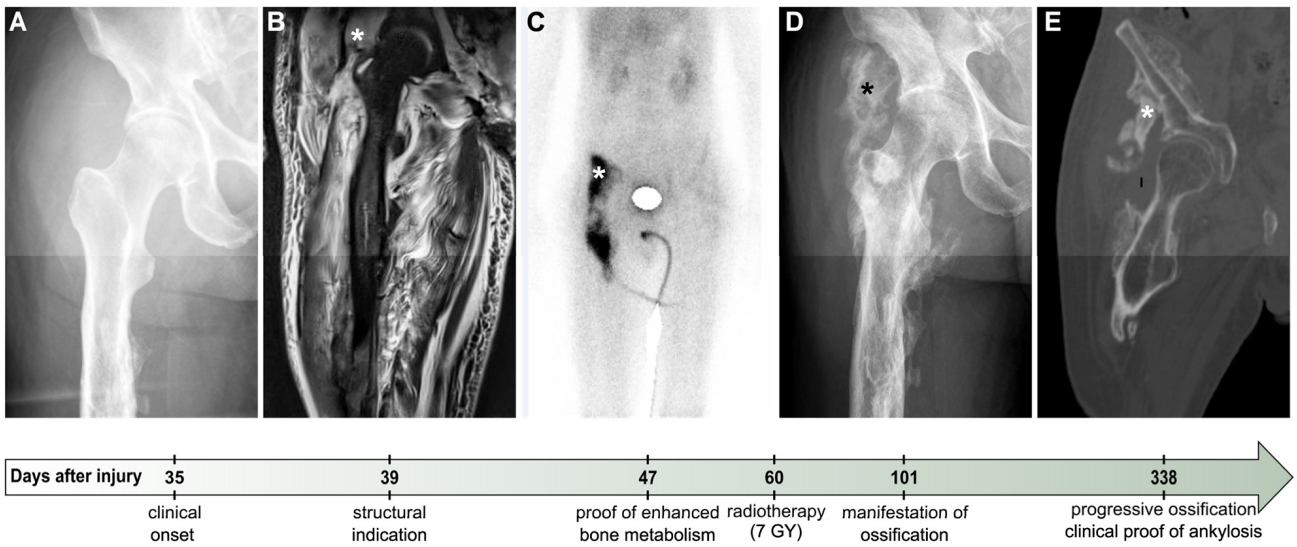

FIGURE 1 | Exemplary clinical course of severe Heterotopic Ossification (ID 13). Plain X-ray **(A,D)**, MRI **(B)**, scintigraphy **(C)**, and CT **(E)**, in chronological sequence of the clinical course from the onset of symptoms to the decision on surgical resection of HO. "Days after injury" denote the time elapsed since SCI. Depicted stars (*) point to areas of ossification. Four days after first clinical indications of potential HO medical diagnostics were initiated (day 39 after SCI), with plain X-ray still lacking reliable proof of ossification **(A)**. On the same day, MRI showed suspicious but rather nonspecific diffuse T2 hyperintense muscle signal behavior in the vicinity of the femoral head **(B)**. Forty-seven days after SCI, enhanced bone metabolism detected by scintigraphy confirmed the previously suspected diagnosis of HO **(C)**. Despite a performed single-time radiotherapy with 7 Gy on day 60, a clinically relevant ossification was proven by CT more than 2 months after the onset of symptoms **(D)**. Ankylosis occurred roughly one year after SCI **(E)** and led to a surgical resection of the ossification (day 408 after SCI), immediately after a second single radiotherapy with 7 Gy the day before.

RESULTS

Of 531 patients from the SCI center at Heidelberg University Hospital enrolled in EMSCI, 25 participants were diagnosed with HO in the 1st year after SCI. Of these, 11 had to be excluded (median age 36 years) from the analysis due to missing data in ISNCSCI, seven of which were characterized by a severely limited range of motion of HO-affected joints within the first 6 months after injury (6× at the hip joint limited to <90° flexion and 1× elbow joint limited to <80° flexion with a median time since the injury to HO diagnosis of 51.5 days, IQR 37.75–60.75). For example, this may have led to "not testable" key muscles and subsequently "not determinable" LEMS according to ISNCSCI (**Figure 2**). Fourteen individuals could be identified for whom a complete dataset was available, i.e., one early (up to 40 days after injury) and one late (150–546 days after injury) ISNCSCI and SCIM assessment. For 13 of these 14 individuals with HO (two females and 11 males, median age 56 years, five AIS A, four AIS B, three AIS C, one AIS D), the identical number of matched partners as controls was identified in the EMSCI database (three females and 10 males, median age 40 years; no significant difference between groups regarding age $p = 0.12$), leading to five mixed and eight male pairs. The matching accuracy for the 13 subjects with HO is reflected by similar baseline characteristics without significant differences regarding relevant ISNCSCI parameters (**Tables 2**, **3**). The median time from onset of SCI to the confirmed diagnosis of HO was 55 days (IQR 46.0–89.5). When HO was suspected, both AP and CRP showed elevated values with a median of 163 (IQR = 114–305) U/l (normal: 35–105 for females and 40–130 U/l for males) for AP and 22 (IQR = 17–39) mg/l (normal: <5 mg/l) for CRP. The diagnosis was confirmed by radiological workup (X-ray or computed tomography) in all but two cases. In these two cases again, MRI, supplemented by either ultrasound or scintigraphy, supported the HO diagnosis. HO was most frequently located at the femur and hip (92%, $n = 3 + 9 = 12$), respectively. One individual with cervical SCI developed HO at the shoulder. A detailed illustration of the diagnostic workflow is presented in **Table 1**.

Neurological Recovery Unaltered by Heterotopic Ossification

Individuals with and without HO—starting with similar if not identical degrees of motor impairment due to the narrow matching process—displayed comparable motor recovery in terms of UEMS and LEMS at the late stage (**Table 2**). Key muscles in the arms added up to a median UEMS of 45.5 (IQR = 14.5–50.0) in subjects with HO vs. 49.0 (16.0–50.0, $p = 0.5$; **Table 2**) in the control group. The same applies to the LEMS, where no differences between the groups are seen, even in the late stages after SCI ($p = 0.1$).

Total light touch scores did not differ in either the early and the late stage of SCI ($p_{early} = 0.87$, $p_{late} = 0.58$; **Supplementary Table 1**), whereas total pin-prick scores differed consistently in both groups with matched controls displaying a median of 1 and 3 points higher scores in the acute as well as chronic stage ($p_{early} = 0.004$, $p_{late} = 0.02$; Hales et al., 2015).

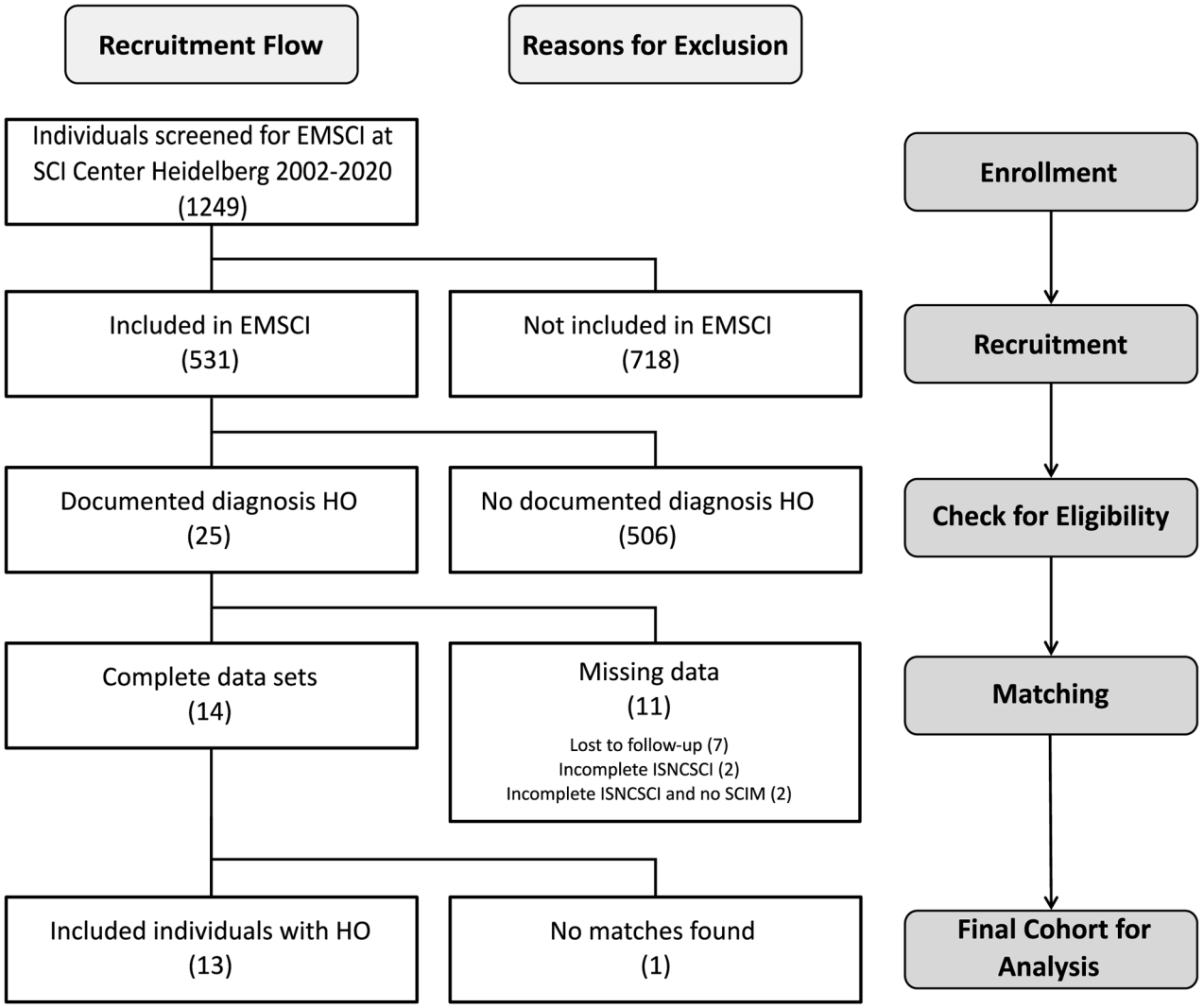

FIGURE 2 | Flow diagram of recruitment process at each stage of the study. Abbreviations: HO, heterotopic ossification; EMSCI, European Multicenter Study about Spinal Cord Injury; ISNCSCI, International Standards for Neurological Classification of Spinal Cord Injury; SCIM, spinal cord independence measure.

Heterotopic Ossification Associated With Impaired Functional Recovery

As opposed to motor recovery, functional recovery as assessed by SCIM revealed differences in outcomes between the two investigated cohorts. While the total SCIM score including all subscales did not differ in the early stage, the median SCIM total score of individuals with HO was 27 (IQR 18–66) points, thus 47.1% lower (p = 0.01) in the late stage of SCI as compared to individuals of the control group who presented with a median total SCIM of 51 (IQR 30–69) in the late stage (**Table 3**). Notably, at the end of the observation period, all subscales of the SCIM showed relevant differences between both groups, albeit to a varying extent (**Table 3**). The self-care subscale median was 4 points lower in the HO group—corresponding to a 30.8% worse outcome (p = 0.02). The median of the respiration and sphincter management subscale was 9 points lower—corresponding to a 37.5% worse outcome (p = 0.03), while the median mobility subscale was 7 points lower—corresponding to 53.8% worse outcome (p = 0.03).

DISCUSSION

Neurological recovery—either naturally occurring or promoted by eventually established interventions and/or restorative therapies—represents the foundation for the restoration of physical abilities, e.g., ambulation or grasping function. However, neurological recovery does not transfer automatically in a meaningful functional gain and can be negatively affected by secondary complications of SCI. As the majority of secondary complications are reversible, e.g., pneumonia, thrombosis, or various pain conditions, they are expected to not interfere with achieving the highest possible functional outcome in the long

TABLE 1 | Individual characteristics of study participants related to spinal cord injury and initial management of heterotopic ossification.

ID	Gender	Age [years]	NLI	AIS	Cause	Localization	Brooker stage	TSI to HO [days]	Diagnostics					TSD to 7 Gy Radiation [days]	TSD to Surgical Intervention [days]
									Abnormal AP/CRP	Ultrasound	MRI	Scinti	X-Ray	CT	
01	m	33	T6	A	I	both hips	n.a.	56	yes/yes	no	no	no	yes	yes	no	1,414
02	m	74	T3	B	T	left femur	n.a.	24	n.a./n.a.	no	no	no	yes	yes	0	no
03	m	63	C5	A	T	left hip	n.a.	47	yes/yes	yes	yes	no	no	no	10	no
04	m	35	T3	B	I	left hip	n.a.	63	n.a./n.a.	yes	yes	no	yes	no	33	no
05	f	43	C5	C	T	left shoulder	n.a.	106	n.a./n.a.	no	no	no	yes	yes	27	no
06	m	65	C5	B	T	right femur	n.a.	120	no/no	no	no	no	yes	yes	14	no
07	m	48	C1	C	T	both hips	III	43	yes/yes	no	no	no	no	yes	16	202
08	m	57	C4	B	T	left hip	I	135	yes/yes	no	no	no	yes	no	21	no
09	m	66	C2	A	T	left hip	I	54	yes/yes	no	yes	no	yes	yes	29	no
10	m	68	C3	C	T	left hip	I	61	no/yes	no	no	no	yes	no	no	no
11	m	38	T4	A	T	both hips	IV	41	yes/yes	no	yes	yes	no	no	28	no
12	f	18	C4	D	T	right femur	II	84	no/yes	no	no	no	yes	no	no	no
13	m	56	T2	A	T	right hip	IV	47	yes/yes	no	yes	yes	yes	yes	13	361
Median (IQR)								56 (47–84)							18.5 (13.25–27.75)	

Abbreviations: AIS, American Spinal Injury Association Impairment Scale; AP, alkaline phosphatase; CRP, C-reactive protein; CT, computed tomography; HO, heterotopic ossification; IQR, interquartile range; I, ischemia; MRI, magnetic resonance imaging; n.a., not available; NLI, Neurological Level of Injury; Scinti, scintigraphy; T, trauma; TSD, time since diagnosis (of HO); TSI, time since injury.

run. In contrast, HO cannot easily be reversed and has the potential to permanently impair the translation of neurological recovery into functional improvement and independence in activities of daily living.

The present study demonstrates that despite a comparable motor recovery, the clinical manifestation of HO predisposes for unfavorable functional outcomes in individuals with SCI as determined by the SCIM assessment. The relatively large effect size supports the confidence in this observation despite a (relatively) small sample size. The limited number of individuals with SCI currently included in EMSCI did not allow to add both age and gender as matching criteria in the present study. The age in the HO cohort, although not significantly higher, may nevertheless have contributed to the observed differences in functional outcomes (Jakob et al., 2009; Kaminski et al., 2017; Geuther et al., 2019; Kirshblum et al., 2021b; Wichmann et al., 2021). Gender, which was not equally distributed in both groups, has not been identified as a confounder in this context. Ankylosis represents the most severe manifestation of HO. Some participants with (HO-associated) ankylosis had to be excluded from the study because essential neurological assessments were thus not feasible, which may have led to an underrating of the negative clinical impact of HO. Therefore, it is essential to use the current revision of the ISNCSCI in future studies, which indeed considers non-SCI conditions, thus preventing the loss of study participants due to missing data (Rupp et al., 2022). Nevertheless, HO as an already known relevant serious complication in daily clinical practice may additionally become a critical adversary in translating gains in neurological recovery—promoted through innovative regenerative therapies—into added functional improvement.

We found inferior functional outcomes for all subscales of the SCIM related to mobility, self-care, respiration, and sphincter management. A structural change in major joints such as the hip joints with a limited range of motion can obviously affect mobility, be it walking function or wheelchair mobility. However, impairment in functions related to self-care and sphincter management is not as obvious in this context. A closer look at respective SCIM items reveals that the performance in these categories also depends on the degree of assistance needed to manage these aspects of daily living successfully. Thus, impaired range of hip joint motion can indeed negatively impact respective SCIM subscales.

Both cohorts, which—based on the matching procedure—started with comparable UEMS and LEMS, did not show any difference in these parameters, even at the late stage. However, HO could theoretically have affected strength training, thus contributing to a less than optimal motor score. Alternatively, expanding bone formation could have compromised peripheral nerves (e.g., sciatic nerve) in the vicinity of the bone formation (Salga et al., 2015; Law-Ye et al., 2016; Onat et al., 2017), which was apparently not the case in the present study considering the similar outcomes regarding motor strength. Interestingly, the pin-prick assessment, reflecting pain sensation mediated by the spinothalamic tract, yielded lower scores in the HO group

TABLE 2 | Detailed comparison of motor (sum) scores between individuals with heterotopic ossification and matched controls.

Pair	Early stage of SCI						Late stage of SCI			
	NLI		UEMS		LEMS		UEMS		LEMS	
	HO	CON	HO	CON	HO	CON	HO	CON	HO	CON
01	T6	T6	50	50	0	0	50	50	0	0
02	T3	T4	50	50	0	0	50	50	10	47
03	C5	C5	9	8	0	0	28	16	0	0
04	T3	T3	50	50	0	0	50	50	0	0
05	C5	C4	8	6	0	0	12	11	3	0
06	C5	C5	44	44	0	0	50	50	0	0
07	C1	C1	12	10	2	7	n.a.	30	n.a.	37
08	C4	C4	10	8	0	0	13	12	5	0
09	C2	C3	6	9	0	0	15	10	0	2
10	C3	C3	0	7	0	8	6	27	15	33
11	T4	T4	50	50	0	0	50	50	0	0
12	C4	C4	26	26	26	29	41	49	39	48
13	T2	T2	50	50	0	0	50	50	0	0
Median	**NA**	**NA**	**26**	**26**	**0**	**0**	**45.5**	**49**	**0**	**0**
(IQR)	(NA)	(NA)	(9–50)	(8–50)	(0–0)	(0–0)	(14.5–50)	(16–50)	(0–6.25)	(0–33)
Mean	**NA**	**NA**	**28.1**	**28.3**	**2.2**	**3.4**	**34.6**	**35.0**	**6.0**	**12.9**
(SD)	(NA)	(NA)	(±20.2)	(±19.8)	(±6.9)	(±7.9)	(±17.5)	(±16.9)	(±11.0)	(±19.3)
p-value	**NA**	**NA**	**0.68**	**0.09**	**0.50**	**0.10**				

In the late stage, cells shaded in dark gray denote worse outcome for the individual with proved HO compared to the matched control, cells in light gray highlight equal results for both matching partners, and white cells indicate a worse outcome for the participant who serves as matched control. Abbreviations: CON, controls; HO, heterotopic ossification; IQR, interquartile range; LEMS, lower extremity motor score; NA, not applicable; n.a., not available; NLI, neurological level of injury; SCI, spinal cord injury; SD, standard deviation; UEMS, upper extremity motor score.

compared to the control group at both the early and the late stage. Of note, pin-prick scores were not part of the matching process. Reduced pain sensation could represent a risk factor for HO since individuals with SCI with severe paresis do not properly sense repetitive micro-traumatic impacts while practicing activities of daily living such as turning in bed, wheelchair transfer, or locomotor training (van Kuijk et al., 2002). This finding would need to be confirmed in a larger prospective study.

In this monocentric analysis, roughly 5% of the SCI individuals included in EMSCI at the SCI center Heidelberg between July 2002 and January 2020 had HO as documented diagnosis. The found incidence is lower than in previous publications (around 20%; Goldman, 1980). This discrepancy is probably due to a selection bias based on the retrospective character of the HO evaluation, the availability of complete datasets, and the fact that previous studies were characterized by a rather unselective inclusion of individuals with acute SCI (Lal et al., 1989; Krauss et al., 2015; Rawat et al., 2019). The rather high median age of 56 years in the HO cohort compared with previous reports (Wittenberg et al., 1992) is likely attributable to the same bias since eligible individuals with HO who had to be excluded were younger. Concerning other debated risk factors, the present individuals with HO represent a rather typical cohort: most of the individuals with HO in the study had marked injury severities (69% AIS A and B), a cervical level of injury (62%), with HO most frequently having occurred in the vicinity of the hip joints (85%) quite early after injury (median 56 days). Existing literature indeed reports SCI-related HO at the hips in about three-quarters to more than 90% of cases (Garland, 1988; Ohlmeier et al., 2017). Triggers for HO are most likely multifactorial, including genetic predisposition to insufficient mobilization early after the injury as well as microinjury to muscles and tendons in paretic limbs during active rehabilitative interventions (van Kuijk et al., 2002; Mitchell et al., 2010). Specific pathophysiological processes beyond inflammatory changes in the beginning and endochondral ossification as the end state are subject to ongoing research (Brady et al., 2018).

Considering the early development of HO after SCI and the serious functional consequences to be expected within the first year after injury, an early and stringent diagnostic and therapeutic regimen is highly desirable. However, in clinical practice, the management of HO is frequently rather heterogeneous due to a sparse evidence base resulting in a lack of effective clinical practice guidelines. None of the diagnostic measures such as basic laboratory diagnostic (alkaline phosphatase, C-reactive protein) or radiological workups (X-ray, CT, MRI, ultrasound, scintigraphy) alone or in combination allow diagnosing HO early on with a high level of confidence (May et al., 2000; Shehab et al., 2002; Estrores et al., 2004; Wick et al., 2005; Rosteius et al., 2017). While prophylactic administration of NSAR is already broadly established in clinical practice (Aubut et al., 2011), the potential to effectively prevent HO formation is limited. Radiation therapy is likely much more effective if applied in the early stage but requires a rather high degree of certainty in respect to the diagnosis considering potentially harmful side effects (Krauss et al., 2015; Museler et al., 2017; Yang et al., 2017). In the presented cohort radiation therapy was applied in more than three-quarters of cases. The detrimental impact of HO on functional outcome in the study cohort was most likely not prevented because radiation therapy was not administered early enough. HO resection surgery was performed in three individuals with

TABLE 3 | Detailed comparison of total SCIM and its subscales between individuals with heterotopic ossification and matched controls.

Pair	Early stage of SCI								Late stage of SCI							
	Self-care		Respiration and Sphincter Management		Mobility		Total		Self-care		Respiration and Sphincter Management		Mobility		Total	
	HO	CON	HO	CON	HO	CON	HO	CON	HO	CON	HO	CON	HO	CON	HO	CON
01	10	10	14	14	0	0	24	24	17	20	30	37	19	19	66	76
02	0	0	10	10	0	0	10	10	13	18	15	40	9	33	37	91
03	0	0	0	4	0	0	0	4	7	9	15	15	5	6	27	30
04	10	8	10	10	1	2	21	20	18	18	36	33	18	18	72	69
05	0	0	0	0	0	0	0	0	2	0	2	15	3	3	7	18
06	3	1	0	2	0	2	3	5	9	20	15	29	6	15	30	64
07	0	0	0	2	0	0	0	2	2	8	13	18	3	11	18	37
08	1	0	6	10	3	0	10	10	0	1	6	19	3	3	9	23
09	0	0	0	0	0	0	0	0	0	0	4	8	4	3	8	11
10	0	0	10	10	0	0	10	10	0	4	15	19	3	13	18	36
11	9	9	13	13	0	0	22	22	19	13	36	27	18	11	73	51
12	0	0	2	0	0	0	2	0	15	20	35	25	32	35	82	80
13	8	7	10	15	7	0	25	22	9	17	10	24	8	14	27	55
Median (IQR)	**0** (0–8)	**0** (0–7)	**6** (0–10)	**10** (2–10)	**0** (0–0)	**0** (0–0)	**10** (0–21)	**10** (2–20)	**9** (2–15)	**13** (4–18)	**15** (10–30)	**24** (18–29)	**6** (3–18)	**13** (6–18)	**27** (18–66)	**51** (30–69)
Mean (SD)	**3.2** (±4.2)	**2.7** (±3.9)	**5.8** (±5.4)	**6.9** (±5.5)	**0.9** (±2.0)	**0.3** (±0.7)	**9.8** (±9.6)	**9.9** (±8.8)	**8.5** (±7.0)	**11.4** (±7.8)	**17.9** (±11.8)	**23.8** (±9.0)	**10.1** (±8.6)	**14.2** (±10.0)	**36.5** (±26.1)	**49.3** (±24.6)
p-value	**0.95**		**0.05**		**0.56**		**0.46**		**0.02***		**0.03***		**0.03***		**0.01***	

*In the late stage, cells shaded in dark gray denote worse outcome for the individual with proved HO compared to the matched control, cells in light gray highlight equal results for both matching partners, and white cells indicate a worse outcome for the participant who serves as matched control. Abbreviations: CON, controls; HO, heterotopic ossification; IQR, interquartile range; SCI, spinal cord injury; SCIM, spinal cord independence measure; SD, standard deviation. *were used to highlight significant differences ($p < 0.05$).*

severe manifestations of HO (Brooker stages of III or higher; Brooker et al., 1973)—towards the end of or beyond the 1-year-post-injury observation period. The impact of surgical HO resection to reverse or even worsen functional deficits cannot be determined, since a standardized functional assessment (SCIM) was not performed subsequently (Garland and Orwin, 1989; Meiners et al., 1997; Melamed et al., 2002; Genet et al., 2011). The effects of prolonged administration of rehabilitative interventions aiming to mitigate HO-induced functional deficits are unknown (Derakhshanrad et al., 2015). Results from the present study can inform the planning of future prospective studies probing early diagnosis and treatment regimens to effectively block function impairing HO formation by providing hints regarding clinically meaningful study endpoints and effect sizes.

Limitations of the Study

EMSCI captures functional outcomes only up to 1 year after injury. Of course, differences in functional outcomes may have disappeared subsequently. Either more effective compensatory strategies or surgical resection of respective bone formations may have contributed to mitigating HO induced deficits.

The rather small sample size and the partly retrospective nature of this study (chart review to obtain information in respect to the diagnosis and treatment of HO) challenge the generalizability of this study. A recently implemented additional registry now provides a solid basis to prospectively record SCI-related secondary disease conditions such as HO (Rupp et al., 2021b). This eventually allows obtaining larger sample sizes combined with high-quality data to critically reflect the findings reported here and will help better understand the causes of deviating neurological and functional recovery profiles.

CONCLUSION

Neurogenic heterotopic ossification represents a complication, which can add substantial secondary disability to the already grave neurological and functional deficits caused by SCI. HO-associated functional impairment as shown in the present study emphasizes the need for effective diagnostic and therapeutic measures, to tackle this condition as early as possible. Precious functional gains achieved through comprehensive SCI care and potentially augmented by effective restorative therapies, once they are available, are at stake.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors upon reasonable request, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee, Medical Faculty of Heidelberg University, Alte Glockengießerei 11/1, 69115 Heidelberg, Germany. Phone: +49 62 21 56 2 64 6-0 Fax: +49 62 21 56 2 64 8-0 ethikkommission-I@med.uni-heidelberg.de. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

RR was significantly involved in the development and establishment of EMSCI. CS and SF were responsible for the study conception and design. LH, LR, RR, and SF the entire EMSCI study group were involved in the data acquisition. CS, LR, and SF contributed to data analysis and data interpretation. SF and NW drafted the manuscript. CS and LH drafted the Figures. CS, NW, and RR revised the final draft of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIALS

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fncel.2022.842090/full#supplementary-material.

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Treadmill Training for Common Marmoset to Strengthen Corticospinal Connections After Thoracic Contusion Spinal Cord Injury

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Spinal cord injury (SCI) leads to locomotor dysfunction. Locomotor rehabilitation promotes the recovery of stepping ability in lower mammals, but it has limited efficacy in humans with a severe SCI. To explain this discrepancy between different species, a nonhuman primate rehabilitation model with a severe SCI would be useful. In this study, we developed a rehabilitation model of paraplegia caused by a severe traumatic SCI in a nonhuman primate, common marmoset (*Callithrix jacchus*). The locomotor rating scale for marmosets was developed to accurately assess the recovery of locomotor functions in marmosets. All animals showed flaccid paralysis of the hindlimb after a thoracic contusive SCI, but the trained group showed significant locomotor recovery. Kinematic analysis revealed significantly improved hindlimb stepping patterns in trained marmosets. Furthermore, intracortical microstimulation (ICMS) of the motor cortex evoked the hindlimb muscles in the trained group, suggesting the reconnection between supraspinal input and the lumbosacral network. Because rehabilitation may be combined with regenerative interventions such as medicine or cell therapy, this primate model can be used as a preclinical test of therapies that can be used in human clinical trials.

Keywords: marmoset, spinal cord injury, rehabilitation, kinematics, locomotion, treadmill training

INTRODUCTION

A thoracic spinal cord injury (SCI) disrupts the connectivity between the brain and lumbar spinal circuits and leads to lower limb paralysis. Rehabilitative approaches are being explored to improve locomotor function, which is an important factor affecting the quality of life after SCI. Mechanisms of rehabilitative effects on locomotor recovery have been gradually studied over the past decade (Fouad and Tetzlaff, 2012; Shinozaki et al., 2016). These mechanisms include the upregulation of neurotrophic factors (Vaynman and Gomez-Pinilla, 2005; Tashiro et al., 2015), an increase in the sprouting and regeneration of neural fibers (Girgis et al., 2007), and the facilitation of cortical map changes (Ishida et al., 2016). Rehabilitation has been shown to improve the recovery of stepping

movements even after a severe SCI in various animal models, including mice (Fong et al., 2005), rats (Cha et al., 2007), and cats (Leon et al., 1998). However, in patients with a severe SCI (A or B on the American Spinal Injury Association Impairment Scale [AIS]), intensive treadmill training improved electromyographic activity but not overground walking (Dietz et al., 2002).

The discrepancy in rehabilitation results between human and animal models may be due to differences in walking recovery mechanisms between these two species. Although the central nervous system (CNS) of rodents is small, their spinal cord accounts for 30% of their net CNS weight, whereas that of humans is only 3% (Swanson, 1995). The recovery of walking after incomplete lesions in humans depends on a descending input from the motor cortex and the ability to strengthen corticospinal connections (Yang and Gorassini, 2006), and the recovery of walking function in patients with SCI seems to be highly dependent on supraspinal input (Cote et al., 2017). For example, the firing of primary motor cortex (M1) neurons is not modulated during feline walking (Drew et al., 2002), whereas cortical EEG signals are modulated during human walking (Petersen et al., 2012). Other studies have shown that stimulation of the corticospinal tract (CST) improves walking in patients with a chronic incomplete SCI (Benito et al., 2012). These reports suggest that walking function by the human spinal cord is highly dependent on cortical commands. Inconsistent results in lower mammals and primates/humans suggest that conducting studies on nonhuman primates is beneficial to bridge this knowledge gap.

Previous studies on nonhuman primates have shown the importance of the motor cortex in locomotion (Rosenzweig et al., 2010; Friedli et al., 2015; Capogrosso et al., 2016) but have created a hemisection model that is not clinically relevant. On the other hand, several studies have been conducted in common marmosets with a contusive SCI to facilitate the successful progress of potential treatments to clinical trials (Iwanami et al., 2005; Kitamura et al., 2011; Kobayashi et al., 2012; Iwai et al., 2015). We tested whether locomotor rehabilitation could lead to a regain of supraspinal input and improved walking recovery after a contusive SCI. To answer this question, we developed a thoracic contusive SCI model of nonhuman primates and investigated their kinematics, electrophysiological, and histological analyses.

MATERIALS AND METHODS

Animals

In the present study, nine common marmosets (*Callithrix jacchus*; female, bodyweight: 280–350 g, age: 2–5 years) were used. All animal experiments were approved by the Animal Research Committee of Keio University School of Medicine (approval number: 11,006) and conformed to the National Institutes of Health (1996) guidelines.

Each marmoset was assigned to one of the two groups: trained ($n = 3$, Marmoset T_K, T_M, and T_D) and untrained ($n = 3$, Marmoset UT_R, UT_W, and UT_G). For scale development and validation of the open field scoring test, UT_Y and UT_H underwent 175 kdyn, and UT_I underwent a 200-kdyn contusive SCI.

Model of SCI

Surgery was performed under general anesthesia induced by intramuscular injection of ketamine (30 mg/kg, Daiichi-Sankyo, Tokyo, Japan), xylazine (2.5 mg/kg, Bayer Healthcare, Monheim, Germany), and atropine sulfate (0.05 mg/kg, Mitsubishi Tanabe Pharma, Osaka, Japan), and was maintained by 1–1.5% isoflurane (MSD, Tokyo, Japan). Pulse and arterial oxygen saturation were monitored during surgical procedures. After laminectomy at the T10 level, a 250-kdyn (UT_Y and UT_H underwent 175 kdyn, and UT_I did 200 kdyn) contusive SCI was inflicted on the exposed dura mater (without durotomy) using a commercially available SCI device (IH Impactor, Precision Systems and Instrumentation, KY, USA).

To maintain a desirable state of health after SCI, intensive daily care was done. To avoid constipation, liquid food or milk was served instead of solid food from 2 days pre-injury to 5 days after injury. Manual bladder emptying was performed two times a day. One week after injury, ceftriaxone (30 mg/kg, Nichi-Iko Pharmaceutical Co., Ltd., Tokyo, Japan) and butorphanol tartrate (5.0 mg/kg, Meiji Seika, Tokyo, Japan) were injected intramuscularly, and saline was administered subcutaneously. Paralyzed animals were given adequate quantities of food and water until they recovered their ability to ingest food and water without assistance. Marmosets regained their bowel, bladder, and autonomic function 1 week after SCI.

Treadmill Training

Animals were acclimatized for 1–2 weeks, so that they would remain calm when walking on a custom-made treadmill device for partial body weight-supported bipedal treadmill walking (**Figure 1**). Prior to intervention, animals were habituated to wear a custom-made jacket with a Velcro® strip (Velcro, London, UK), which was used to attach the marmoset to a weight support and to grip the bar in front of them. The treadmill device was inclined at 15°, and the speed of the treadmill was set to 10.0 cm/s. We know empirically that marmosets that were able to move ankle joints 2 weeks after injury would subsequently regain locomotor functions without rehabilitation. Hence, to ensure that the contusive injury was correctly formed, rehabilitation was initiated after confirming little or no ankle movement 2 weeks after injury. Rehabilitation sessions were performed 5 days/weeks for 3 consecutive weeks (2–4 weeks after injury) and for 30 min with manual assistance. During the stance phase, the experimenter helped the animals swing by holding their ankles and carefully pressing their plantars firmly against the floor.

Marmoset Motor Scale

All animals showed paraplegia after injury, and their locomotor functions gradually recovered. Because reliable outcome measures are required to assess the recovery of locomotor functions in marmosets, we developed original scoring scales based on the previous locomotion scale in rodents (BBB; Basso et al., 1995) and mice (BMS; Basso et al., 2006). This scale was named Marmoset Motor Scale for Locomotion (MMS). **Table 1** describes how the motor characteristics of marmosets differ

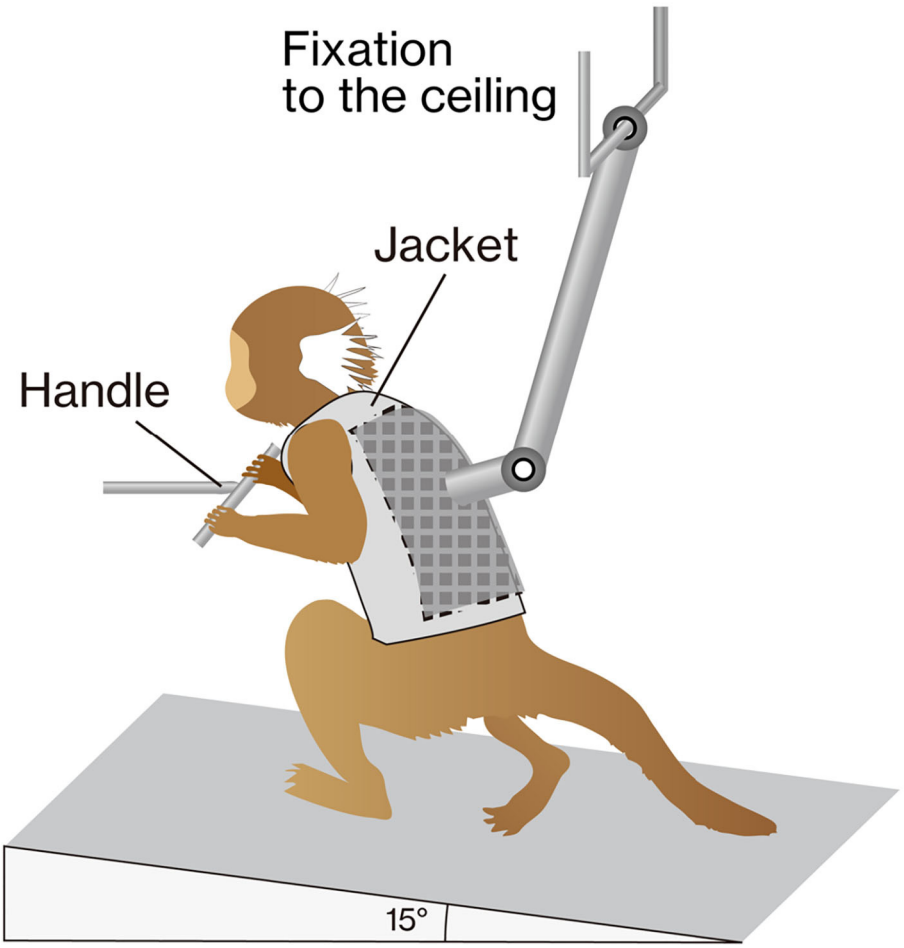

FIGURE 1 | The training apparatus. The marmoset was placed on a treadmill (inclined by 15°) with a custom-made jacket attached to the ceiling for weight support. The gripping handle was positioned in front of the animal. During training, marmosets were rewarded to keep them stable.

from those of rodents when assessing the recovery of marmoset motor function. Because marmosets move faster than rodents and have a relatively long moving span, evaluation requires video recording. In rats, toe clearance was assessed based on auditory cues with their short leg lengths, whereas in marmosets, it was visually assessed due to their larger size and longer legs. We used a large, open field enclosed in a plexiglass sidewall (0.3 m sidewall height and a 4 × 2 m rectangle).

TABLE 1 | Characteristics of locomotion in the open field.

	Mouse	Rat	Marmoset
Moving Speed	Relatively rapid	Slow	Rapid
Moving span	Short bouts	Relatively long	Long
Recovery of FL-HL coordination	Simultaneously recover with paw position	Precede recovery of paw position	Precede recovery of paw position
Leg length	Short	Relatively long	Long
Toe clearance	Hard to assess	Assessed from auditory cues	Assessed visually

Kinematics Analysis

All animals walking at a self-speed were recorded before the injury and at 2, 5, 8, and 11 weeks after injury (**Figure 1**). All of the procedures used have been previously described in detail (Shimada et al., 2017). Briefly, a clear acrylic walkway (1,350 mm in length, 90 mm in width, and 180 mm in height) with a black rubber floor sheet was used to avoid slipping while walking. Animals were habituated to the walkway before recording to ensure their stability while walking straight on it. The walking of the animals was recorded using two cameras (MEMRECAM GX-3; NAC Imaging Technology, Tokyo, Japan), which were synchronized through an internal trigger (150 or 200 Hz). Before each recording, a 4-mm reflective marker was attached bilaterally to the shaved skin, overlying the following specific bony landmarks: acromion of the scapula (shoulder), greater trochanter of the femur (hip), femoral condyle (knee), lateral malleolus of the fibula (ankle), and the fifth metatarsal head (MTP).

Each marker was tracked from each video, and *x*- and *y*-coordinates were automatically quantified using the KinemaTracer software (Kissei Comtec Co., Ltd., Nagano, Japan). The walking cycle was defined as one foot contact to the following foot contact and was subdivided into the stance and swing phase by foot-lift. Each foot contact was visually determined and fed into the KinemaTracer software.

The body was modeled as an interconnected chain of rigid segments and was visualized as stick pictures. In addition, overlaying the trajectory of the MTP in the swing phase for each trial made it easier to visually compare the changes in hindlimb movements.

Each joint angle was defined as the narrower angle between the two segments across each joint. The amplitude of angular joint movements was measured as the difference between the maximum positions in flexion and extension. To quantify the similarity of joint angles before and after injury, the cross-correlation was applied to the time-normalized waveform, and the highest correlation coefficient determined the similarity. Furthermore, based on previous studies (Takeoka et al., 2014; DiGiovanna et al., 2016; Sato et al., 2021), 47 kinematic parameters were extracted (**Table 2**) and principal component analysis (PCA) was applied for a comprehensive evaluation of changes. Note that UT_G was not included in the kinematics analysis because it was not possible to extract gait parameters because the hindlimb of UT_G was dragged until 11 weeks after SCI. Therefore, the kinematics data of the untrained group only included the other two marmosets.

TABLE 2 | Kinematic parameters used for principal component analysis (PCA).

Temporal features of gait	**Joint angle and segmental oscillation**	
Cycle duration (s)	Maximal angle (deg.)	Thigh
Stance duration (s)		Shank
Swing duration (s)		Foot
Relative stance duration, %		Hip
		Knee
Endpoint trajectory		Ankle
Stride length (cm)	Minimal angle (deg.)	Thigh
Step length (cm)		Shank
Maximal forward position of foot (cm)		Foot
Maximal backward position of foot (cm)		Hip
Maximal forward position of foot (x) (cm)		Knee
Maximal backward position of foot (x) (cm)		Ankle
Step height (cm)	Angle Amplitude (deg.)	Thigh
Maximal speed during swing (cm/s)		Shank
Time of maximal velocity during swing, %		Foot
Acceleration at swing onset (cm/s^2)		Hip
Endpoint velocity (x) (cm/s)		Knee
		Ankle
Lags with maximum cross correlation between segments	Maximal angle velocity (deg./s)	Hip
		Knee
Thigh vs Shank		Ankle
Shank vs Foot	Minimal angle velocity (deg./s)	Hip
Hip vs Knee		Knee
Knee vs Ankle		Ankle
Ankle vs Foot	Angle velocity amplitude (deg./s)	Hip
		Knee
		Ankle

Intracortical Microstimulation

Before the injury, the head pipe was implanted in a manner similar to that previously described (Kondo et al., 2018). Marmosets were anesthetized by an intramuscular injection of ketamine (30 mg/kg) and xylazine (2.5 mg/kg). Body temperature and oxygen saturation levels were monitored. Anesthetized animals were mounted on a custom-made stereotaxic apparatus (IMPACT-1000B; Muromachi Kikai, Tokyo, Japan), the skull was exposed, and two polyetheretherketone (PEEK) pipes (Muromachi Kikai, Japan) were attached to the skull with dental cement (UNIFAST II, GC, Japan). Two pipes were parallelly placed over the frontal and occipital areas, and both pipes were flanked by small stainless steel bars.

The next day, marmosets fitted with head pipes were sedated with an intramuscular injection of ketamine (30 mg/kg), atropine sulfate (0.05 mg/kg), ceftriaxone (30 mg/kg), and dexamethasone (0.30 mg/kg, MSD, Tokyo, Japan), and placed in a custom-made stereotaxic chair (Muromachi Kikai, Tokyo, Japan). The abovementioned two pipes were used for head fixation; this allowed us to avoid the use of painful tooth/eye and ear bars. Therefore, marmosets received intracortical microstimulation (ICMS) under natural conditions without xylazine treatment. A large craniotomy (7 × 7 mm square) was performed in the area of the right motor cortex, and tungsten electrodes were implanted to deliver ICMS (10 biphasic pulses composed of 0.2-ms cathode and 0.2-ms anode pulses at 333 Hz). The locations of the hindlimb and forelimb M1 areas were estimated based on the observation of evoked motor responses. At the end of ICMS, artificial dura (5 × 5 mm) (PPX-03060; Gore, Tokyo, Japan) was applied to the brain surface, and a Kwik-Sill® silicone elastomeric adhesive (World Precision Instruments, FL, USA) was mounted.

Histological Analysis

At 11 weeks after injury, marmosets were deeply anesthetized with an overdose of sodium pentobarbital (50 mg/kg; Kyoritsu Seiyaku, Tokyo, Japan) and intracardiac perfusion of 0.1 M potassium phosphate-buffered saline (PBS; pH 7.3), followed by 4% paraformaldehyde (PFA; Merck, NY, USA) in 0.1 M PBS. The spinal cords were removed, post-fixed overnight in 4% PFA in 0.1 M PBS, soaked overnight in 10% sucrose, followed by 30% sucrose, and then cut serially into 50-μm thick coronal sections using a freezing microtome (Retoratome, REM-710; Yamato, Saitama, Japan). The sections located at the lesion epicenter were stained with Luxol fast blue (LFB) to evaluate the myelinated area after injury. For NF-H immunohistochemistry, the sections located 500 μm above the lesion epicenter were incubated overnight at 4°C with the primary mouse monoclonal anti-SMI-32 antibody (1:2,500 in PBS-T; 801701, Biolegend, CA, USA). Sections were incubated with AlexaFluor 488

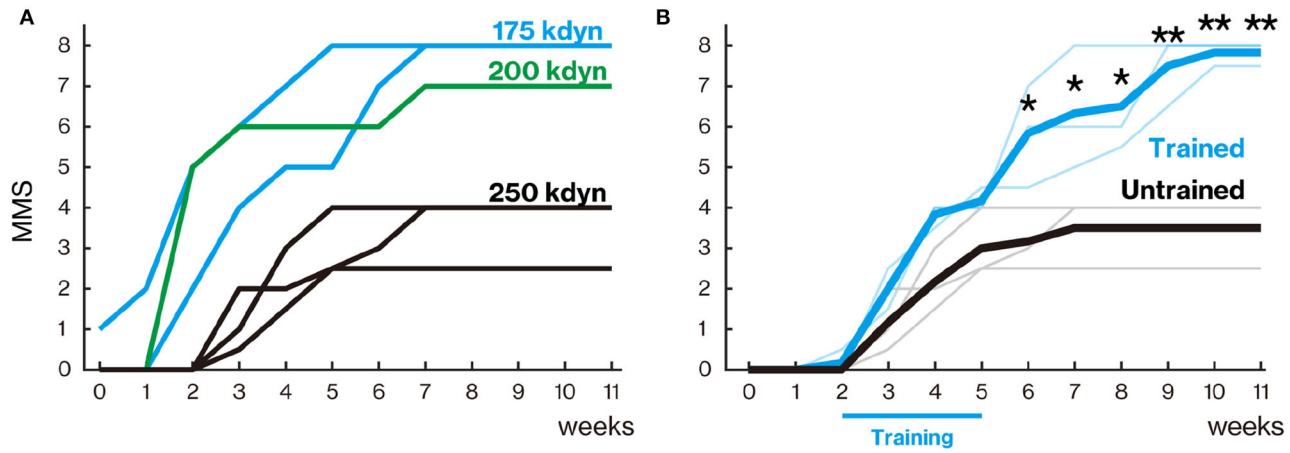

FIGURE 2 | Treadmill training promoted locomotor function recovery. **(A)** Marmoset Motor Scale for Locomotion (MMS) with mild (175 and 200 kdyn) and severe (250 kdyn) contusion groups. Animals with mild contusion gradually recovered their stepping ability, while animals with a severe injury could hardly move their hindlimb until 2 weeks after spinal cord injury (SCI). A detailed explanation of MMS is described in **Table 3**. **(B)** MMS for the trained and untrained group with severe contusions. Thin and thick lines indicate MMS for each animal and their mean, respectively. Animals in the untrained group gradually recovered and reached a plateau around 5 weeks. In the trained group, the difference in locomotor performance was not seen during the training period (from 2 to 5 weeks after injury); they gradually recovered even after 6 weeks. $^{*}p < 0.05$; $^{**}p < 0.01$; Welch's *t*-test.

conjugated secondary goat anti-mouse IgG antibody (1:1,000 in PBS-T; Jackson ImmunoResearch, PA, USA) for 3 h at room temperature. Images of LFB and NF-H staining were obtained using a fluorescence microscope (BZ-9000; Keyence Co., Osaka, Japan). The sectional spinal area was determined using LFB-stained images of axial sections from the center of the lesion, captured at ×10 magnification ($n = 3$ in each group). ImageJ software (National Institutes of Health, MD, USA) was used to quantify the LFB- and NF-H-stained sections.

RESULTS

Spontaneous Recovery of Locomotion After Contusive Injury

Locomotor recovery was scored weekly for up to 11 weeks using the MMS (**Figure 2A**). The MMS and its definitions are presented in **Table 3**. In the early phase of recovery after a 250-kdyn contusive SCI, marmosets showed no or slightly isolated joint movements. During this phase, movements of hip and knee showed variability: these joints showed contracture in most cases or flaccid hindlimb paralysis in other cases, whereas movements of the ankle showed less variability, and the extent of ankle movement could be readily classified. Consequently, we assessed only ankle movements in the MMS. Ankle movement was classified as slight or extensive based on whether the joint motion is less or more than half of the range of motion. Marmosets with a 250-kdyn contusive SCI showed no or slight ankle joint movements until 2 weeks after injury, and as they recovered, extensive ankle movement was demonstrated. At the end of this early phase, marmosets with a 250-kdyn contusive SCI moved their hip, knee, and ankle extensively and simultaneously.

TABLE 3 | Marmoset Motor Scale for Locomotion.

Score	Description
0	No ankle movement
1	Slight ankle movement
2	Extensive ankle movement
3	Plantar placing of the paw with or without weight support -OR-
	Occasional, frequent or consistent abnormal stepping but no plantar stepping
4	Occasional plantar stepping
5	Frequent or consistent plantar stepping, no coordination -OR-
	Frequent or consistent plantar stepping, some coordination, paws rotated at initial contact and lift off (R/R)
6	Frequent or consistent plantar stepping, some coordination, paws parallel at initial contact (P/R, P/P) -OR-
	Frequent or consistent plantar stepping, mostly coordinated, paws rotated at initial contact and lift off (R/R)
7	Frequent or consistent plantar stepping, mostly coordinated, paws parallel at initial contact (P/R, P/P) -OR-
	Frequent or consistent plantar stepping, mostly coordinated, paws parallel at initial contact and lift off (P/P), and no or occasional toe clearance during forward limb advancement
8	Frequent or consistent plantar stepping, mostly coordinated, paws parallel at initial contact and lift off (P/P), mostly toe clearance during forward limb advancement, and tail down or up and down
9	Frequent or consistent plantar stepping, mostly coordinated, paws parallel at initial contact and lift off (P/P), mostly toe clearance during forward limb advancement, and tail always up

Slight, Moves less than half of the ankle joint excursion; Extensive, Moves more than half of the ankle joint excursion; Occasional, Stepping less than or equal to half of the time moving; Frequent, Stepping more than half the time moving; Consistent, Plantar stepping almost all of the time moving.

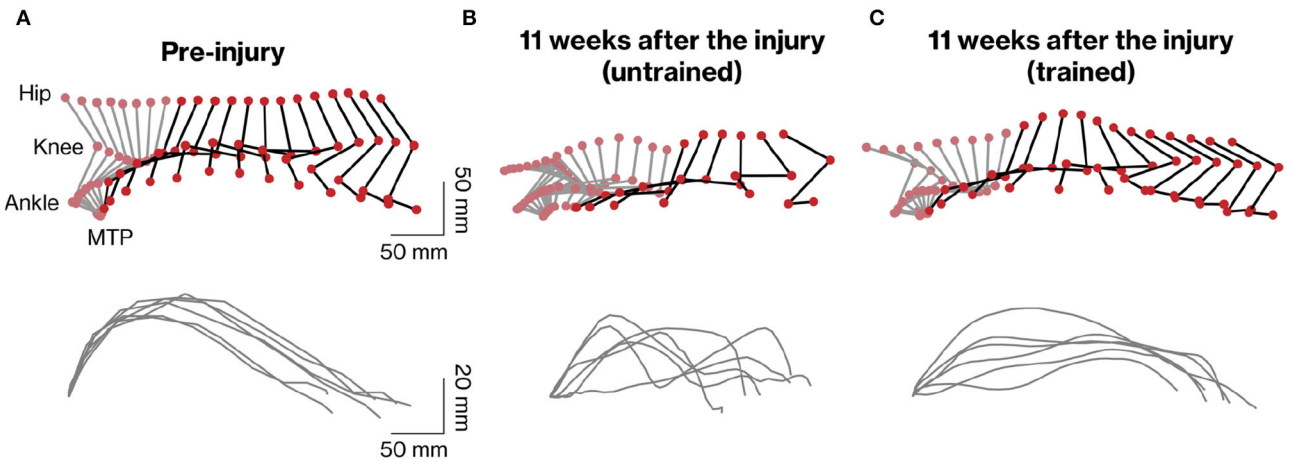

FIGURE 3 | Kinematic analysis of hindlimb movements assessed during quadrupedal walking. Representative stick diagrams of hindlimb movements in pre-injury **(A)** and 11 weeks after injury in untrained **(B)** and trained **(C)** groups are shown (upper panels). Gray and black sticks indicate stance and swing phases, respectively. The time between individual sticks is 35 ms. Successive trajectories of the fifth metatarsal (MTP) are shown for the six consecutive steps in each group (lower panels).

When the ankle, knee, and hip joint can move well, marmosets can place the plantar surface on the ground, allowing stepping movements. Weight support during plantar placement was scored when the hindquarters were elevated in response to paw placement. Marmosets with a 250-kdyn contusive SCI began to stand 4–6 weeks after injury, but the frequency of plantar stepping was occasional (less than half of the time moving forward).

In the late recovery phase, mildly injured marmosets showed improvements in the finer aspects of locomotion, such as forelimb–hindlimb (FL–HL) coordination, paw position during stance, and toe clearance during swing. These aspects of locomotor recovery were not sustained in marmosets with a 250-kdyn contusive SCI. FL–HL coordination was defined as a one-to-one correspondence between forelimb and hindlimb steps, as in BMS and BBB for rodents. The paw position at the beginning and end of the stance phase, initial contact, and liftoff were determined by assessing whether the middle digits of the hind paw were parallel to the long axis of the body. Tail position during locomotion was rated according to whether it was always down, up and down (held up at least once during locomotion), or up (always held up). The occurrence of any FL–HL coordination was 3–4 weeks after injury in the mild injury group (175 and 200 kdyn; **Figure 2A**). FL–HL coordination in marmosets as well as in rats preceded the recovery of paw position; on the other hand, coordination and paw position recovered simultaneously in mice.

Treadmill Training Promoted Significant Locomotor Function Recovery

Animals in the untrained group gradually recovered and reached a plateau at approximately 5 weeks after injury (**Figure 2B**). In the trained group, the degree of recovery did not change from the untrained group during the training period (from 2 to 5 weeks after injury), but significantly higher recovery was observed from 6 weeks after injury than in the untrained group (6–8 weeks, $p < 0.05$; 9–11 weeks, $p < 0.01$).

Kinematics Analysis

To analyze the stepping ability of marmosets with SCI with and without rehabilitation, the trajectory patterns of the ankles and MTP during stepping were plotted (**Figures 3A–C**). To clarify the difference in motor function between the groups, the stepping patterns of trained and untrained marmosets were compared at 11 weeks after injury (compare **Figures 3B,C**). Without rehabilitation, the stepping patterns remained erratic. In contrast, in the trained group, the trajectory before the injury was almost constant.

To assess motor recovery at 11 weeks after injury, we measured the angular movement of the three hindlimb joints. The walking pattern displayed by the hindlimb impacted by the contusion was still deficient in untrained marmosets but was well organized in trained marmosets (**Figures 4A–C**). To comprehensively evaluate the changes, 47 kinematic parameters (**Table 2**) were extracted in each cycle and PCA was applied. The results show that pre-training, trained and non-trained groups are clustered at different positions on the plane created by PC1 and PC2 (**Figure 4D**). In particular, PC1 scores, which explain 33% of the variance, were significantly different among the groups, with the trained group closer to the pre-training group than the non-trained group. The maximal forward position of foot and ankle angle amplitude are shown as representative examples of kinematic parameters, which were significantly different between pre-training and trained groups, but are closer than in the nontrained group as well as PC1 scores (**Figure 4E**).

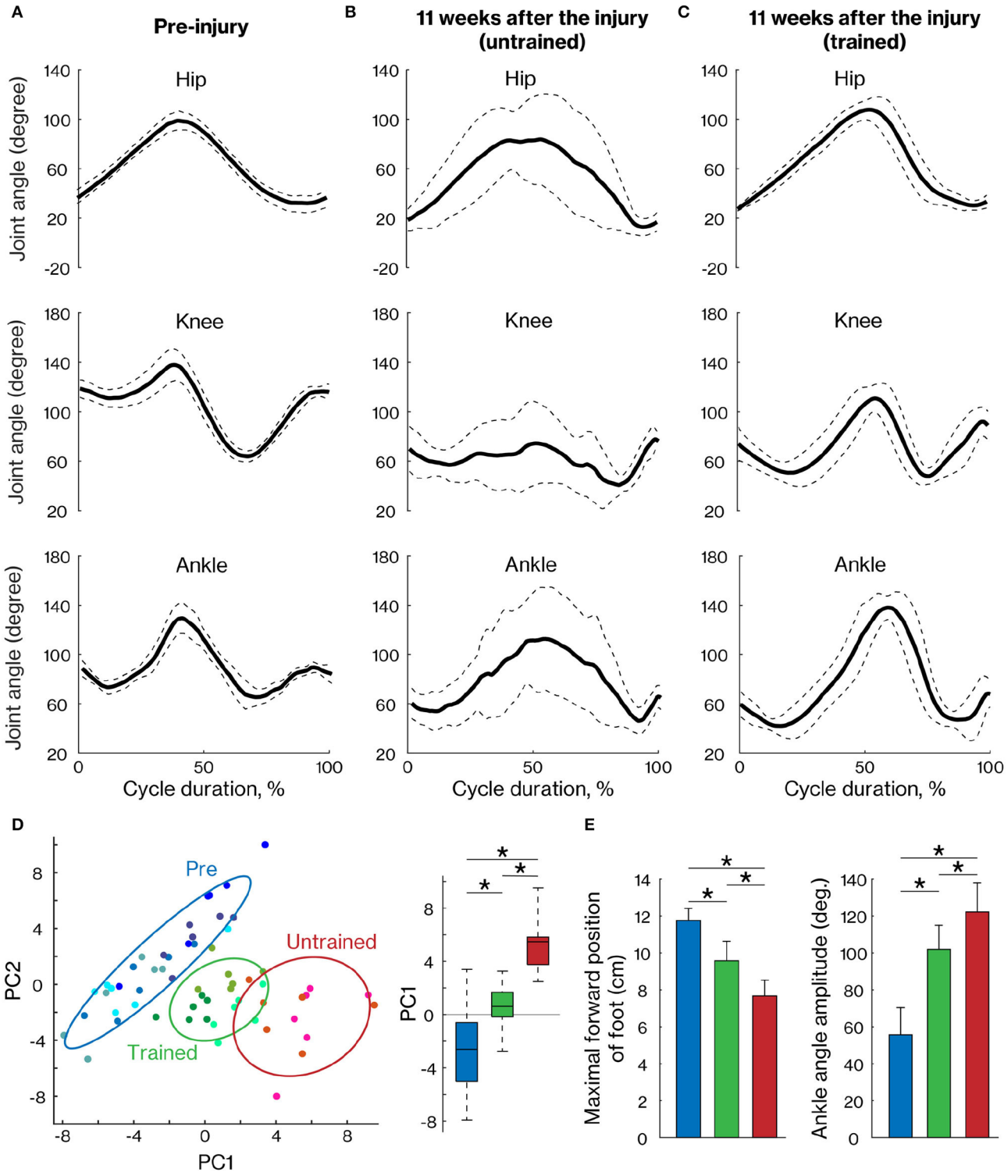

FIGURE 4 | Comparison of the right hindlimb locomotor pattern between trained and untrained groups. The mean (±SD) of angular excursion of the three right hindlimb joints during quadrupedal locomotion in pre-injury **(A)** and 11 weeks after injury in untrained **(B)** and trained groups **(C)**. The solid and dotted lines indicate the mean and SD, respectively. **(D)** The plane was created by PC1 and PC2 (left) and also by the box plots of PC1 scores (right), which were calculated with 47 kinematic parameters. Each plot indicates each cycle data. Least-squares circles help visualize the clusters. Blue, green, and red indicate the pre-training, trained, and untrained groups, respectively. **(E)** Representative kinematic parameters with a high loading factor of PC1. * < 0.05; one-way ANOVA followed by Tukey *post hoc* comparison.

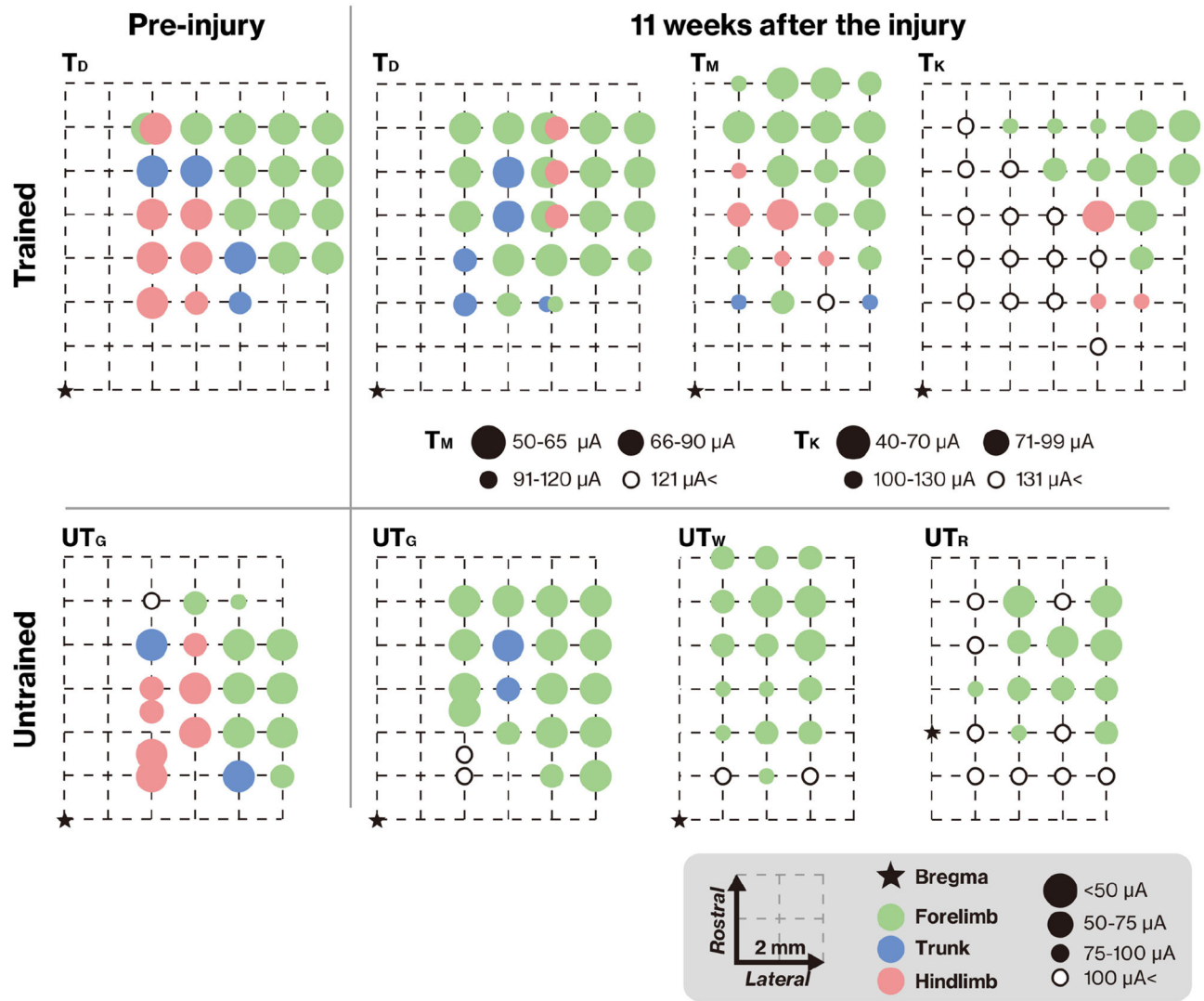

FIGURE 5 | Training-induced changes in the topographic map. The topographic map represents the electrode penetration sites. The corresponding positions of the electrodes are displayed as colors indicating the body territory activated under the lowest intracortical microstimulation (ICMS) current that still causes a movement. The results show that hindlimb movements could be evoked at positions close to the bregma in both T_D and UT_G (left column). Pre-injury ICMS maps are shown in the right panel. The hindlimb areas in the trained group remained unchanged (upper panel), but after the lesion, the hindlimb areas on the cortical surface in the untrained group were diminished (lower panel). In both groups, the M1 forelimb areas remained after the lesion. Note that different intensities were used for T_M and T_K.

Training-Induced Changes in Connectivity Between the Motor Cortex and Lumbar Spinal Cord

To verify connectivity between the motor cortex and lumbar spinal cord across the lesion, we analyzed the representative hindlimb area of the motor cortex using ICMS. In T_D and UT_G, ICMS was also performed before the lesion (**Figure 5**, left column) to verify the reorganization of motor maps. The motor maps derived from the ICMS of this study were consistent with those of previous reports (Burish et al., 2008; Burman et al., 2008); the hindlimb motor area was located in the medial part of the motor cortex. Eleven weeks after injury, the hindlimb areas decreased in the untrained group (**Figure 5**, right panels). In contrast, in the trained group, hindlimb muscle movements were elicited by ICMS. In the T_M, the emerged hindlimb area seems to be the original hindlimb area; however, in T_D and T_K, hindlimb muscle movements were not elicited in original hindlimb areas and were elicited in ectopic areas (i.e., in the original forelimb area). This indicates that training promotes reorganization of the connection between newly emerged hindlimb areas and the lumbar spinal cord.

Histological Analysis

To demonstrate the effects of rehabilitation on the prevention of demyelination or the promotion of remyelination after SCI, LFB staining was performed 11 weeks after injury

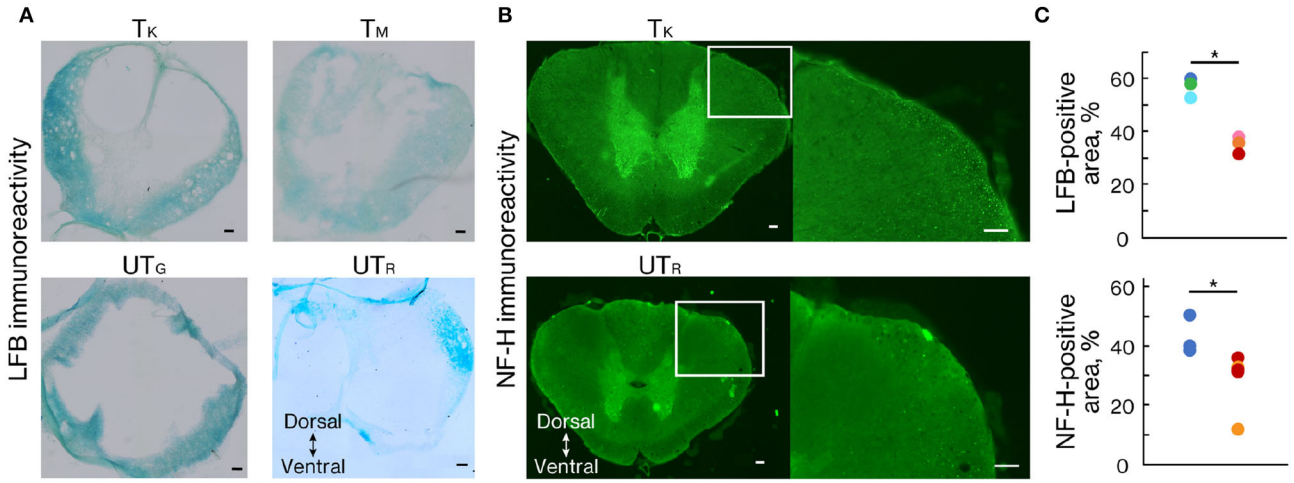

FIGURE 6 | Rehabilitation enhanced myelinated areas and fibers. **(A)** Luxol fast blue (LFB) and **(B)** NF-H staining 11 weeks after injury. **(C)** Percentage of the positive area in trained ($n = 3$) and untrained ($n = 3$) groups. The LFB-positive area was significantly more prominent at the lesion epicenter in the trained group than in the untrained group. The NF-H-positive area was significantly larger in the trained group than in the untrained group. Conventions are the same as in **Figure 4D**. *$p <$ 0.05; Welch's t-test. Scale bars = 100 μm.

(**Figure 6A**). LFB-positive areas within the lesion epicenter were significantly larger in the trained group than in the untrained group (**Figures 6A,C**; $p < 0.01$). We also performed an immunohistochemical analysis of NF-H to quantify the neuronal fibers passing through the lesion related to locomotor recovery. The NF-H-positive areas were comparable between the two groups in animals with chronically injured spinal cords at all tested levels (**Figures 6B,C**; $p < 0.05$).

DISCUSSION

In this study, we developed a contusive SCI model with therapeutic intervention for locomotor rehabilitation in nonhuman primates. ICMS elicited hindlimb movement in the trained group, and significant locomotor recovery was detected on both the original open-field scale and kinematics analysis. Histological analysis showed that marmosets in the training group had less tissue damage than those in the non-training group, suggesting that there might be a neuroprotective effect.

Rehabilitation regained the connection between supraspinal input and the lumbar spinal cord. Previous studies have shown that rehabilitative training causes the secretion of neurotrophic factors, such as brain-derived neurotrophic factors (BDNF; Fouad and Tetzlaff, 2012). BDNF is involved in neuroprotection, axonal sprouting, and regeneration (Kafitz et al., 1999; Vavrek et al., 2006). Although the present study did not assess these factors because of the small number of samples, our histological findings with larger myelinated and axonal areas in the trained group indicate that these trophic factors may be upregulated and promote neural protection.

As the recovery of locomotor functions depends on the preservation of supraspinal input (Cote et al., 2017), the degree of connection between supraspinal input and the lumbar spinal cord is also a possible mechanism for recovery in this study. Most clinical studies examining the influence of the supraspinal tract have been performed using transcranial magnetic stimulation of the motor cortex (Kamida et al., 1998; Friedli et al., 2015), and ICMS (Alstermark et al., 2004; Schmidlin et al., 2004), leading to preferential activation of the CST (Yang and Gorassini, 2006). In this study, the effect of supraspinal input was tested by ICMS, and the muscle activity of the lower limbs was induced in the training group (**Figure 5**). To date, there is no evidence to address the changes in the functional map induced in the hindlimb representation of the motor cortex in primates. In rodent models, it is generally accepted that the expansion of motor maps is related to functional recovery (Fouad and Tetzlaff, 2012) and can be facilitated by rehabilitative training (Girgis et al., 2007; Ishida et al., 2016). The main reason for this restoration may be increased collateral sprouting of CST fibers in parallel with cortical map changes (Fouad and Tetzlaff, 2012). Because primate species have greater potential for CST regeneration than rodents do (Friedli et al., 2015), the cortical map changes that occurred in trained marmosets in our study may have been caused by CST plasticity, which in turn may have contributed to motor functional improvement. However, our results showed that the restoration of corticospinal connectivity did not directly indicate the sprouting and regeneration of a CST. For example, a study of rehabilitation after CST injury in rats showed that muscle movements were elicited by ICMS, *via* the cortico-rubral tract (Ishida et al., 2016). To validate CST plasticity, it is necessary to visualize CST using neural tracers.

A Severe Contusion Model With Rehabilitation in Marmosets

Nonhuman primates may provide many advantages in investigating the safety and efficacy of various therapies

to promote functional recovery after SCI. In the field of rehabilitative areas, several primate studies have examined the effectiveness of using incomplete transection models to test the effects of a treatment that promotes sprouting from spared axons and, perhaps, axon regeneration (Cote et al., 2017). However, these transection models do not resemble human SCI and are therefore not clinically relevant (Courtine et al., 2007; Kwon et al., 2011). Hence, it is important to establish rehabilitation methods in primate contusion models to obtain clinically applicable knowledge.

Furthermore, a few contusive SCI studies have been carried out in large primates because they are difficult to handle, labor-intensive, and require indispensable skilled daily care (Babu and Namasivayam, 2008). In addition, weight-supported treadmill rehabilitation requires an extremely hard step of putting a weight support jacket on monkeys and some other special rehabilitative setups (Krucoff et al., 2017). On the other hand, small marmosets are easy to handle, care for, and rehabilitate (Okano, 2021). This advantage over the use of macaques enabled the development of a rehabilitation model for SCI.

In general, the recovery effect is limited if rehabilitation therapy is applied alone, and its efficacy depends on the severity of the injury. Patients with motor incomplete SCI (AIS C or D) showed some improvement in overground walking (Cote et al., 2017), while patients with more severe injury (AIS A and B) showed increased electromyographic amplitude and improved mutual activation of the extensor and flexor muscles, but no recovery of independent stepping ability after rehabilitation (Dietz et al., 2002; Forrest et al., 2008). These studies suggest that functional recovery after a severe SCI requires a combination of rehabilitation and biological repair strategies, such as pharmacological (Ito et al., 2018) and stem cell therapies (Shinozaki et al., 2021; Tashiro et al., 2021).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Research Committee of Keio University School of Medicine.

AUTHOR CONTRIBUTIONS

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

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Advancing Peripheral Nerve Graft Transplantation for Incomplete Spinal Cord Injury Repair

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Peripheral nerves have a propensity for axon growth and regeneration that the central nervous system lacks (CNS). However, CNS axons can also grow long distances if introduced to a graft harvested from a peripheral nerve (PNGs), which is the rationale for using PNGs as repair strategy for injuries of the spinal cord. From a clinical perspective, PNGs provide interesting possibilities with potential to repair the injured spinal cord. First, there are numerous options to harvest autologous grafts associated with low risk for the patient. Second, a PNG allow axons to grow considerable distances and can, by the surgical procedure, be navigated to specific target sites in the CNS. Furthermore, a PNG provides all necessary biological substrates for myelination of elongating axons. A PNG can thus be suited to bridge axons long distances across an injury site and restore long tracts in incomplete SCI. Experimentally, locomotor functions have been improved transplanting a PNG after incomplete injury. However, we still know little with regard to the formation of new circuitries and functional outcome in association to when, where, and how grafts are inserted into the injured spinal cord, especially for sensory functions. In this perspective, we discuss the advantages of PNG from a clinical and surgical perspective, the need for adding/repairing long tracts, how PNGs are best applied for incomplete injuries, and the unexplored areas we believe are in need of answers.

Keywords: spinal cord injury, peripheral nerve graft, axon regeneration, repair strategy, chronic injury, sensorimotor functional recovery

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INTRODUCTION

Spinal cord injury (SCI) affects millions world-wide and each year there are more than 500 thousand new cases, caused by accidents, violence, and surgical intervention (National Spinal Cord Injury Statistical Center, 2021). In comparison to many other disorders, the functional losses as a consequence of the injury remain permanent and there is no clinically available therapeutic treatment. Most injuries are incomplete, with partial loss of function below the injury. Hence, the loss of function among the majority of patients can be quite heterogeneous, which is further exaggerated by difference in the potential for spontaneous recovery depending on the injury location and the secondary sequalae that hinders repair (Kjell and Olson, 2016). Despite some spontaneous recovery in the first months after an incomplete injury, most of the initially lost functions will remain as chronic deficits (Fawcett et al., 2007; Kirshblum et al., 2021). Development

of potential new treatments for chronic injuries are in the minority, while most interventions are aimed at the acute and subacute stage. For clinical translation acute-subacute treatments are very challenging as large groups of patients must be studied due to highly variable outcome in patients with similar acute symptoms. There are also ethical concerns about asking newly injured, often desperate patients, to participate in clinical trials. Hence, there is a need for treatments that can address obstacles and complexities associated to various chronic conditions.

Although the tracts and neurons of the spinal cord have a fair ability for shorter regeneration and plasticity, it is important to keep in mind that functional recovery remains dependent on spared long tracts that maintain adequate conductive properties (Rossignol, 2006; Chew et al., 2012). Therefore, we believe it is fair to hypothesize that a combination of circuit rearrangement and an addition of long tracts would maximize the potential for recovery of functions below the injury site. However, long distance growth within the injured spinal cord is a challenge that seems difficult to overcome (Chew et al., 2012). Given the original spinal circuitry architecture, 10 cm and beyond of robust axonal growth may be needed for directly targeting and providing any recovery of specific functions. One of the few treatment interventions that have shown the potential of providing this type of repair is transplantation of long axon growth-promoting peripheral nerve grafts (PNGs) into the spinal cord (Côté et al., 2011). However, although it has unique potential, several details regarding the new circuitry formation and function, outcome as a result of various surgical placements, and application at a chronic stage remain undetermined. In this perspective, we wish to discuss the need for adding/repairing long tracts and also put it in the context of the last decades progress with regard to our understanding of spontaneous recovery and circuit rearrangements. Also, we will discuss the advantages of PNGs from a clinical and surgical perspective, explain our perspective of the most advantageous application of PNG for incomplete SCIs, and highlight unexplored areas we believe are in need of answers.

The Obstacle at the Center of It All

The uninjured human spinal cord carries sensorimotor signals back and forth from the periphery to the brain through long and short neuronal connections through the of 43–45 cm length of the spinal cord. Axons and dendrites of the spinal cord typically only regenerate short distances, while the nerves in the periphery can heal great lengths (Scheib and Höke, 2013). In the injured spinal cord, there will be progressive scarring at the injury site that inhibits regeneration and becomes a wall that the spinal circuitry has to circumvent for any spontaneous recovery and regeneration (Norenberg et al., 2004; Silver et al., 2014; Kjell and Olson, 2016). In many cases, if there is edema at the injury site, a cyst may develop instead or as an addition to the scar. The injury site therefore remains a chronic obstacle that will vary in form and shape for each individual that sustained an incomplete injury. Our observations delineate that the injury site in traumatic injuries typically span 1.5–6 cm in length, whether the injury is complete or incomplete (Frostell et al., 2012). Hence, therapeutic transplant has to be able to adjust to various lengths of the injury, while enabling long axonal regeneration or long neuronal relay connections within the spinal tissue. This is a fact that makes injury site-directed surgical interventions complicated. The components of the spinal scar have been extensively reviewed elsewhere and although experimental treatments that are promising for dealing major inhibitory components, regeneration of tracts for longer distances within the spinal cord remain a daunting task (Silver et al., 2014; Bradbury and Burnside, 2019). Instead of attempting to bring long tracts through the scar, taking axons around the injury site and past long stretches of spinal cord tissue would be a way to provide repair of sensorimotor functions below the injury site. In fact, this type of repair occurs after incomplete injuries when spared tracts or long propriospinal circuitry take over some of the functions previously performed by severed tracts during the spontaneous recovery that ensues during the first year after injury (Rossignol, 2006; Fawcett et al., 2007; Kirshblum et al., 2021).

Spontaneous Recovery and Intact Long Circuitry

In the last two decades, we have made many advances in our understanding of the neurobiology behind spontaneous recovery after SCI. After an incomplete SCI, there are great amounts of new shorter sprouting, plasticity, and compensation both in propriospinal and supraspinal circuitry (Bareyre et al., 2004; Courtine et al., 2008; Flynn et al., 2011; Zörner et al., 2014). Firstly, after incomplete injuries new intraspinal relay circuits provide spontaneous recovery (Bareyre et al., 2004; Courtine et al., 2008). Secondly, spared axons may sprout new collaterals connecting to adjacent circuitry (Hagg, 2006). New connections may also form at various levels supraspinally to adapt to new input (Zörner et al., 2014). Lastly, there is synaptic plasticity that adapts to functional need and new signaling. Overall, this allows signals and functions to relocate to new circuitry setups. Recently, it has been found that locomotor recovery after an incomplete injury is also dependent functional local proprioceptive feedback circuits at various levels below the injury (Takeoka et al., 2014; Takeoka and Arber, 2019). Nevertheless, spontaneous functional recovery after incomplete SCI remains dependent on intact longer tracts that take signals to and from the brain.

Importantly, the spontaneous recovery after SCI is in itself limited, primarily demonstrated by the chronic loss of function by patients with SCI. However, it is possible to enhance process associated to spontaneous recovery and promote further functional repair (Duffell and Donaldson, 2020). The classic way of enhancing functional ability is through, often arduous, rehabilitation programs (Fouad and Tetzlaff, 2012). Lately, using our understanding of these systems and how electric stimulation can activate them, we may in the future reliably push spontaneous recovery further with the circuitry architecture that remains (Wagner et al., 2018). Nevertheless, the ability to enhancing spontaneous recovery also depend on various intact and long circuitry in the spinal cord. To what degree a tract, either extending supraspinally or part of the propriospinal network, can compensate and support functional signaling, and

whether there is a ceiling or diminishing return is unknown. However, it has been reported that either spared or new connections comprising about 5–10 percent of the original tract can compensate for large parts of the original function (Hollis et al., 2015). Hence, regenerating or adding even a smaller part of long reaching tracts may potentially provide valuable functional recovery.

Peripheral Nerve Graft Repair

Experimentally, long circuitry reconstruction after SCI has only been achieved with PNGs transplantation (Côté et al., 2011). This unique position rests on the fact that axons of the central nervous system (CNS) can grow great lengths in a peripheral nerve, very much similar to the repair seen with peripheral axons (Scheib and Höke, 2013). PNG as a method for spinal cord repair and its history has been reviewed in detail by Houle and colleagues and we will focus on describing some key concepts here (Côté et al., 2011). Importantly, the axons in the PNG will be myelinated by the Schwann cells, in contrast to many regenerating axons in the spinal cord that remain unmyelinated or poorly myelinated (Tan et al., 2007; James et al., 2011). The grafts are autologous and do not need additional immunosuppression to avoid graft rejection. Furthermore, surgical placement of a PNG can reduce the distances axons need to travel when entering or exiting the graft. Axons entering and exiting the graft can be increased by improving the microenvironment around the insertion points with various co-treatments, such as adding FGF1 in the tissue glue used during grafting (Cheng et al., 1996; Lee et al., 2008; Nordblom et al., 2012). Therapeutic PNG techniques have primarily been advanced toward clinical application as a treatment intended for complete SCI (Nordblom et al., 2009; Frostell et al., 2018).[1] In injuries with no function below the injury site, the transplantation involves first completely removing the spinal scar and then reconnecting the disconnected spinal cord with several short PNG assigned in locations that will direct spinal tract regeneration from white matter into the opposing spinal gray matter (Cheng et al., 1996). In the case of incomplete SCIs, the intervention typically involves grafting one end of a peripheral nerve at or close to the injury site and the severed tracts, and the other end close to neuronal targets. This effectively makes the PNG a bridge that may allow spinal cord axons access to distant locations. In this manner, a single PNG transplanted as a "bridge" connecting spinal locations two spinal levels apart has been shown effective in improving either locomotor or respiratory recovery (Houle et al., 2006; Alilain et al., 2011). However, advancement of various other transplant strategies have reached a stage where it is reasonable to believe they may also be able to provide some repair in motor functions two spinal sections below the injury (Rosenzweig et al., 2018). Nevertheless, we would like to argue that PNG still is an interesting repair strategy for incomplete injuries with its unique capability to provide long axon growth-promoting bridges that can be surgically placed at various location on the spinal cord. However, there are still several gaps in our knowledge of using long PNGs "bridges" for repair of various types of incomplete spinal cord injuries.

Peripheral Nerve Graft for Incomplete Injuries

For incomplete injuries, surgery and PNG transplantation would be performed in an individual with some functions remaining below the injury site. Prior to a surgical proceedure associated to any such injury, a proper assessment of the injury-outcome, the potential benefits, and risks would be necessary. Such an assessment, asserted with great confidence, is only really feasible in the chronic stage. Therefore, we believe implementation of a surgical intervention such as PNG would be most practical and reasonable in the chronic state, when spontaneous recovery has ended and scarring has matured. We reason that any application in humans would depend on minimizeing transplantation-associated injury by positioning one end of the graft at the border of the scar, while the other end would be grafted close to gray matter either sub-pially or longitudinally between white matter tracts. With this method, the scar is bypassed and tissue damage to the spinal cord is minimized. Nevertheless, the small injuries to the spinal cord associated to the transplantation may be considered a draw back and an unavoidable potential risk. Notably, experiments in rodents suggest removing the scar may be possible without functional harm (Sandrow et al., 2008). Although, removing the spinal scar in an incomplete injury would be clinically unfeasible today, it may be considered an acceptable risk in the future if proper techniques are developed that can, with certainty, delineate the spinal scar borders. Lastly, we deem it possible to surgically place the graft itself in the spinal column, above or below the dura, so it remains protected. With regard to the various steps in the surgery method, it could be advantageous to develop the surgical application in large animal models.

From the clinical stand-point, an important advantage of using PNG to bypass the injury site is that the surgical intervention can be adapted depending the injury of the individual. The intervention can be adjusted based on the injury location, results from neurological examination, and the intended area of functional recovery. We have shown in a previous study that the upper and lower level of an injury can be determined with high precision in the thoracic spine (Frostell et al., 2012). In order to extract such information, we used standard neurophysiological evaluation in combination with MRI. MRI alone will not give this information since it doesn't tell anything about the functional status of the cord. We believe that functional data is valuable when any surgical approach to the injured cord is considered. In particular, when transplantation procedures like cell therapy or corresponding is considered to minimize risk by having precise information about the level of injury mapped before surgical intervention. Technically, we solved this using a bipolar needle for EMG registrations stepping through all intercostal muscles. Voluntarily activated motor units were registered above the injury, thus giving data on the upper level. Normal motor units can also be generated below the injury by inducing spasticity in the in lower limbs in parallel with

[1] https://clinicaltrials.gov/ct2/show/NCT02490501

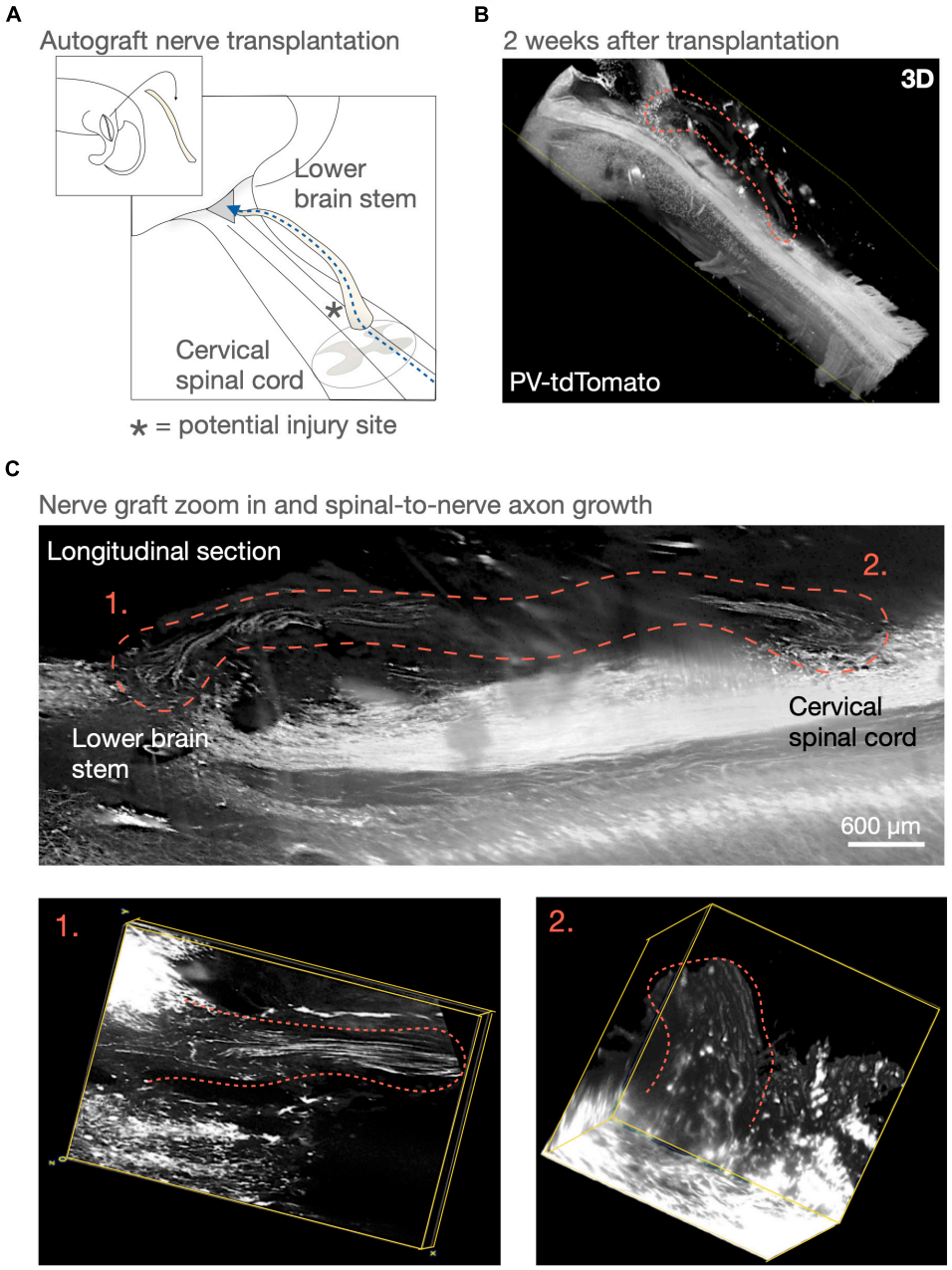

FIGURE 1 | Peripheral nerve graft (PNG) and repairing the sensory tracts of the dorsal column. In 1981, David and Aguayo (1981) demonstrated long distance axon growth using a peripheral nerve as a bridge for dorsal column axons. This experimental model aimed at demonstrating long-distance regeneration. However, using one or more PNGs as an axon bypass in a similar manner could be a potential repair strategy for incomplete spinal cord injuries that wouldn't necessitate removing the spinal scar. **(A,B)** Here in a similar surgery model to David and Aguayo's (1981) PNG "bridge" model, we visualize parts of the sensory tracts of the dorsal column in the lower brain stem and cervical spinal cord using a parvalbumin-reporter mouse (PV-tdTomato mouse). **(C)** An autologous PNG is transplanted and 2 weeks after transplantation we can visualize axons throughout the PNG. To follow axons throught the cord in 3D was not possible at the time of Aguayo's (1985) experiment and our understanding of using PNG as a repair strategy for the spinal cord has advanced. Such techniques and knowledge may today allow us to obtaining necessary answers for potentially considering advancing PNG transplantation as a long-distance axonal tract repair for incomplete spinal cord injuries.

EMG registration, which allows mapping of the lower border of the injury zone.

With the clinical application in mind, questions regarding the potential for functional repair would need an experimental model that aims to reconnect long distances and remove/injure less tissue than done before, while primarily performing transplantations in the chronic stages (Houle, 1991; Alilain et al., 2011; Côté et al., 2011; see Figure 1). Among the first PNG experiments in the spinal cord, David and Aguayo (1981) inserted a PNG bridging the sensory tracts of the dorsal column of the

cervical spinal cord back up to the cuneate and gracile nuclei of the brains stem (David and Aguayo, 1981; Aguayo, 1985). The PNG allowed, at that time, unprecedented growth from adult CNS axon and it also encouraged the idea that parts of severed spinal tracts perhaps could be restored. Although functional regeneration has been demonstrated for motor function since then, it still remains to be demonstrated that PNG can restore sensory function in an incomplete SCI (Houle et al., 2006). For investigating such a sensory axon bypass, a similar model used by Aguayo et al. could be advantageous (see **Figure 1**). Notably, the dorsal column lends itself well to various modern imaging and trancing tools (Attwell et al., 2018; Susaki et al., 2019) (**Figure 1**). Moreover, and clinically incomplete SCI often involve the dorsal columns of the spinal cord. Also, to recover some sensory function below the injury could be valuable, since it may for example allow individual with SCI to prevent pressure ulcers. Furthermore, the clinical experience concerning injuries of the dorsal column involves severe neurological deficits with significant gait disturbance (Nathan, 1994). In rodents, the functional loss is also severe initially. However, with time, rodents recover and compensate for most of the functional loss. Recently, it was discovered that this recovery is due to approximately 6% of the dorsal column tracts that sprout ventrolaterally to intact tracts that are part of the same circuitry network and also reaches the thalamus in the brain (Hollis et al., 2015). Putting PNG in context of this type of spontaneous recovery will be important for its implementation and it will be necessary to understand how and if this affects the propensity for a PNG to provide functional recovery in the context of a chronic injury.

A robust model of PNG transplantation for incomplete injury will allow investigation of other important questions regarding the details of axon growth through the graft and functional connectivity at the nuclei. After Aguayo's (1985) original experiments, it was found that axons in the graft originated both from axons of the ascending tracts and local propriospinal neurons close to the graft insertion point (David and Aguayo, 1981; Aguayo, 1985). There were both axons running up and down the graft and their identity seemed varied from electrophysiological measurements. Today, new PNG surgery techniques have been developed, for example using different grafting time-points for the caudal and rostral end of the inserted graft (Houle et al., 2006; Alilain et al., 2011; Côté et al., 2011), which can reduce the number of axons in the opposite direction. In the successful experiments with complete SCI, the graft was apposed the white matter and directed onto gray matter (Cheng et al., 1996). Whether precise apposition of PNG onto white matter tract such as the dorsal column and other end directly apposed the gray matter of the cuneate or gracile nuclei will provide more axon growth from primarily axonal tracts, rather than local neurons, is unknown. Furthermore, the neuronal identity of axons entering and exiting the graft, as well as functional connectivity and conduction properties, will need to be studied in detail. Whether graft source makes a difference and in what ways graft length makes a difference also remains to be studied.

Lastly, with the answers to the above questions in one model system, the crucial conceptual question of; "Does circuitry repair using PNG vary for different tract and/or at different levels?" remains. Hence, comparing the outcome of PNGs for some different and clinically relevant incomplete injuries is necessary. It would provide answer to the overall question of; What could surgical PNG treatment mean for functional recovery of incomplete injuries? One thing to consider here is, given what we have learned from spontaneous recovery and introducing new relay neurons, that it may not be necessary to repair tracts in their original form in order to provide robust functional improvements (Courtine et al., 2008; Fischer et al., 2020; Zavvarian et al., 2020). By understanding the potential or need for relay circuitry for PNG better, the grafts technique may be found useful in unexpected ways. Perhaps, spinal locations distant to the injury site can be reconnected by simply reaching other tracts able to pass the injury site. Or perhaps PNG in combination with growth prone-relay circuits derived from transplanted neural stem cell can provide better outcome, especially in a chronic SCI (Rosenzweig et al., 2018; Fischer et al., 2020). Furthermore, combining PNG with exercise already proven beneficial (Houle and Côté, 2013). Hence, site-directed electrical stimulation may be a way to boost both axon growth and new circuitry formation with PNGs (Jack et al., 2020).

CONCLUSION

Peripheral nerve graft as an experimental therapeutic treatment for SCI has demonstrated that it can provide improved recovery even for incomplete injuries. Transplantation of PNGs has a unique potential to bypass damaged long tracts, providing myelination, and be surgically directed toward specific sites of the spinal cord. Hence, it could potentially provide a functional recovery for patients simply not possible with other experimental treatments. However, we believe that this implementation and its advancement toward clinical application hinges on addressing some conceptual questions and developing of appropriate surgery. Nevertheless, given that the above discussed knowledge gaps are addressed in the future we believe that a very large group of individuals would benefit from the type of repair that PNG implants may offer.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by the regional Animal Ethics Committee in Stockholm.

AUTHOR CONTRIBUTIONS

JK and MS wrote the perspective. MS conceived the project and performed surgery. JK conceptualized and planned the project

and performed tissue analysis. Both authors contributed to the article and approved the submitted version.

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REVIEW
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The Role and Modulation of Spinal Perineuronal Nets in the Healthy and Injured Spinal Cord

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Rather than being a stable scaffold, perineuronal nets (PNNs) are a dynamic and specialized extracellular matrix involved in plasticity modulation. They have been extensively studied in the brain and associated with neuroprotection, ionic buffering, and neural maturation. However, their biological function in the spinal cord and the effects of disrupting spinal PNNs remain elusive. The goal of this review is to summarize the current knowledge of spinal PNNs and their potential in pathological conditions such as traumatic spinal cord injury (SCI). We also highlighted interventions that have been used to modulate the extracellular matrix after SCI, targeting the glial scar and spinal PNNs, in an effort to promote regeneration and stabilization of the spinal circuits, respectively. These concepts are discussed in the framework of developmental and neuroplastic changes in PNNs, drawing similarities between immature and denervated neurons after an SCI, which may provide a useful context for future SCI research.

Keywords: perineuronal nets, CSPGs, spinal cord injury, plasticity, stability, ChABC

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INTRODUCTION

The proper functioning of the nervous system strongly depends on a balance between plasticity and stability. On the one hand, plasticity is needed in both development and adulthood to constantly adapt to changing physiological demands and environmental stimuli. In contrast, stability is required to sustain appropriate network connectivity throughout life. In the mature nervous system, these two processes are dynamically controlled since their imbalance, in either direction, can lead to pathological outcomes (Takesian and Hensch, 2013). In the spinal cord, those spinal networks involved in the respiratory and locomotor control are a good example of this plasticity-stability trade-off. Both spinal circuitries produce stable and synchronized synapses to ensure the generation of an appropriate output: breathing or walking. Nevertheless, considering the environmental challenges that can perturb normal locomotion and respiration, a degree of flexibility is needed to permit correct reorganization of these networks, without leading to instability. This synaptic regulation is complex and not only requires the interplay of neurons and glial cells, but also the extracellular matrix (ECM), establishing the so-called tetrapartite synapse (Dityatev and Schachner, 2003; Dityatev and Rusakov, 2011). Particularly, there is a highly condensed ECM called the perineuronal net (PNN) that surrounds some neurons in the central nervous system (CNS) with a widely known role in synaptic stabilization. This reticular

net dynamically controls plasticity and stability processes by rearranging their structure in an activity-dependent manner (Kalb and Hockfield, 1988; Dityatev et al., 2007). Although they were first described by Camillo Golgi in 1898, using a modified recipe of his silver impregnation technique (Celio et al., 1998), interest in PNNs was tempered until about a century later. Initially, his discovery was controversially discussed and finally dismissed by Ramón y Cajal who argued that PNNs were an artifact of his preparation. Thus, PNNs research was ceased until 1966, when histological techniques gradually improved and enabled researchers to visualize again PNNs using periodic-acid-Schiff (PAS) staining (Glegg and Pearce, 1956), lectins (Brauer et al., 1984), colloidal iron hydroxide (Seeger et al., 1994), and finally, specific antibodies to detect chondroitin sulfate proteoglycans (CSPGs) (Härtig et al., 1994). These histological advances helped to understand PNNs composition, but it was not until the twenty-first century that the role of PNNs in plasticity regulation was reported (Pizzorusso, 2002). Later, the interest in PNNs exponentially increased, and, in 20 years, PNNs have been linked to many other functions in the healthy and diseased brain.

Their presence in the spinal cord was firstly described in the cat in 1983 (Hockfield, 1983). Nevertheless, the function of spinal PNN was neglected compared with the encephalic ones. It is possible that the application of ChABC, an enzyme that degrades CSPGs, at the injured spinal cord to overcome the inhibitory environment created by the glial scar (McKeon et al., 1991) has blinded us to other roles of spinal PNNs under normal conditions due to ChABC impressive efficacy. Somehow, the presence of PNNs around denervated neurons far from the injury site becomes a barrier to axonal reinnervation and therefore, to functional recovery in SCI models (Massey et al., 2006; Alilain et al., 2011). However, recent research has revealed several differences between cortical and spinal PNNs regarding their composition (Vitellaro-Zuccarello et al., 2007; Irvine and Kwok, 2018), modulation (Smith et al., 2015), and the type of neurons surrounded by PNNs (Irvine and Kwok, 2018). These divergences have increased the interest in spinal PNNs and raised concern about the assumption that cortical and spinal PNNs play similar roles in both anatomical locations.

Overall, this review is focused on describing the role of PNNs in the CNS, highlighting the current knowledge of spinal PNNs and their relevance after an SCI.

PERINEURONAL NETS

Distribution

Perineuronal nets are specialized ECM that encapsulate the soma and the proximal dendrites of some neurons in the CNS. This mesh-like structure has been described in various mammals [e.g., rodents (Kalb and Hockfield, 1988; Galtrey et al., 2008), cats (Hockfield, 1983), dogs (Atoji et al., 1997), sheep (Härtig et al., 2017), primates (Mueller et al., 2016), and humans (Jäger et al., 2013)] and non-mammal species [e.g., fish (Takeda et al., 2018), birds (Balmer et al., 2009), frogs (Gaál et al., 2014), and chickens (Morawski et al., 2009)]. Among all of them, spinal PNNs have been characterized in fish, rodents, cats, primates, and humans.

Perineuronal nets are irregularly distributed throughout the brain (Brückner et al., 1996) and spinal cord (Vitellaro-Zuccarello et al., 2007). In the brain, they mainly ensheath fast-spiking, gamma-aminobutyric acid (GABA)ergic parvalbumin (PV) interneurons (extensively reviewed in Härtig et al., 1992; van't Spijker and Kwok, 2017). However, PNNs also surround pyramidal neurons in the hippocampus (Carstens et al., 2016), visual, somatosensory and motor cortex (Hausen et al., 1996; Alpár et al., 2006), PV-positive and PV-negative neurons in the striatum (Lee et al., 2012), and excitatory neurons in the amygdala and deep cerebellar nucleus (Carulli et al., 2006; Morikawa et al., 2017). PNNs' heterogeneity increases even more between species. For instance, while PNNs are mainly found in sensory brain areas in the rat, primates present a larger proportion of neurons with PNNs in motor areas (Mueller et al., 2016).

Along the spinal cord, PNNs surround motoneurons (MNs) and spinal interneurons (**Figure 1**). Remarkably, there are differences in the proportion of PNN-enwrapped neurons throughout the spinal laminae (**Figure 1B**). In the dorsal horn, 20% of neurons present PNNs and specifically, none in the laminae I and II (Galtrey et al., 2008). The lack of PNNs in those laminae correlates with the grade of synaptic plasticity found in that region after injury, which is related to neuropathic pain development (Woolf et al., 1992). In contrast, in laminae VII and VIII, 50% of neurons have PNNs. This proportion has been associated with spinal interneurons including both PV- and calbindin-positive cells (Renshaw cells) (Vitellaro-Zuccarello et al., 2007). Finally, in contrast to the brain, PNNs located in the ventral horn surround large neural somas: spinal MNs (Vitellaro-Zuccarello et al., 2007; Galtrey et al., 2008). Notably, only α-MNs present PNNs in the spinal cord, unlike γ-MNs (Al'joboori et al., 2020). Possibly, the different PNNs expression around α- and γ-MNs could be explained by the electrophysiological properties of each MN type (Manuel and Zytnicki, 2019). Since PNNs formation during development is activity-dependent, the level of activity in each MN type seems to determine the presence or absence of this net. In fact, we have reported that vestigial PNNs modify the physiological properties of α-MNs (Sánchez-Ventura et al., 2021), suggesting a role in MN maturation. Considering the percentage of α-MNs enwrapped by PNNs, it was initially thought that only 30% of α-MNs had PNNs when they were stained with Wisteria floribunda lectin (WFA) (Galtrey et al., 2008). Nonetheless, selectively staining for the expression of aggrecan offers a different perspective on spinal PNNs, detecting around 80% of α-MNs with dense aggrecan-positive PNNs in rat (Irvine and Kwok, 2018) and 76% in primates (Mueller et al., 2016). These results certainly reinforce the physiological relevance of spinal PNNs.

Composition and Structure

Perineuronal nets are condensed ECM rich in CSPGs with holes at the site of synaptic contacts (Celio et al., 1998). PNNs are composed of hyaluronan (HA), CSPGs, link proteins, and tenascin-R (tn-R) (Kwok et al., 2011), which are highly organized in a ternary stable structure (**Figure 2**). PNNs' composition makes them different from the diffuse ECM, widely present

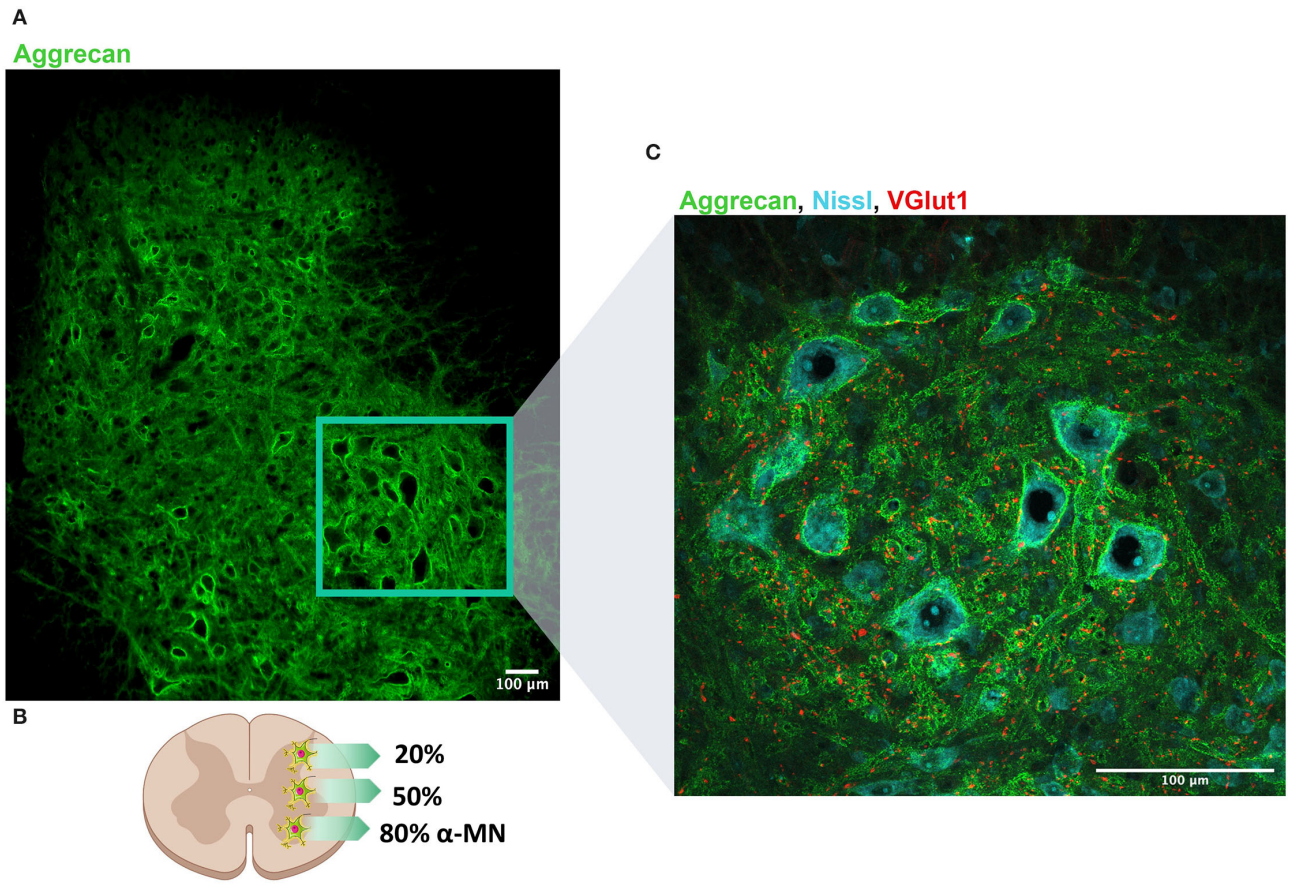

FIGURE 1 | Perineuronal nets (PNNs) distribution along the dorso-ventral axis of the lumbar spinal cord. **(A)** PNNs are labeled by aggrecan (denoted in green) and observed along the whole lumbar spinal cord. **(B)** Schematic representation of the distribution of PNNs in the spinal cord: 20% of neurons in the dorsal horn present PNNs, whereas 50% of neurons located in the intermediate column are wrapped by PNNs. Considering α-MN, around 80% are surrounded by PNN. **(C)** Magnification of the region of the ventral horn where most of MNs are localized. Neurons are labeled in blue (Nissl) and proprioceptive afferents (VGlut1) that typically project to MN are labeled in red. Scale bar: 100 um.

throughout the CNS. Although both types of ECM contain HA, tn-R, and some CSPGs, the diffuse matrix lacks link proteins.

Among all PNNs components, link proteins are crucial for their formation. *In vitro*, the lack of the Crtl1 gene, which encodes the link protein 1, prevents PNNs formation around PNN-bearing cells (Kwok et al., 2010). *In vivo*, it generates attenuated PNNs in the visual cortex (Carulli et al., 2010) and aberrant PNNs in the spinal cord, with an altered proportion of its components (Sánchez-Ventura et al., 2021). Another important element in PNNs structure is aggrecan (Rowlands et al., 2018). While mice deficient for the lecticans neurocan (Zhou et al., 2001) or brevican (Brakebusch et al., 2002) present organized PNNs, aggrecan-deficient mice show altered PNNs with no WFA staining (Giamanco et al., 2010). The importance of tn-R in PNNs formation is observed in the tn-R knockout (KO) mouse whose PNNs structure is affected in both development and adulthood (Weber et al., 1999; Brückner et al., 2000; Haunsø et al., 2000).

Most PNNs components, including aggrecan, hyaluronan synthase (HAS), and link proteins (Matthews et al., 2002), are produced by neurons, although astrocytes and oligodendrocytes also contribute to PNNs formation through synthesizing neurocan (Jones et al., 2003) and tn-R (Galtrey et al., 2008), respectively. PNNs are anchored to neurons by the enzyme HAS, which produces an HA polymer chain on the neuronal surface, constituting the backbone of PNNs. This conclusion was reached in view that HA receptors such as CD-44 are expressed in glial cells but not in neurons (Aruffo et al., 1990), dismissing the idea that HA receptors are the link between PNNs and neurons and accepting that HAS are the ones that attach PNNs to the neural membrane. In the spinal cord, HAS 3 is expressed in PNN-enwrapped neurons located in both the dorsal and ventral horn in adulthood. In contrast, HAS 1 is only expressed in the developing spinal cord (Galtrey et al., 2008).

Hyaluronan provides a scaffold for the binding of CSPGs. CSPGs are the major components of PNNs representing only 2% of all the CSPGs of the nervous system (Fawcett, 2015). Among CSPGs, aggrecan is the major constituent of spinal PNNs, and three other CSPGs (i.e., brevican, neurocan, and versican) are present in PNNs in variable degrees. All types of CSPGs are composed of two parts, namely, a core protein

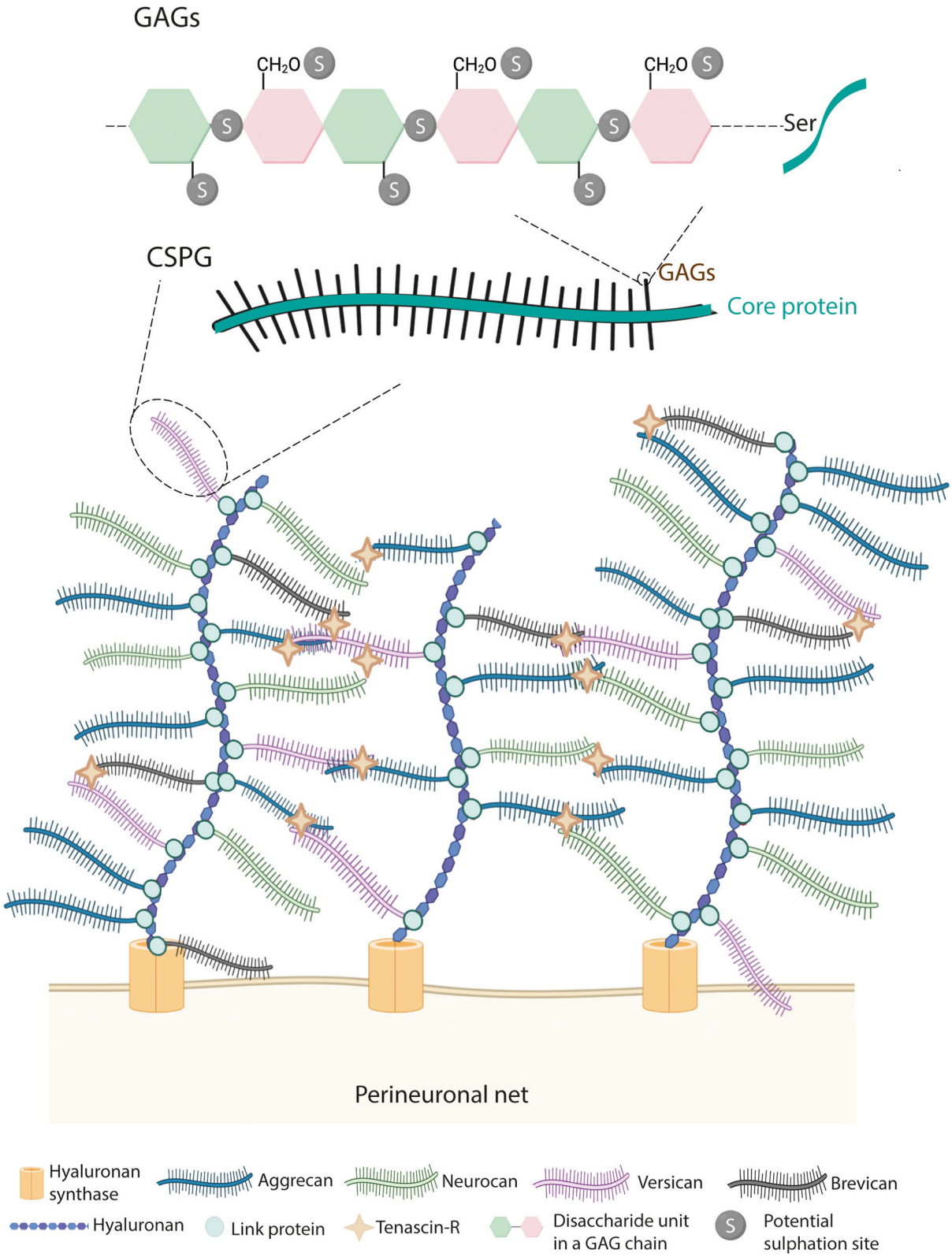

FIGURE 2 | Structure of PNNs. PNNs are composed by hyaluronan, link proteins, chondroitin sulfate proteoglycans (CSPGs) and tenascin-R. Hyaluronan, secreted by the hyaluronan synthase (HAS), binds to members of the lectican family of CSPGs (i.e., aggrecan, brevican, versican, and neurocan) *via* link proteins. Then, tenascin-R further cross-links lecticans generating a lattice-like structure. CSPGs are formed by a core protein in which GAGs are covalently attached through serine residues. GAGs are composed of disaccharide units of chondroitin sulfate chains, which can be sulfated at the 2nd, 4th, and 6th positions.

and a variable number of glycosaminoglycan (GAG) chains. The core proteins are a tridomain structure formed by a N- and C-terminal globular domain, necessary for the binding of HA and tn-R, respectively, and a central region (Yamaguchi, 2000). In the N-terminal, the interaction between HA and CSPGs is maintained by link proteins (HAPLN family genes). Although HAPLN1, 2, and 4 are found in the nervous system, in the spinal cord, only HAPLN1/Crtl1 and HAPLN4/Bral2 are described in PNN-bearing neurons (Galtrey et al., 2008). Tn-R binds to the C-terminal of the CSPGs and, thanks to its trimeric structure, facilitates the cross-link between HA and CSPGs (Lundell et al., 2004). In the central region, GAGs are covalently attached to the core protein through serine residues.

Although PNNs disposition is quite preserved, slight changes in CSPGs conformation contribute to PNNs heterogeneity. Their heterogeneity arises from the type of core protein found (Dauth et al., 2016) and/or the number and sulfation pattern of GAG chains (Miyata and Kitagawa, 2017). Antibodies against different CSPGs core protein showed a different distribution compared with the brain and spinal cord sections (Galtrey et al., 2008). The heterogeneity of the aggrecan core protein can increase even more since different glycosylation variants of this lectican also display differential distribution in different neuronal types in the CNS (Matthews et al., 2002). The GAGs can be sulfated at different positions including the 4th (CS-A), 6th (CS-C), 2nd−6th (CS-D), and 4th−6th (CS-E). Changes in this sulfation pattern can modify CSPGs charge and thus, provide binding properties to PNNs, a characteristic that also differs from the diffuse ECM (Carulli et al., 2006). Interestingly, CS-A and CS-C have different characteristics, while CS-A is inhibitory to axon growth and suppresses plasticity, CS-C is permissive to axon growth and increases plasticity (Wang et al., 2009; Miyata et al., 2012). Furthermore, this sulfation pattern may vary depending on the CNS region since WFA, which detects the non-sulfated GalNAc residues (Nadanaka et al., 2020), shows a low percentage of detection around spinal PNNs (Irvine and Kwok, 2018) compared with that in cortical ones.

Function

Although PNNs are widely known for their role in plasticity inhibition and synaptic stabilization, other functions have been attributed to cortical PNNs such as ionic buffering, neuroprotection, and neural maturation (extensively reviewed in Kwok et al., 2011; van't Spijker and Kwok, 2017; Lorenzo Bozzelli et al., 2018; Fawcett et al., 2019). PNNs functions are tightly related to the structural properties of their CSPGs. Hence, manipulation of PNN's CSPGs has a direct impact on the function of PNNs and the neuron surrounded (Gama et al., 2006). The highly negative charge of GAGs is one of the most important factors determining PNNs functions (Brückner et al., 1993), and this can be considered in several different ways:

- PNN's negative charges provide a suitable microenvironment around fast-firing neurons due to their buffering capacity of local ions. PNNs can control the diffusion of ions serving as a fast cation exchanger to provide rapid neuronal responses (Härtig et al., 1999). Besides, in the brain (Härtig et al., 1999; Carulli et al., 2006) and the dorsal and intermediate zone of the spinal cord (Deuchars et al., 2001), there is a good correlation between the expression of the potassium channel Kv3.1b, a marker of fast-firing neuron (Rudy and McBain, 2001) and PNNs. Interestingly, this is not the case for spinal MNs, and thus, PNNs function in these neurons needs to be further investigated.
- The negative charges of GAGs can buffer cations produced after oxidative stress or toxic metal ions, conferring neuroprotective capabilities to PNNs (Morawski et al., 2010; Suttkus et al., 2014).
- The negative milieu, provided by the charge, around neurons determines the membrane capacitance, which affects neural excitability (Tewari et al., 2018). Indeed, Glykys et al. (2014) found a negative correlation between PNNs intensity and internal Cl^- concentration, suggesting that PNNs are involved in setting the local Cl^- levels. Indeed, PNNs degradation resulted in increased neural excitability (Hayani et al., 2018).
- Negative charges can, directly and indirectly, inhibit neural regeneration. Although the mechanisms used by CSPGs to inhibit neural regeneration are not completely understood, the GAG chains generate a steric hinderance for regrowth. This is observed in the fibro-glial scar formed after an SCI (McKeon et al., 1999) and overcome after ChABC application (Bradbury et al., 2002). Indirectly, their inhibitory properties can also be triggered after interacting with specific receptors (Shen et al., 2009; Lang et al., 2015). Moreover, GAGs chains disulfated at the 4th−6th position facilitate the binding of the repulsive guidance molecule semaphorin 3A (Sema3A), which inhibits neural outgrowth and regeneration (Dick et al., 2013,Vo et al., 2013).

Importantly, the sulfation pattern can contribute to other neural functions such as maturation. After the binding to the PNNs through CS-E chains, the Otx2 protein can translocate into neurons and facilitate PV positive-neurons maturation (Sugiyama et al., 2008; Beurdeley et al., 2012). PNNs components also interact with ion channels and receptors and thus, regulate synaptic activity and membrane current. Tn-R binds to GABA receptors (GABAr) through the HNK-1 motif (Saghatelyan et al., 2000). This PNN component also interacts with subunits of voltage-gated Na^+ channels (Xiao et al., 1999), while brevican interacts with K^+ channels and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAr) (Favuzzi et al., 2017). Consequently, their removal can alter the excitatory/inhibitory balance. Furthermore, the lateral mobility of some receptors is also restricted by PNNs. ECM digestion with ChABC increases the mobility of AMPAr (Frischknecht et al., 2009).

Overall, while PNNs have been classically defined as a barrier for plasticity, they also regulate numerous different neuronal functions. These functions that appear to be crucial to neural development may play adaptive or maladaptive roles after SCI, and effort is needed to improve our understanding of these functions in the spinal cord.

DEVELOPMENT: PNN FORMATION

During postnatal development, there is a window of plasticity, called the critical period, during which neural circuits are sensitive to environmental stimuli (Hubel and Wiesel, 1970; Berardi et al., 2000). This sensory experience increases neuronal activity resulting in a boost of plasticity that favors synaptic wiring and fine-tunes spinal neuron properties (Kalb and HockField, 1994; Cameron and Núez-Abades, 2000). During this period, excitatory circuits predominate over inhibitory ones, as the high Cl^- levels within immature GABAergic neurons result in depolarizing responses. However, this sensory experience progressively produces some intracellular and extracellular changes around immature neurons. On the one hand, it gradually lowers Cl^- inside GABAergic neurons, switching from depolarizing to hyperpolarizing actions (Ben-Ari et al., 2012). The developmental upregulation of the KCC2 K^+/Cl^- cotransporter also contributes to this shift (Rivera et al., 1999). In contrast, it progressively increases the expression of some PNNs components until reaching a peak in which the link protein 1 is upregulated and condensed PNNs appear (Carulli et al., 2010). At that time point, there is also an increase in the sulfation CS-A/CS-C ratio of PNNs' CSPGs (Carulli et al., 2006; Miyata et al., 2012). This increase is comparable among the three spinal regions, although it appears earlier in the cervical segment than in the thoracic and lumbar one (Takiguchi et al., 2021). In the spinal cord of rodents, PNNs formation occurs in the second postnatal week (Kalb and Hockfield, 1988; Galtrey et al., 2008), marking the end of the critical period for plasticity. In fact, digestion of PNN by the enzyme ChABC reopens this window of plasticity (Pizzorusso, 2002; Pizzorusso et al., 2006). Indeed, the GABAergic shift occurs at the end of the critical period too, coinciding with PNNs deposition around neurons (Berardi et al., 2000; Frischknecht et al., 2009; Takesian and Hensch, 2013). A negative correlation between PNNs intensity and intracellular Cl^- levels was reported and further confirmed after digesting PNNs and measuring increased intracellular Cl^- levels (Glykys et al., 2014).

Once PNNs are fully formed, they mold the new connections generated into a meaningful manner to prepare the circuitry for adulthood: while active neurons would be wrapped by PNNs to strengthen their connections, their absence around unused synapses would lead to their pruning (reviewed in Murakami et al., 1992). In the spinal cord, around 50% of synapses are lost during development (Ronnevi et al., 1974). Interestingly, transgenic mice lacking the Crtl1 gene have aberrant PNNs and an increased number of excitatory synapses around spinal MNs (Sánchez-Ventura et al., 2021). Likewise, transgenic mice lacking tn-R, neurocan, and brevican also exhibited an increase in excitatory synapses around cortical neurons (Silver and Miller, 2004). Therefore, an adequate synaptic balance in mature neurons depends on the presence of proper PNNs.

Thus, the formation of PNNs is activity-dependent and coexists with the end of the critical period and the maturation of the CNS in which synaptogenesis, synaptic refinement, and neural maturation occur (**Figure 3**) (Pizzorusso, 2002; Carulli et al., 2006; Galtrey et al., 2008). Alterations in this activity can produce changes in the morphology, connectivity, and electrophysiological properties of MNs. In the spinal cord, all the excitatory drive that MNs received during development is provided by proprioceptive and supraspinal inputs (Kalb and HockField, 1994). Hence, sciatic nerve injury or thoracic hemisection before PNNs deposition suppresses MN activity and disrupts aggrecan expression (Kalb and Hockfield, 1988). Consequently, this reduced MN activity decreases MN pruning (Pittman and Oppenheim, 1979) and size (Brandenburg et al., 2018). Reduced MN size during development changes MN excitability and consequently affects motor neuron recruitment, since small MN cell bodies are more excitable and are recruited before large MNs (Henneman et al., 1965), directly impacting neural circuits (Kalb and HockField, 1994).

Overall, PNNs are instrumental in the transition from a permissible to a restricted milieu in the adult. The GABA potential shift and PNNs formation contribute to the well-known inhibitory environment found in the mature nervous system.

MATURE NERVOUS SYSTEM: PNN FUNCTION AND MODULATION

Once the critical period ends and PNNs have fully emerged, plasticity is not permanently lost but rather regulated more rigorously under the dynamic control of PNNs. This dynamism is explained by the activity-dependent modulation of PNNs still present in the adult: changes in the activity of mature neurons would structurally rearrange PNNs, and consequently, plasticity would be restricted or facilitated (Smith et al., 2015; Favuzzi et al., 2017). This modulation is mediated by endogenous and exogenous mechanisms. Exogenous mechanisms are mainly used in experimental studies and consist in the application of degrading enzymes like ChABC and hyaluronidase (Pizzorusso, 2002; Massey et al., 2006; Pyka et al., 2011; Starkey et al., 2012). Endogenous mechanisms are the main physiological modulators of PNNs, adjusting the synthesis and degradation of the different PNNs components. This constitutive remodeling is controlled by several protease families such as metalloproteases (MMPs) and A Disintegrin and Metalloproteinase with Thrombospondin motifs (ADAMTS) (Cawston and Young, 2010). Furthermore, the sulfation of CSPGs is another endogenous way through which activity can alter PNNs condensation and conformation (Miyata et al., 2012).

The regular turnover of PNNs persists throughout life, and it is crucial for many physiological processes. Interestingly, activity differently modulates PNNs depending on their anatomical location (Smith et al., 2015). In the cerebellum, an enriched environment (EE) reduced the synthesis of PNNs components and enhanced their degradation by increasing the activity of MMP2 and MM9 (Foscarin et al., 2011). In contrast, in the spinal cord, Wang et al. (2011) were the first to demonstrate that activity, in terms of rehabilitation, increased spinal PNNs in an SCI model. This specific finding, the impact of physical activity on spinal PNNs, did not receive much attention, despite clearly demonstrating that activity differentially modulates spinal and encephalic PNNs. Afterward, two studies corroborated that

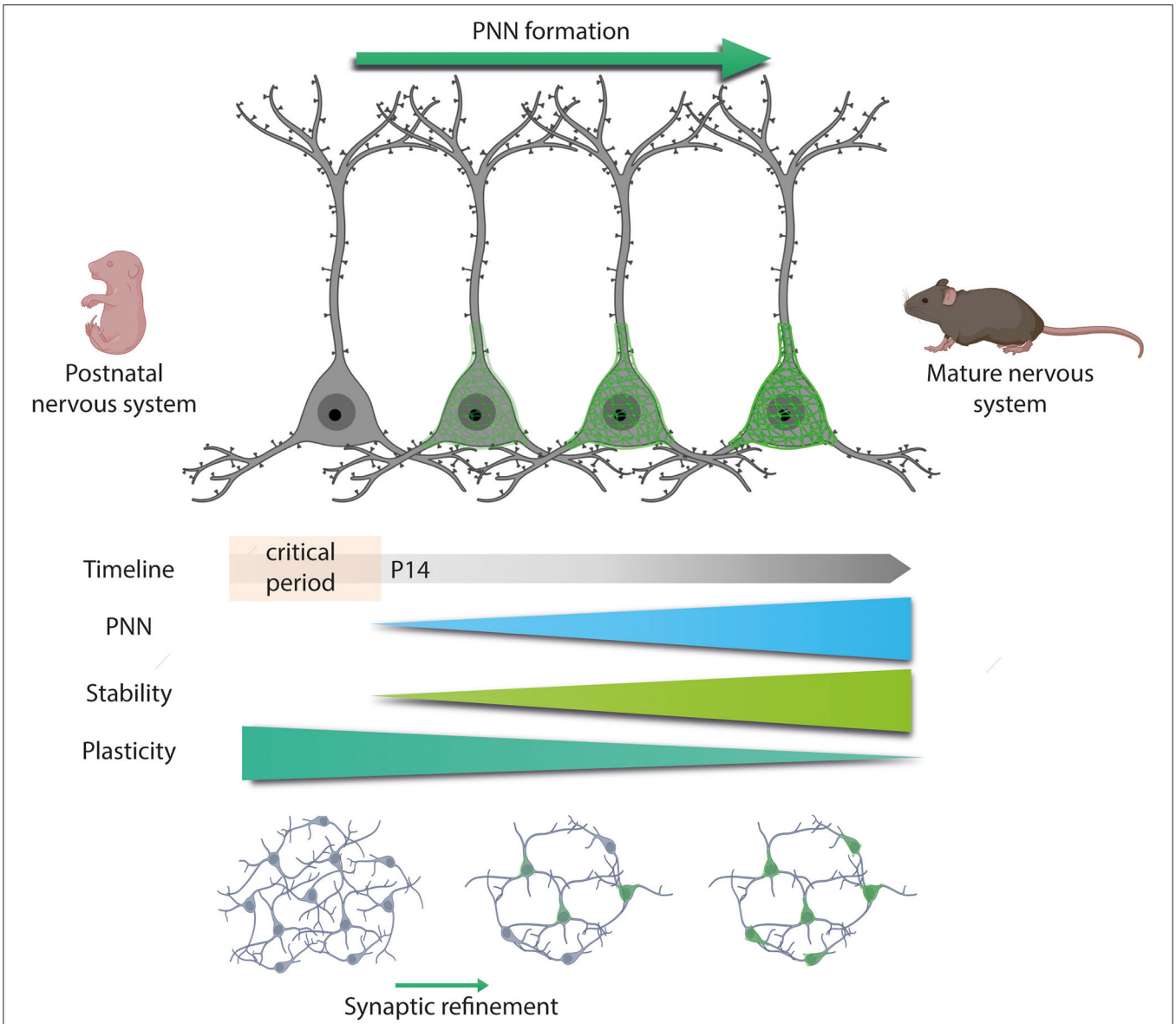

FIGURE 3 | Developmental PNNs formation. PNNs first appear during development, specifically at the end of the critical period, P14 in the spinal cord. The critical period is a plastic phase in which neurons increased their activity and generate new synaptic contacts. Once PNNs appear, they would wrap active neurons and stabilize these new connections formed. However, the absence of PNNs around unused synapses would lead to their pruning. Thus, as long as PNNs are formed, the plasticity of the central nervous system (CNS) decreases while stability increases.

physical exercise increased PNNs around intact spinal MNs (Arbat-Plana et al., 2015; Smith et al., 2015). Thus, the same physical activity that decreases PNNs' thickness around neurons located in the brain increases PNNs' thickness in the spinal cord (Smith et al., 2015). This differential activity-dependent modulation could have a biological significance, as most spinal cord functions rely on the stability of spinal circuits, whereas plasticity is essential for most brain functions. The relevance of spinal reflexes' stability in spinal cord function has been demonstrated in transgenic mice with aberrant PNNs that display motor impairment (Sánchez-Ventura et al., 2021).

Finally, the malleability of PNNs maintains the nervous system in an equilibrium whose imbalance in any direction can have broad implications in terms of neurological diseases. Excessive proteolytic processing of PNNs can lead to excessive plasticity as well as increased vulnerability to neurotoxic stimuli, which can trigger neurological disorders mainly studied in the brain. Indeed, cortical PNNs alterations have been linked with seizures (Tewari et al., 2018), CNS infection (Belichenko et al., 1997), traumatic brain injury (Hsieh et al., 2017), and stroke (Hobohm et al., 2005), among others. Nonetheless, very few studies have evaluated whether

changes in PNNs can lead to pathological conditions in the spinal cord.

INJURED NERVOUS SYSTEM: SPINAL CORD INJURY

Similar to the brain, insults to the spinal cord such as a traumatic SCI result in alterations in the ECM that consequently break the plasticity-stability balance. The most evident changes in ECM following SCI are related to the formation of the fibro-glial scar at the injury site, mainly produced by astrocytes and fibroblasts. However, less is known about the changes that spinal PNNs suffer due to the injury and the impact of these alterations on spinal circuits.

Fibro-Glial Scar in the Injury Site

After an SCI, there is an upregulation of CSPGs, at the injury site, forming the so-called fibro-glial scar. Specifically, there is a significant increase in lecticans such as neurocan, versican, phosphacan, NG2, and brevican but not aggrecan (Lemons et al., 2001; Jones et al., 2003; Buss et al., 2009). These CSPGs persist in the glial scar (Silver and Miller, 2004) and are remodeled by MMP, which expression significantly increases after injury (de Castro Jr et al., 2000; Zhang et al., 2011). Apart from CSPGs upregulation, selective changes in the sulfation pattern of GAGs have been reported. Following SCI, a large increase in the 4-sulfated (CS-A) GAG chains in the injury site have been observed, with no changes in the 6-sulfated (CS-C) GAG ones (Wang et al., 2009; Hussein et al., 2020).

The glial scar cannot be simply defined as beneficial or detrimental to CNS repair since it walls the injured area but also forms a barrier for axonal regeneration (Fawcett and Asher, 1999; Sofroniew, 2010). A lot of effort has been made to find an effective strategy to overcome the inhibitory influence of GAGs and thus, promote regeneration. These strategies are extensively exposed in the last section.

Spinal PNN After SCI

The fate of PNNs located below the level of the injury is controversial since different studies have reported a reduction (Lemons et al., 2001), no changes (Al'joboori et al., 2020) or an increase (Alilain et al., 2011) in PNNs thickness. Alilain et al. (2011) showed an upregulation of CSPGs, labeled by WFA, associated with PNNs around phrenic MNs after a cervical SCI. However, they did not specifically quantify PNNs around the neuronal soma, which is needed to differentiate PNNs from the loose ECM found in the grey matter. In addition, since an upregulation of CSPGs in the brainstem was also observed after a dorsal column section (Massey et al., 2006), it was assumed that SCIs increase PNNs from any type of neuron denervated by the injury, reducing plasticity and limiting axonal regeneration. Later studies tried to address the fate of spinal PNNs far from the injury site. Reduced staining of aggrecan around lumbar MN was reported 35 days after a thoracic SCI, recovering normal levels at later stages (Sánchez-Ventura et al., 2020). Similar findings were described after hemisection in goldfish (Takeda et al., 2018). A study evaluating aggrecan synthesis and degradation found a significant decline in aggrecan levels after SCI too (Lemons et al., 2001). In contrast, a recent study described no change in the amount of WFA+ CSPGs in the lumbar ventral horn after a thoracic SCI at early time points. However, at 67 days post injury, a significant increase was found (Al'joboori et al., 2020). Taken together, these findings point to some controversy regarding the fate of spinal PNNs after SCI not only because of the marker used to assess CSPGs' changes (WFA vs. aggrecan) but also to the type of neuron evaluated. PNNs surrounding phrenic MNs can behave differently than lumbar ones, given their differing functions (breathing vs. locomotion) and innervation [low vs. high proprioceptive Ia afferents (Alvarez et al., 2004; Nair et al., 2017)]. Besides, neurons in the brainstem are not located in the spinal cord, and hence, their PNNs can present the same activity-dependent modulation than encephalic PNNs (Sánchez-Ventura et al., 2020). Finally, the chronicity and type of injury may also impact on PNNs modulation. Long-term changes can be masked by the spontaneous recovery observed in some SCI models. Indeed, the progressive locomotor recovery in injured animals can modulate PNNs, since physical activity increases PNNs around spinal MNs (Wang et al., 2011; Arbat-Plana et al., 2015; Sánchez-Ventura et al., 2020). This increase is linked to the type and intensity of the activity (Arbat-Plana et al., 2015, 2017; Sánchez-Ventura et al., 2020), as well as the ability of the animal to be active besides the physical protocol (Arbat-Plana et al., 2015, 2017; Al'joboori et al., 2020). Thus, the denervated state of neurons below the injury produced by the disruption of descending inputs and proprioceptive inactivity due to muscle paresis might reduce PNNs, reverting neurons to their developmental state before PNNs first appear. In fact, PNNs reduction after injury is accompanied by changes that resembled those seen during the critical period, including increased synapse formation and altered neuronal excitability (Arbat-Plana et al., 2015; Sánchez-Ventura et al., 2020). Hence, developmentally immature neurons and denervated neurons post-SCI share similarities in PNNs integrity and neuronal properties (**Figure 4**). Lumbar PNNs disintegration after SCI was accompanied by reduced expression of the KCC2 co-transporter (Sánchez-Ventura et al., 2020), probably due to the role of PNNs sustaining receptors in the cell membrane (Frischknecht et al., 2009), modifying Cl^- intracellular levels. In parallel, the loss of negative charges provided by the CSPGs of spinal PNNs can shift the GABAergic response of spinal neurons and thus, increase spinal excitability (Sánchez-Ventura et al., 2020). Therefore, the increased collateral sprouting and neuron excitability observed after SCI (review in Oudega and Perez, 2012) are also reported in transgenic mice with aberrant PNNs. Animals lacking the link protein 1 presented an altered ratio of excitatory and inhibitory synapses leading to hyperexcitability of spinal circuits. After SCI, these animals presented increased sprouting of the corticospinal tract (CST) (Sánchez-Ventura et al., 2021), corroborating that spinal PNNs play an important role in stabilizing synapses and limiting plasticity in the mature nervous system. Overall, while the absence of PNNs before the end of the critical period is necessary for developmental synaptogenesis, PNNs reduction after SCI can lead to uncontrolled sprouting and hyperexcitability in the spinal cord that leads to maladaptive

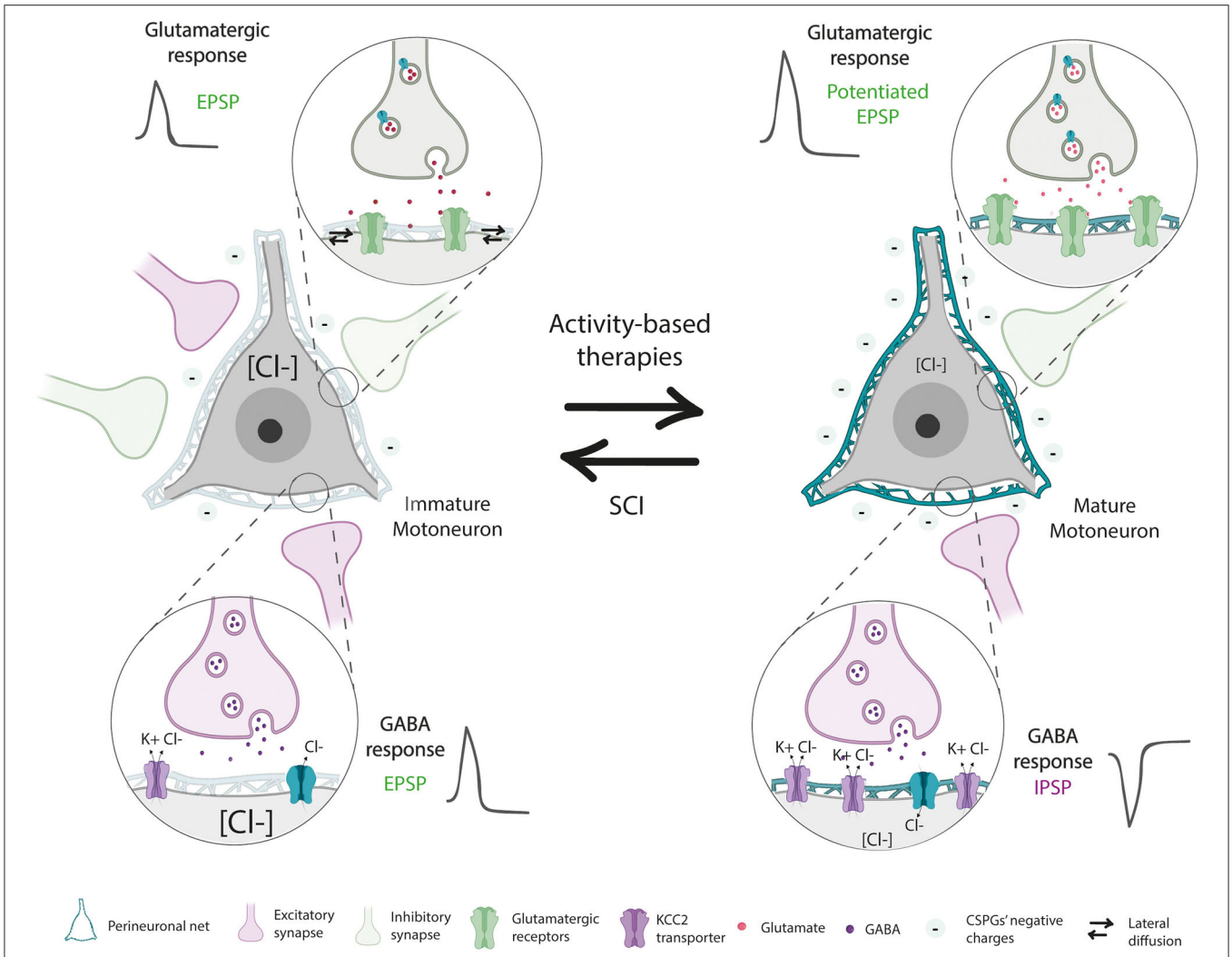

FIGURE 4 | Developmentally immature neurons and denervated neurons post-spinal cord injury (SCI) share similarities in PNN integrity and neuronal properties. Mature neurons present stable PNN, which generates a high density of negative charges around neurons that maintain ionic homeostasis necessary for proper synapse functioning. Besides, the physical barrier provided by PNNs maintains receptors in the cell surface and stabilizes synapses. However, after SCI, denervated neurons reduced PNNs thickness, shifting the GABAergic response of inhibitory neurons due to a reduction of the KCC2 co-transporter and a decrease of their negative chargers around the neuron. Besides, PNNs decline facilitates the mobility of receptors, generating inefficient excitatory synapses. These characteristics resemble those seen in developmentally immature neurons. The application of activity-dependent therapies prevents PNNs reduction and restores mature neuron properties.

symptoms such as neuropathic pain and spasticity (Costigan and Woolf, 2000; Boulenguez et al., 2010; Pitcher and Cervero, 2010; Côté et al., 2014; Mòdol et al., 2014). Interestingly, the blockage of MMP-9 and MMP-2 in the spinal cord inhibits the early and late phases of neuropathic pain, respectively (Kawasaki et al., 2008).

Finally, altered spinal PNNs are also observed in other pathologies such as amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA). Indeed, disorganized and vestigial PNNs are found in the ventral horn of terminal SOD1 mice (Forostyak et al., 2014), an experimental model of ALS. *In vitro* and *in vivo* studies revealed that patients with SMA present a downregulation of the Ctrl1 gene, altering their PNNs (Dayangac-Erden et al., 2018).

Therapeutical Manipulations of the ECM After SCI

The application of the enzyme ChABC in the fibro-glial scar is one of the most successful strategies to overcome the inhibitory influence of GAGs and consequently, promote regeneration (McKeon et al., 1991). The effectiveness of ChABC in degrading CSPGs have been tested *in vitro* and *in vivo* (Lee et al., 2010). *In vivo*, most of its efficacy has been evaluated in rodents, though it has also been studied in larger mammals such as cats and non-human primates (Tester and Howland, 2008; Rosenzweig et al., 2019). In most cases, ChABC has demonstrated huge potential in promoting regeneration of dopaminergic (Moon et al., 2001) and sensory axons (Shields et al., 2008), as well as, inducing sprouting of both intact and injured serotoninergic

(Tom et al., 2009), corticospinal (Barritt et al., 2006; García-Alías et al., 2009), and sensory fibers (Massey et al., 2006). However, controversial results do appear in the literature too. When CST regeneration was compared in hemisected and contusion models, ChABC injections only enhanced regeneration of the CST in the hemisection model (Iseda et al., 2008). Accordingly, ChABC's effect may depend on the severity and location of the lesion, in addition to the number of spared axons and the time of the ChABC injection. Indeed, Warren et al. (2018) described that ChABC was more effective when applied chronically than acutely after a cervical hemisection.

Although axonal growth (regeneration or sprouting) is typically necessary for recovery, it is not always sufficient. Despite some studies have demonstrated improved motor, sensory and bladder function after ChABC application (Bradbury et al., 2002; Caggiano et al., 2005; Massey et al., 2006; Cafferty et al., 2008), many others found limited results. The administration of a Sema3A inhibitor enhanced axon regeneration but not recovery due to a lack of functional connections (Zhang et al., 2014). Similarly, some works have reported limited recovery after ChABC application since the plasticity promoted by this enzyme needs an appropriate interaction with their target to make functional networks (García-Alías et al., 2009; Tom et al., 2009; Harris et al., 2010; Alilain et al., 2011; Wang et al., 2011).

Indeed, the combination of ChABC or Sema3A inhibitor with specific rehabilitation further enhanced functional recovery compared with applying them alone. In this regard, it has been proposed that the plasticity achieved by ChABC or Sema3A inhibitor only establishes meaningful connections when is combined with rehabilitative training, which activates spinal circuits, increases spinal PNNs around active neurons (Arbat-Plana et al., 2015; Smith et al., 2015; Sánchez-Ventura et al., 2020), and stabilizes these connections (García-Alías et al., 2009; Wang et al., 2011; Zhang et al., 2014). The ability of rehabilitation to rewire and stabilize synapses in a functionally meaningful manner after SCI has been already reviewed (reviewed in Torres-Espín et al., 2018). Nevertheless, many questions remain unanswered regarding the mechanisms involved. Since this synaptic stabilization is comparable to that found at the end of the critical period, in which PNNs contribute, it is plausible that these nets participate in the recovery promoted by rehabilitation after SCI. As stated before, activity increases spinal PNNs' thickness, in contrast to the brain (Smith et al., 2015). Physical activity and EE following SCI resulted in increasing spinal PNNs around lumbar MN whereas reducing PNNs in the brainstem sensory nuclei (Sánchez-Ventura et al., 2020). At the spinal level, physical activity prevented PNNs decline caused by the injury (Wang et al., 2011; Arbat-Plana et al., 2015; Smith et al., 2015; Sánchez-Ventura et al., 2020), which probably contributes to the synaptic stabilization of the newly formed connections and hence promotes functional recovery (García-Alías et al., 2009; Wang et al., 2011; Zhang et al., 2014; Sánchez-Ventura et al., 2020).

The importance of PNNs integrity after SCI is also observed in the CST. Digestion of cortical PNNs located in the boundary between motor and sensorimotor cortex by ChABC perturbed anatomical and functional CST reorganization after injury, resulting in an aggravation of motor deficits (Orlando and Raineteau, 2015).

Overall, combinatorial therapies appear to be the most effective approach to enhance functional recovery after an SCI. This combination can include therapies to widen the window of plasticity and modify the glial scar, such as the enzyme ChABC, and rehabilitation to shape the newly formed connections. However, the application of this enzyme can indirectly digest spinal PNNs and entail deleterious effects on the physiology of spinal neurons. Therefore, the ChABC approach presents limitations in the context of human therapy not only due to its non-specific nature but also due to the multiple injections or large volumes needed. Hence, more precise and selective manipulations than ChABC have been proposed to enhance axonal regeneration. Transgenic mice expressing ChABC under the GFAP promoter, thus limiting ChABC expression on astrocytes, showed enhanced corticospinal regeneration at the injury site (Cafferty et al., 2007). However, given that astrocytes can also contribute to PNNs' turnover, this type of approach can also indirectly act on spinal PNNs. Similarly, targeting the mRNA of critical enzymes in CSPGs' glycosylation and elongation has been tested *in vitro* (Grimpe and Silver, 2004; Laabs et al., 2007) and *in vivo* (Grimpe and Silver, 2004) with encouraging results in regeneration around the lesion site. However, the selectivity of these molecules in the glial scar is unclear. To solve this non-specificity, antibodies against specific structures are found in the literature. On the one hand, antibodies that neutralize the CSPG NG2 have demonstrated effectiveness in increasing regeneration of sensory axons after injecting into the dorsally transected spinal cord (Tan et al., 2006). Considering that no work has previously described the presence of the lectican NG2 in spinal PNNs, the application of this neutralizing antibody could offer an alternative way to specifically target the glial scar (Galtrey et al., 2008; Irvine and Kwok, 2018). In contrast, the application of selective antibodies against 4-sulfated GAG chains improved neurite growth (Yang et al., 2017), suggesting that modulating the sulfation pattern of proteoglycans can be an alternative to ChABC. In fact, while 4-sulfated GAG is distinguished by its inhibitory properties, the 6-sulfated GAG can also be beneficial for regeneration (Gilbert et al., 2005).

The therapeutic modulation of the PNNs must strike an accurate balance. Widely, PNNs digestion can generate excessive plasticity and increase neural vulnerability to neurotoxic stimuli, whereas excessive PNNs deposition can generate increased levels of synaptic stability. Apart from the modulation provided by activity-dependent therapies such as rehabilitation or exposure to an enriched environment, PNNs can be also modulated pharmacologically. In this sense, targeting specific PNNs components, such as Sema3A protein or the sulfation patterns of GAGs, are promising approaches.

CLOSING REMARKS

Growing evidence sheds light on the potential of PNNs in controlling the proper function of the CNS, especially in

the brain. However, more spinal cord research is needed to improve our understanding of spinal PNNs dynamics and function. It will be important to clearly define functional changes in the PNNs, the loose ECM, and the glial interface that accumulate in injured tissues, to completely understand the consequences of non-specific CSPG digestion. Digestion with ChABC alone does not reveal the full extent to which spinal PNNs can adaptively or maladaptively regulate changes after SCI, since only 2% of all CSPGs are found in PNNs (Fawcett, 2015). Future work harnessing the strengths of transgenic animals with aberrant PNNs, specifically those lacking the Crtl1 and aggrecan gene (Carulli et al., 2010; Giamanco et al., 2010; Rowlands et al., 2018), will help to elucidate the PNNs role in the healthy and injured spinal cord. An enhanced knowledge of spinal PNNs would facilitate the development of more effective and targeted strategies to properly treat SCI and other neuronal disorders, where both protection of synaptic integrity and controlled plasticity are needed.

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Fighting for recovery on multiple fronts: The past, present, and future of clinical trials for spinal cord injury

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Through many decades of preclinical research, great progress has been achieved in understanding the complex nature of spinal cord injury (SCI). Preclinical research efforts have guided and shaped clinical trials, which are growing in number by the year. Currently, 1,149 clinical trials focused on improving outcomes after SCI are registered in the U.S. National Library of Medicine at ClinicalTrials.gov. We conducted a systematic analysis of these SCI clinical trials, using publicly accessible data downloaded from ClinicalTrials.gov. After extracting all available data for these trials, we categorized each trial according to the types of interventions being tested and the types of outcomes assessed. We then evaluated clinical trial characteristics, both globally and by year, in order to understand the areas of growth and change over time. With regard to clinical trial attributes, we found that most trials have low enrollment, only test single interventions, and have limited numbers of primary outcomes. Some gaps in reporting are apparent; for instance, over 75% of clinical trials with "Completed" status do not have results posted, and the Phase of some trials is incorrectly classified as "Not applicable" despite testing a drug or biological compound. When analyzing trials based on types of interventions assessed, we identified the largest representation in trials testing rehab/training/exercise, neuromodulation, and behavioral modifications. Most highly represented primary outcomes include motor function of the upper and lower extremities, safety, and pain. The most highly represented secondary outcomes include quality of life and pain. Over the past 15 years, we identified increased representation of neuromodulation and rehabilitation trials, and decreased representation of drug trials. Overall, the number of new clinical trials initiated each year continues to grow, signifying a hopeful future for the clinical treatment of SCI. Together, our work provides

a comprehensive glimpse into the past, present, and future of SCI clinical trials, and suggests areas for improvement in clinical trial reporting.

KEYWORDS

clinical trial, spinal cord injury, systematic analysis, trends, outcomes, interventions

Introduction

Spinal cord injury (SCI) is a devastating event, typically resulting in lifelong neurological deficits, which affects an estimated 253,000–378,000 persons in the US alone (National Spinal Cord Injury Statistical Center, 2022). Individuals living with SCI and their loved ones face physical, emotional, social, and financial strain. It is estimated that the lifetime cost of SCI ranges from $1.2 to $5.4 million USD per person, with 30% of people undergoing re-hospitalizations one or more times during any given year following injury (National Spinal Cord Injury Statistical Center, 2022). To date, a large number of clinical trials have been initiated in an effort to improve the lives of individuals with SCI. However, there remain no FDA-approved treatments that can even partially improve neurological dysfunction after injury (Ahuja et al., 2016, 2017a, 2020; Elizei and Kwon, 2017; Hachem et al., 2017). In recent years, the establishment of various animal models has redefined our understanding of the mechanisms underlying SCI pathophysiology (Jakeman et al., 2000; Metz et al., 2000; Basso, 2004; Iwanami et al., 2005; Nout et al., 2012; Cheriyan et al., 2014; Kwon et al., 2015; Sharif-Alhoseini et al., 2017; Alizadeh et al., 2019; Fouad et al., 2020b). In addition, novel engineering applications ranging from cellular reprogramming (Fehlings and Vawda, 2011; Khazaei et al., 2016; Bartlett et al., 2020), to the development of sophisticated technology (Collinger et al., 2013; Courtine and Sofroniew, 2019; Squair et al., 2021), have opened new promising therapeutic avenues.

Since 2016, the National Institutes of Health has spent over $530 million on SCI research, and a substantial portion of that has gone toward supporting SCI clinical studies. Indeed, in 2021 more than 25% of NIH-funded projects related to spinal cord injury involved human subjects as reported by report.nih.gov/funding/categorical-spending#/. While there is still no FDA-approved, proven effective treatment for SCI, some clinical studies have shown great promise, and research priorities of individuals living with SCI have been identified (Anderson, 2004). There have been several excellent reviews published discussing advances in key areas of SCI therapeutics, such as stem cell transplantation and neuromodulation (Hawryluk et al., 2008; Gensel et al., 2011; James et al., 2018; Hofer and Schwab, 2019; Bartlett et al., 2020; Platt et al., 2020). However, these reviews typically focus on outcomes and not general conclusions about the priorities, or evolution, of SCI clinical trials. To address this, we have conducted a systematic review of 1,149 SCI clinical trials using data extracted from ClinicalTrials.gov and annotated by a team of investigators. We reviewed clinical trial characteristics including enrollment, phase, results, status, types and numbers of interventions and primary/secondary outcomes, as well as trends over time for the past 15 years. Collectively, this data provides the first comprehensive, systematic analysis of spinal cord injury clinical trials that will be of broad use for researchers, community members, and clinicians. Ultimately, the insights gained from this information highlight the need to continue pushing toward therapeutic interventions in such a way that is more efficient, held to higher reporting standards, and is overall more informative to the broad community.

Methods

Search parameters and exclusion criteria

On January 10, 2022, a search was performed on ClinicalTrials.gov using "spinal cord injuries" as the keyword under the "Condition or disease" category. This broad search resulted in 1,411 clinical trials. We downloaded and exported all 1,411 studies with all available data columns as tab-delimited text files. The exported 'raw' data included the following data categories: Rank, NCT Number, Title, Acronym, Status, Study Results, Conditions, Interventions, Outcome Measures, Sponsor/Collaborators, Gender, Age, Phases, Enrollment, Funded Bys, Study Type, Study Designs, Other IDs, Start Date, Primary Completion Date, Completion Date, First Posted, Results First Posted, Last Update Posted, Locations, Study Documents, and URL. Data was reviewed, classified, and annotated by a team of six investigators (V.A.D., N.R., K.K., S.M., M.P., J.N.D.), with each clinical trial listing reviewed by at least two independent investigators. Any discrepancies during this process were resolved through consultation between the reviewing investigators and a third reviewer from the team.

Prior to screening, we first excluded listings with Status that was classified as "Withdrawn," "No longer available," or "Temporarily unavailable," as well as trials that were classified as Study Type "Observational" (Figure 1). Clinical trials with the status "Withdrawn" are defined by ClinicalTrials.gov as a trial that ended early before enrolling its first patient. Next, we excluded clinical trial listings that were targeted toward caregivers or healthcare providers, but not individuals with SCI.

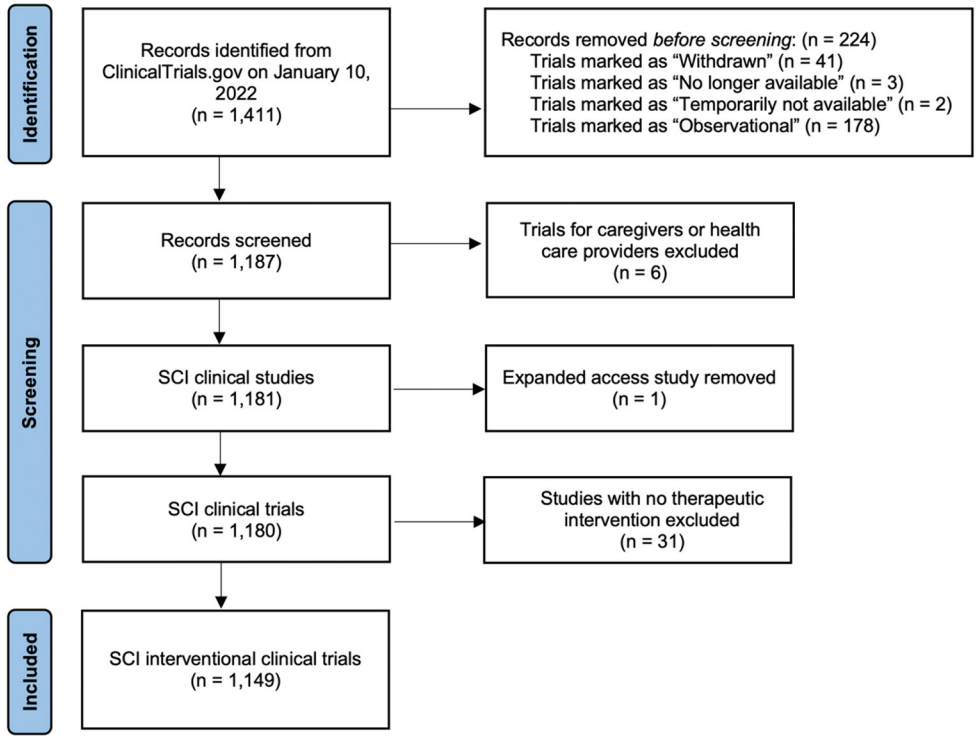

FIGURE 1
PRISMA flow diagram of the search strategy used in this study. SCI, spinal cord injury (Page et al., 2021).

We removed one listing that was not a clinical trial but rather an expanded access program for an investigational new drug. Finally, we refined the list of clinical trials to exclude those that did not include a therapeutic intervention (intended to have a therapeutic or beneficial effect on patients with SCI), as judged by the investigating team. This led to the exclusion of trials that were focused on generation or validation of a diagnostic tool, identification of biomarkers, or development of an intervention without testing the effects of the intervention. A total of 262 clinical trial listings were excluded based on these criteria, leaving 1,149 clinical trials used for analysis.

Clinical trial annotation and classification

We generated categories for interventions and outcomes based on common themes that emerged upon reviewing the list of clinical trials. Categories are defined with examples in Tables 1, 2. For intervention type, we formulated 14 unique categories: Acupuncture/needle therapy, Antibody therapy, Assistive/wearable technology, Behavioral, Biomaterials transplantation, Cell or tissue transplantation, Drug, Implanted/internal medical device, Nerve transfer/tendon transfer, Neuromodulation/electrical stimulation, Radiation therapy/laser therapy, Rehab/training/exercise, Surgical intervention/medical procedure, and Other (Table 1). The "Drug" category was further broken down into 15 subcategories according to the class or group of drug being tested. For types of primary and secondary outcome measures, we formulated 37 unique categories: Activity Level, Autonomic dysreflexia, Biomechanics/kinematics, Bladder function/bladder health, Blood pressure/cardiovascular function, Body mass/composition, Bone health, Bowel function/bowel health, Cognition, Depression/Anxiety, Employment/occupational performance, Fatigue, Fertility/sexual function, Independence, Medical imaging, Metabolism, Motor (lower extremities/locomotor function), Motor (not specified), Motor (trunk), Motor (upper extremities/hand function), Muscle and/or nerve function, Neurological score, Pain, Pharmacokinetics, Pressure injuries/pressure sores/wound healing, Psychological/social, Pulmonary function/breathing/cough, Quality of life, Safety, Sensory function, Sleep, Spasticity, Survival, Thermoregulation, Usability/feasibility/satisfaction of the intervention, Wheelchair propulsion/mobility, and Other (Table 2).

The 1,149 clinical trials that met our inclusion criteria were then annotated according to the types of interventions used and the types of primary and secondary outcomes

TABLE 1 Intervention categories.

Intervention type	Definition and examples
Acupuncture/needle therapy	**Definition**: Puncturing or pricking the skin with needles as a therapeutic practice.
Antibody therapy	**Definition**: Treatment with a monoclonal antibody.
Assistive/wearable technology	**Definition**: Any technology that is worn on the person or used by the person, which does not provide electrical stimulation or directly modulate the nervous system. **Examples**: Wearable garments, robotic gloves, prosthetics, orthoses, vibration/mechanical stimulation devices, CPAP, tongue-control devices, exoskeleton, adaptive robotic devices, adapted furniture, adapted environment.
Behavioral	**Definition**: Interventions that require the individual to modify their behavior, either short-term (during a study visit) or long-term (at home throughout the duration of the study), to produce a desired therapeutic effect. **Examples**: Phone apps, wellness or therapy groups, telemedicine programs, counseling programs, music therapy, educational programs, community programs, modifying diet or exercise routines, self-management routines, cognitive behavioral therapy, hypnosis, virtual reality programs presenting a different environment, visual illusions (e.g., phantom hand).
Biomaterials transplantation	**Definition**: Transplantation of a bioengineered material or biological scaffold, which may or may not contain cells or tissue, into the spinal cord. **Examples**: NeuroRegen scaffold, polyethylene glycol, hyaluronic acid.
Cell or tissue transplantation	**Definition**: Transplantation of living tissue or cells, either into the spinal cord or somewhere else into the body. This excludes biomaterials. **Examples**: Neural stem cells, bone marrow stem cells, mesenchymal stem cells, umbilical cord blood-derived cells, Schwann cells, oligodendrocyte precursor cells.
Drug	**Definition**: A pharmaceutical compound, medicine, supplement, or biological compound that is ingested or delivered into the body. Definitions for some of the subcategories are included below. **Subcategories**: **Adenosine receptor agonist/antagonist**: A compound that modulates activity of adenosine receptors. **Adrenergic receptor agonist/antagonist**: A compound that modulates activity of adrenergic receptors. **Anti-inflammatory**: Non-steroidal anti-inflammatory drugs. **Antibiotic** **Botulinum toxin** **Cannabinoid**: Natural or synthetic compounds within the cannabinoid family. **Growth factor**: Recombinant growth factor such as FGF, EGF, NGF, BDNF. **Herbal/natural/supplement**: Includes vitamins, homeopathic treatments, probiotics, dietary supplements, herbal supplements. **Hormone** **Lidocaine** **Neuromodulatory**: A drug, not falling into the other subcategories, that exerts a direct effect on the nervous system; examples include neurotransmitter reuptake inhibitor or a compound that mimics the effect of a neurotransmitter. **Opioid** **Statin** **Vasoactive**: A drug that exerts effects on blood vessel dilation/constriction and blood pressure. **Other:** Any drug not falling into one of these subcategories.
Implanted/internal medical device	**Definition**: An implanted device that is worn inside the body, but does not provide electrical stimulation. This does not include software or assistive devices that are not worn, or worn on the outside of the body. The implanted device can either be permanent or removable. **Examples**: Indwelling catheters, bowel irrigation devices, recording or monitoring devices, colonic tubes, implanted array to monitor but not stimulate brain activity.
Nerve transfer/tendon transfer	**Definition**: A surgical procedure in which either nerves or tendons are surgically cut and transferred to another nerve or muscle.
Neuromodulation/electrical stimulation	**Definition**: An intervention in which electrical or magnetic stimulation is used to elicit activity of the nervous system. Electrodes or electrical fields can be used. The effect is that some part of the nervous system is stimulated. **Examples**: Functional electrical stimulation, epidural stimulation, peripheral nerve stimulation, transcranial magnetic stimulation, direct current stimulation, transcutaneous stimulation, transcranial stimulation with ultrasound.

(Continued)

TABLE 1 (Continued)

Intervention type	Definition and examples
Radiation therapy/laser therapy	**Definition**: Treatment with ionizing radiation, UV light, X-ray, or lasers.
Rehab/training/exercise	**Definition**: Any type of intervention comprised of exercise, activity-based training, or physical rehabilitation.
	Examples: Exoskeleton-mediated walking, treadmill training, stepping training, walking training, upper limb cycling, intermittent hypoxia, breathing training, high-intensity interval training, exercise regimens, passive motion exercises.
Surgical intervention/medical procedure	**Definition**: Surgical manipulations, surgical interventions, medical procedures, or procedure done during a spinal cord decompression surgery, except for nerve and tendon transfers. The surgery or procedure must be the primary intervention to be performed/evaluated.
	Examples: Surgical decompression, controlled surgical lesions of the nervous system, bladder surgeries, comparing or validating different methods of performing surgery, sustained induced hypertension/hypotension, hypothermia, bronchoscopy.
Other	**Definition**: Any intervention that does not clearly fit into the above categories.
	Examples: Passive heat stress, hypothermia, extracorporeal shockwave therapy, ischemic conditioning.

List of 14 classes of intervention used to classify spinal cord injury clinical trials. Each intervention type is defined and in some cases, examples of interventions are listed. Note that the "Drug" category encompasses 15 subcategories for different types of drugs and biological compounds.

assessed (Supplementary Table 1). For each trial, annotation was performed by at least two independent investigators. Only the information that was listed on the ClinicalTrials.gov webpage for a given clinical trial was used to categorize interventions and outcomes; no outside information (for example, information on other websites or published papers) was used to annotate trials. Interventions, primary outcome measures, and secondary outcome measures were annotated independently of each other, using the information available on the provided URL. If a clinical trial used multiple intervention types, each intervention type was listed once. For a given trial, if multiple outcome measures fell into the same category, that category was listed only once as an outcome for that trial. For example, a trial that lists several different measures of sexual function under Primary Outcomes on ClinicalTrials.gov would have "Fertility/sexual function" listed only once as a primary outcome type in our dataset. Primary and secondary outcomes are independent from one another, so it is possible that, e.g., "Fertility/sexual function" could be listed once under primary outcomes and once under secondary outcomes.

Results

General attributes and demographics of spinal cord injury clinical trials

Of the 1,411 clinical trial listings identified, we excluded 262 trials that did not meet our eligibility criteria (Figure 1). We identified a total of 1,149 interventional clinical trials for spinal cord injury listed on ClinicalTrials.gov from 1996 to 2021, which we annotated according to types of intervention and outcome measures (Supplementary Table 1). We first analyzed general demographics and other attributes of the clinical trial data. We found that the numbers of new clinical trials per year have steadily increased over time, with 50% of all SCI clinical trials initiated between 2016 and 2021 (Figure 2A). In 2021, 112 new clinical trials were initiated, the most of any year in history.

We next analyzed enrollment. ClinicalTrials.gov lists either estimated enrollment or actual enrollment; however, it is not clear whether estimated enrollments were actually met for most listings, if results are not posted. The majority of clinical trials have low enrollments; 73.0% of trials had enrollment of 50 subjects or less (Figure 2B). Notably, only 9 of the 1,149 clinical trials had enrollment of over 500 participants. Among these were studies examining behavioral community wellness programs on the effects of lifestyle changes and transitions after injury (e.g., NCT03653390, "A Community Wellness Program for Adults Living With Long-term Physical Disability"; NCT02746978, "A Patient-centered Approach to Successful Community Transition After Catastrophic Injury"), as well as prospective studies examining the effects of surgical manipulations on outcomes such as survival rate (NCT01188447, "Evaluation of the Safety of C-Spine Clearance by Paramedics"; NCT03632005, "Negative Pressure Wound Therapy vs. Sterile Dressing for Patients Undergoing Thoracolumbar Spine Surgery"). Only three clinical trials ranked in the top 20 of enrollment are focused on testing the effects of experimental interventions (methylprednisolone,

TABLE 2 Outcome measure categories.

Outcome type	Definition and examples
Activity level	**Definition**: Assessments of physical activity level.
	Examples: Level of physical activity; the Physical Activity Scale for Individuals with Physical Disabilities (PASIPD); Physical Activity Questionnaire for People with Spinal Cord Injury (LTPAQ-SCI), International Physical Activity Questionnaire.
Autonomic dysreflexia	**Definition**: Adverse events resulting from overactivity of the autonomic nervous system in response to stimulation. This does not include autonomic function-related outcomes such as autonomic classification, autonomic control of respiratory or cardiovascular function.
Biomechanics/kinematics	**Definition**: Measurements of joint position, joint angles, torque, forces, and/or movement of the limbs during motor activity.
	Examples: Torque, resistance to stretching, degrees of flexion/extension of the arm or leg muscles, foot trajectory, propulsion, echogenicity ratio, load, contact time, muscle activity patterns during motion, joint forces.
Bladder function/bladder health	**Definition**: Measurements of bladder function or bladder health.
	Examples: Bladder filling, bladder voiding, bladder emptying, bladder pressure, compliance, leakage, frequency of urination, frequency of catheterization, neurogenic bladder, urinary tract infections.
Blood pressure/cardiovascular function	**Definition**: Measurements of blood flow, blood pressure, or heart function.
	Examples: Blood pressure, systolic blood pressure, hypotension, hypertension, heart rate, cerebral blood flow, arterial stiffness, Cerebral Vascular Resistance Index, VO_2 peak (peak oxygen consumption), autonomic control of cardiovascular function, head-up tilt test, aerobic capacity.
Body mass/composition	**Definition**: Assessments of body mass or body composition.
	Examples: Body weight, body mass index, whole body skeletal muscle and fat mass, percentage of body fat, fat mass/fat-free mass.
Bone health	**Definition**: Assessments of bone health.
	Examples: Bone mineral density, bone health, bone mass, DXA scanning, osteoporosis, fracture, integral volumetric bone mineral content.
Bowel function/bowel health	**Definition**: Assessments of bowel function or health.
	Examples: Bowel function, bowel emptying, frequency of bowel movements, bowel management, bowel care routine, constipation, Knowles Eccersley Scott Symptom (KESS), Patient Assessment of Constipation Quality Of Life scale (PAC-QOL), Neurogenic Bowel Dysfunction (NBD) score.
Cognition	**Definition**: Assessments of cognitive ability.
	Examples: Memory, d2 Test of attention, any cognitive tests including, verbal learning test, word association tests, Stroop test, Cognitive Functioning as Measured by PASAT, Performance on Cognition Battery Tests, Performance on tests of information processing (WAIS-IV and Digit Span) and working memory (SDMT).
Depression/anxiety	**Definition**: Assessments of depression and/or anxiety.
	Examples: Depression symptoms, Anxiety symptoms, Hamilton Depression Rating Scale, HAM-D, 16-Item Quick Inventory of Depressive Symptomatology-Self Report (QIDS-SR16), Depression Scale of the Patient Health Questionnaire (PHQ-9), Change in Patient Health Questionnaire-9 for measure of patient depression severity.
Employment/occupational performance	**Definition**: Assessments or indices of employment or performance of occupational tasks.
	Examples: Ability to perform occupational tasks, rate or success in employment, perform work-related tasks, Canadian Occupational Performance Measurement (COPM).
Fatigue	**Definition**: Assessments of physical or cognitive fatigue or exertion level.
	Examples: Physical fatigue, cognitive fatigue, exertion level, perceived exertion, muscle fatigue.
Fertility/sexual function	**Definition**: Assessments of sexual function, sexual health, or fertility.
	Examples: Sexual health, sexual function, male sexual function, female sexual function, sexual quality of life, sexual dysfunction, fertility, sperm count, sperm viability, sperm health, ejaculation, erectile function, best method to obtain semen.

(Continued)

TABLE 2 (Continued)

Outcome type	Definition and examples
Independence	**Definition**: Assessments of the subject's level of independence in daily life. **Examples**: Independence, Spinal Cord Independence Measure (SCIM or SCIM-III), Spinal Cord Independence Measure-Self Reported (SCIM-SR), Craig Handicap Assessment and Reporting Technique (CHART), Functional Independence Measure (FIM), Wheelchair independence, performance of daily tasks.
Medical imaging	**Definition**: Non-invasive measurements of brain activity or anatomical parameters. **Examples**: Functional magnetic resonance imaging (fMRI), BOLD signal, MRI, X-ray, CT scan, DXA scan.
Metabolism	**Definition**: Assessments of body metabolism at the molecular level. **Examples**: Metabolic health, metabolism, resting metabolic rate, measurement of metabolites in the blood plasma or other body fluids, expression of gene products or metabolites, fasting insulin, fasting glucose, hemoglobin, insulin or glucose sensitivity, oxygen uptake, lipid measurements, circulating markers, inflammatory markers, blood assays, metabolic panels, energy expenditure.
Motor (lower extremities/locomotor function)	**Definition**: Assessments of lower body motor functions such as walking, ambulation, stepping, standing, or any other motor function of the lower extremities. **Examples**: Ten meter walk test, 6 minute walk test, WICSI-II, FIM gait score, Spinal Cord Injury Functional Ambulation Index (SCI-FAI), Berg Balance Scale (BBS), Lower-Extremity Motor Scores (LEMS), walking function, stepping function, standing, sit-to-stand.
Motor (not specified)	**Definition**: Assessments of motor function that are not specified as lower body, upper body, or trunk function. **Examples**: Strength, voluntary movement, task completion, physical function, motor function.
Motor (trunk)	**Definition**: Assessments of trunk motor function including trunk stability, trunk coordination, and sitting balance.
Motor (upper extremities/hand function)	**Definition**: Assessments of upper body and arm/hand motor functions. **Examples**: Graded Redefined Assessment of Strength, Sensibility, and Prehension (GRASSP) strength subscale, upper extremity muscle strength, Manual Muscle Testing (MMT), Hand Held Dynamometry (HHD), Grasp-Release Test, Activities of Daily Living Test, hand grasp, grip strength, upper motor strength, Disabilities of Arm, Shoulder, and Hand (DASH) scores, Michigan Hand Questionnaire (MHQ), Hand Function Tests.
Muscle and/or nerve function	**Definition**: Physiological assessments of muscle, nerves, and reflexes; not including motor functional outcomes. **Examples**: Muscle area, muscle cross-sectional area, motor evoked potentials (MEPs), H-reflex, nerve conduction velocity, muscle stretch reflexes, reflex activity, excitability, muscle activation, resting motor threshold (RMT), Physiology Measurements, electromyography (EMG), Single pulse transcranial magnetic stimulation, nerve action potential latency of nerve conduction studies.
Neurological score	**Definition**: This is a specific terminology that refers to the scores of a neurological exam or the level/degree of neurologic lesion. **Examples**: The ASIA impairment scale (AIS) score, the International Standards for Neurological Classification of Spinal Cord Injury (ISNC-SCI) exam.
Pain	**Definition**: Assessments of pain or pain relief. **Examples**: Pain reduction, Pain severity, Pain interference on quality of life, Mean Pain Intensity, Numeric Rating Scale (NRS), Neuropathic pain scale, International Basic Pain Dataset, mechanical allodynia, Patient-generated Index (PGI), Pain unpleasantness, Wheelchair User's Shoulder Pain Index (WUSPI), musculoskeletal pain.
Pharmacokinetics	**Definition**: Measurements of drug pharmacokinetics. **Examples**: Tolerability, blood serum and cerebrospinal fluid (CSF) levels of the drug, Pharmacokinetic (PK) profile, dosing concentration and drug levels over time, Area Under the Concentration-Time Curve.
Pressure injuries/pressure sores/wound healing	**Definition**: Measurements of pressure injuries, sores, or ulcers, or related parameters. **Examples**: Incidence of pressure ulcers/injuries/sores, wound healing, skin irritation, pressure on skin, bleeding.
Psychological/Social	**Definition**: Assessments of psychological and/or social health and well-being, not related to depression/anxiety. **Examples**: Mood, loneliness, Neuropsychological Tests, social integration, caregiver burden, social problem solving, self-esteem, life satisfaction, self-efficacy, social connectedness, perceived stress, The Ways of Coping Scale- Revised (WOC-R), Community Integration Questionnaire (CIQ), Stage of change Scales (SOC), resilience.

(Continued)

TABLE 2 (Continued)

Outcome type	Definition and examples
Pulmonary function/breathing/cough	**Definition**: Assessments of lung function, breathing, or cough.
	Examples: Pulmonary function, postoperative pulmonary complications, Lung volume, lung capacity, air flow, airway pressure, respiratory motor control, inspiratory/expiratory pressure, inspiratory/expiratory duration, inspiratory/expiratory function, autonomic control of respiratory function, forced vital capacity (FVC), peak inspiratory/expiratory flow, Exhaled Breath Condensate, forced expiratory volume, peak cough flow.
Quality of life	**Definition**: Questionnaires or surveys that allow the patient to self-assess their quality of life (QoL) and/or overall satisfaction with life.
	Examples: Quality of Life Index SCI version (QOLI-SCI), quality of life, satisfaction with life, Satisfaction with Life Scale (SWLS), Life satisfaction Checklist (LiSat-11), World Health Organization Quality of Life (WHQOL), RAND-36 questionnaire to measure health-related quality of life, Quality of Life on the SCI QL-23, EuroQoL.
Safety	**Definition**: This refers to the safety of the intervention being tested. Safety may be assessed by the number or frequency of adverse events (hospital visits, complications, infections, toxicity).
Sensory function	**Definition**: Assessments of sensory function or sensation anywhere in the body, except for pain.
	Examples: Pinprick sensory test (sharp vs. dull with a safety pin), touch sensory test (with a cotton ball), sensory discrimination, Sensation of urinary bladder filling, sensation in the legs, Thermal sensation, sensory examination, Graded Redefined Assessment of Strength, Sensation and Prehension (GRASSP), Semmes Weinstein monofilament sensation test.
Sleep	**Definition**: Assessments of sleep quality.
	Examples: Sleep quality, sleep apnea, apnea index.
Spasticity	**Definition**: Assessments of spasticity.
	Examples: Participant reported spasticity, severity of spasticity, Modified Ashworth Scale (MAS), Portable Spasticity Assessment Device (PSAD), Modified Penn Spasticity scale, Spinal Cord Injury Spasticity Evaluation Tool (SCI-SET).
Survival	**Definition**: Survival of patients at defined timepoints after treatment.
Thermoregulation	**Definition**: Measurements of body temperature and ability to regulate body temperature.
	Examples: Core Body Temperature, thermal comfort, skin temperature, sweating, thermal sensitivity.
Usability/feasibility/satisfaction of the intervention	**Definition**: Measurements of how well the intervention can be used by the patient.
	Examples: Device usability, level of assistance needed to use the intervention, success rate of task performance, Standardized Usability Questionnaire, any questionnaire that rates the ease of using the device, task completion time, System Usability Scale (SUS).
Wheelchair propulsion/mobility	**Definition**: Assessments of how well the patient is able to use a wheelchair.
	Examples: Wheelchair transfer, wheelchair mobility, Wheelchair Skills Test (WST), wheelchair propulsion test, wheelchair independence and mobility, 6-minute Push Test (6MPT), Wheelchair Outcome Measure (WhOM), figure 8 protocol (fatigue intervention).
Other	**Definition**: Any outcome that does not clearly fit into the above categories.
	Examples: Spinal alignment, spinal cord perfusion pressure, expression of genes or gene products, appraisal of disability, nutrition knowledge, skin moisture level.

List of 37 classes of outcome measure used to classify spinal cord injury clinical trials. Each outcome type is defined and examples of measurements or scores related to the outcome type are provided.

NCT00004759; minocycline, NCT01813240; methadone, NCT00006448) on neurological outcomes.

There are five phases of clinical trial, defined on ClinicalTrials.gov as "*Early Phase 1 (formerly listed as Phase 0), Phase 1, Phase 2, Phase 3, and Phase 4.*" Some trials were also listed as combined Phase 1/2 or combined Phase 2/3. According to the ClinicalTrials.gov website, "Not Applicable" describes "*trials without FDA-defined phases, including trials of devices or behavioral interventions,*" and this category should be chosen if the trial does not involve drugs or biological products (clinicaltrials.gov/ct2/about-studies/glossary). We found that 62.8% of trials were classified as "Not applicable," and the second

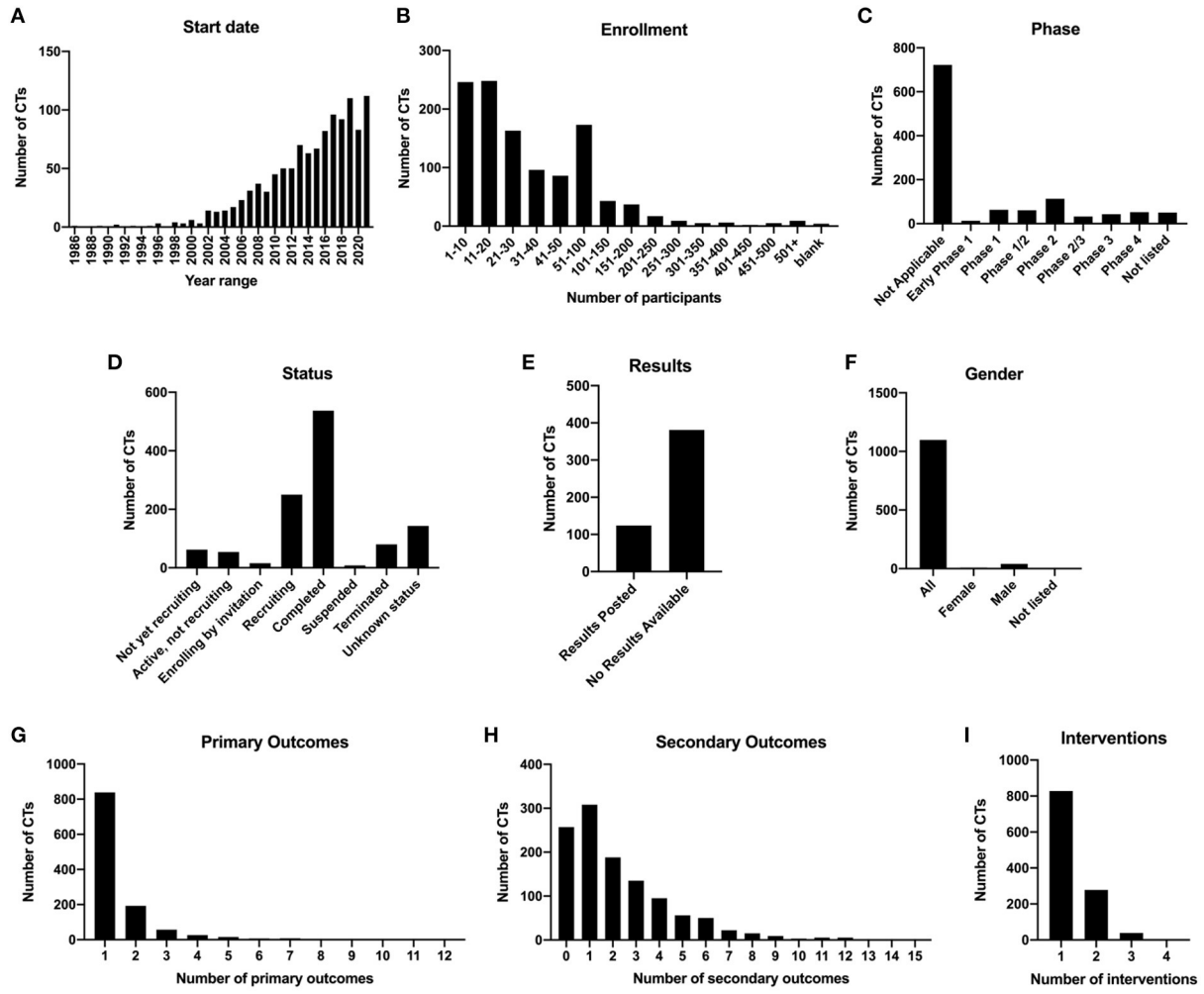

FIGURE 2
Demographics and statistics for 1,149 spinal cord injury clinical trials. **(A)** Numbers of clinical trials initiated per year from 1986 to 2021. **(B)** Number of clinical trials binned by actual or estimated enrollment of patients. **(C)** Number of clinical trials in each phase category. **(D)** Number of clinical trials in each status category. **(E)** Clinical trials marked as Completed and at least 1 year past the completion date, with results posted or no results available. **(F)** Number of clinical trials according to gender of enrolled subjects. **(G)** Number of clinical trials with 1, 2, 3, or 4 interventions. **(H)** Number of clinical trials with one or more types of primary outcome. **(I)** Number of clinical trials with one or more types of secondary outcome.

highest category was Phase 2, at 9.83% (Figure 2C). Fifty trials did not have any data listed for the Phase category ("Not listed").

We further analyzed the types of intervention that were represented in each Phase of trial (Supplementary Figure 1). For trials that were classified as "Not applicable," 42.4% involved rehab/training/exercise, 33.1% involved neuromodulation/electrical stimulation, 19.5% involved assistive/wearable technology, and 18.7% involved behavioral interventions. Surprisingly, 38 of these trials did involve drugs, cells, or biomaterials, so it is unclear how phase classification is not applicable to these trials. One strong trend is that the representation of the Drug category increases with advancing phase. For example, drug-related interventions represent 27.0% of Phase 1 trials, 64.6% of Phase 2 trials, 76.7% of Phase 3 trials, and 84.6% of Phase 4 trials (Supplementary Figure 1). Other interventions decrease with advancing phase; for example, cell or tissue transplantation represents 31.7% of Phase 1 trials, 14.2% of Phase 2 trials, but only 2.33% of Phase 3 trials and 0% of Phase 4 trials.

With regard to status, we found that 46.7% of the 1,149 trials were categorized as completed, whereas 23.1% were either recruiting or enrolling by invitation (Figure 2D). 10.1% of the 1,149 trials were not recruiting, and 7.66% were either suspended or terminated. Of the trials that were completed and at least 1 year post-completion date at the time of the search, 75.4% of them (381/505) had no results posted to ClinicalTrials.gov, whereas only 24.6% had results (Figure 2E). Of the 124 completed trials that had results, only 5 of

those trials did not meet the primary endpoints; thus, 95.9% of completed trials with results posted were successful at meeting the primary endpoints. This information is indicated in Supplementary Table 1. When we analyzed gender, we found that the overwhelming majority (95.6%) of 1,149 clinical trials were targeted toward all genders, while 3.57% listed only males and only 0.78% listed only females (Figure 2F). Of the female-only trials, 8/9 of these were focused on women's health; for example, NCT02398331 "Sexual Health of Spinal Cord Injured Females" and NCT04872569 "Pilot Testing a Pregnancy Decision Making Tool for Women with Spinal Cord Injury". Many of the male-only trials were focused on men's health, including reproductive and sexual health (10/41; NCT00223873, "The Use of Penile Vibratory Stimulation to Decrease Spasticity Following Spinal Cord Injury"; NCT00421983, "Efficacy and Safety of Tadalafil in Subjects with Erectile Dysfunction Caused by Spinal Cord Injury), catheterization (8/41; NCT02230540, "Intermittent Catheterization in Spinal Cord Injured Men"), or testosterone replacement therapy (7/41; NCT00266864, "Testosterone Replacement Therapy in Chronic Spinal Cord Injury"). A subset of male-only trials did not focus specifically on men's health (NCT02703883, "Body Weight Support in Spinal Cord Injury"; NCT01274975, "Autologous Adipose Derived MSCs Transplantation in Patient With Spinal Cord Injury").

Representation of intervention and outcome types

Types of primary and secondary outcomes were also analyzed. Outcome types are listed in Table 2. We found that the majority of the 1,149 trials (73.0%) examined 1 type of primary outcome, 16.8% examined 2 types of primary outcomes, and 4.96% examined 3; the remaining 5.22% of trials examined 4 or more types of outcomes, with a maximum of 12 types of primary outcomes tested in a single trial (Figure 2G). Inclusion of a single primary outcome in most of these studies is consistent with the goal of addressing a focused research question (Vetter and Mascha, 2017), while inclusion of multiple primary outcomes can inflate the false positive rate (Othus et al., 2022). For secondary outcomes, most trials (26.8%) examined only 1 type, though 22.4% did not examine any secondary outcomes (Figure 2H). 34.5% of trials examined 3 or more types of secondary outcomes, with a maximum of 15 types in a single trial.

We next analyzed the numbers of intervention types and outcome types per trial. Intervention types are listed in Table 1. Of the 1,149 clinical trials, 72.1% listed only one intervention, and 24.2% listed two interventions; <5% of trials listed 3 or 4 interventions (Figure 2I). Of the clinical trials testing more than one intervention, 74.8% of these featured Rehab/training/exercise as one of the interventions. Top combinatorial interventions included Assistive/wearable technology + Rehab/training/exercise (25.5%), and Neuromodulation/electrical stimulation + Rehab/training/exercise (34.6%). Four trials had 4 interventions; for example, NCT02136823, "Impact of Persistent Conductances on Motor Unit Firing in SCI," tested the effects of three different drugs plus a stretching exercise on muscle reflex excitability.

We sought to quantify the number of clinical trials according to the types of intervention used, and the types of outcomes assessed. We first quantified the number of the 1,149 trials that used each of 28 classes of intervention, with Drug subcategories collapsed (Figure 3A). We found that the highest-ranking category was Rehab/training/exercise with 386 clinical trials, followed by Neuromodulation/electrical stimulation (284 trials), Drug (all categories; 263 trials), Assistive/wearable technology (172 trials), and Behavioral (155 trials). We further broke down the Drug category into 15 sub-categories and found that neuromodulatory drugs were the most highly represented (70 trials) (Supplementary Figure 2). In addition to ranking interventions by the number of trials, we also calculated total human subject enrollment in all of the trials utilizing each intervention type (Figure 3B). Using this approach, Rehab/training/exercise and Behavioral ranked highest with 15,824 and 15,650 enrolled, respectively. Drug (all subcategories; 15,753 enrolled) also had among the highest enrollments of any intervention. Some of the lowest categories by enrollment are Biomaterials transplantation (150), Nerve transfer/tendon transfer (237), and Acupuncture/needle therapy (421).

The primary outcomes associated with the greatest number of the 1,149 clinical trials were Motor (lower extremities/locomotor function) with 159 trials, Safety with 136 trials, Pain with 111 trials, and Motor (upper extremities/hand function) with 108 trials (Figure 3C). Among the least-represented primary outcomes were Autonomic dysreflexia (3 trials), Thermoregulation (8 trials), and Sleep (9 trials). Upon calculating total enrollment for primary outcomes, we found that the highest enrollments were associated with Safety with 9,236 enrolled, Pain with 6,692 enrolled, Motor (lower extremities/locomotor function) with 6,147 enrolled, and Neurological score with 5,249 enrolled (Figure 3D). Autonomic dysreflexia was still the lowest-ranked outcome by enrollment, with only 77 subjects enrolled in trials that evaluated it as a primary outcome measure. For secondary outcomes, we found that Quality of life was listed for the greatest number of trials (190 trials), followed by Pain with 190 trials, Other with 158 trials, and Motor (lower extremities/locomotor function) with 155 trials (Figure 3E). Upon analyzing actual enrollment associated with secondary outcome measures, we found that there was much greater enrollment represented for secondary outcomes; the highest-ranked categories were Other with 15,115 enrolled, Quality of life with 12,765 enrolled, Usability/feasibility/satisfaction with

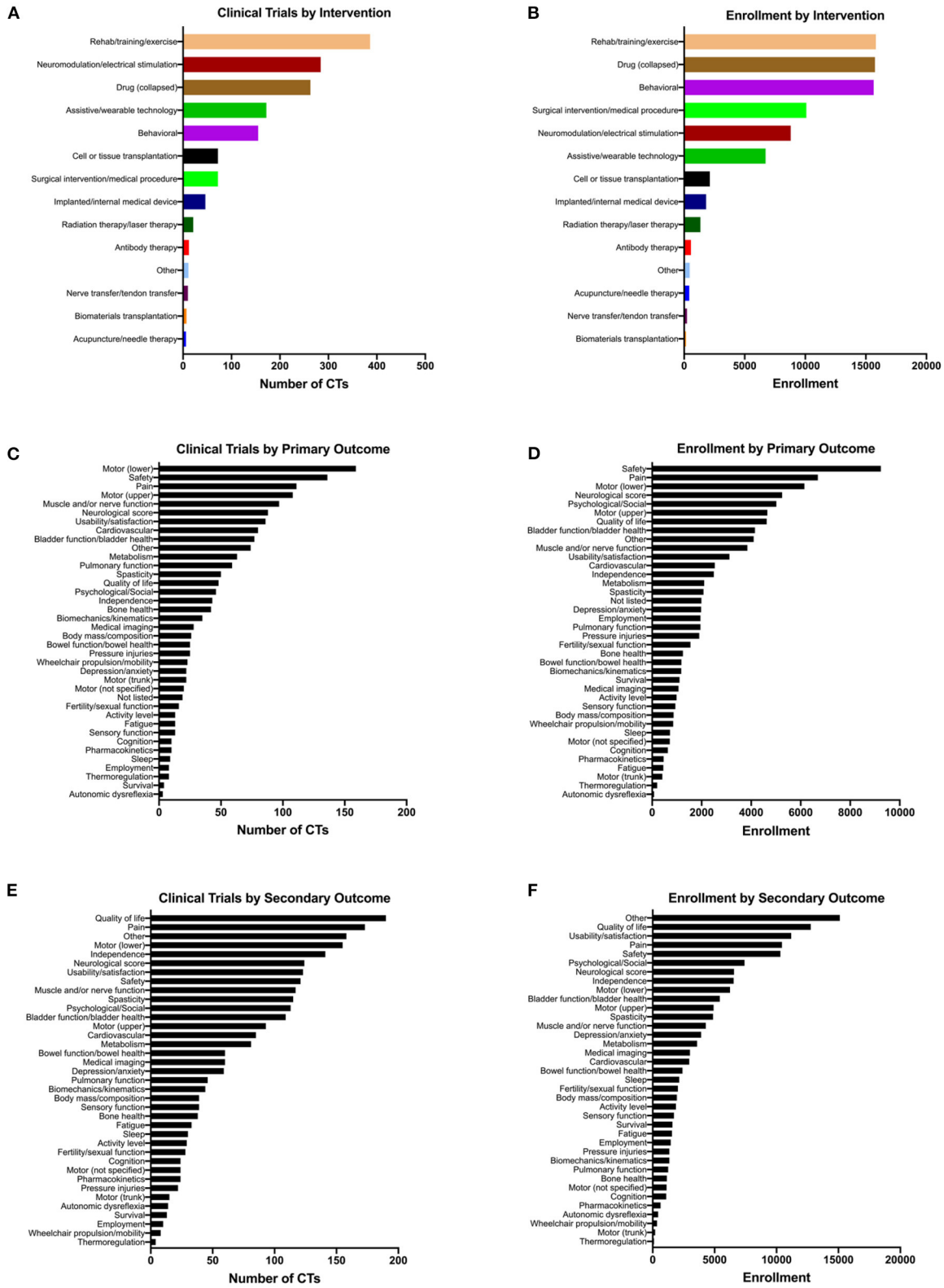

FIGURE 3
Therapeutic spinal cord injury clinical trials classified according to intervention and outcome types. Note that a given trial may have more than one intervention and multiple outcomes, so the total numbers of clinical trials in **(A,C,E)** add up to more than 1,149. **(A)** The total number of clinical trials for each class of intervention. **(B)** The cumulative enrollment for all clinical trials that use each type of intervention. **(C,E)** The total number of clinical trials listing each type of **(C)** primary and **(E)** secondary outcome. **(D,F)** The cumulative enrollment for all clinical trials that list each type of **(D)** primary and **(F)** secondary outcome.

11,188 enrolled, and Pain with 10,438 enrolled (Figure 3F). This reflects the finding that trials were likely to have a greater number of secondary outcomes listed compared to primary outcomes (Figures 2G,H).

Trends in interventions and outcomes over time

We next sought to understand how interventions and outcomes have changed over time. Because of limited data availability for clinical trials initiated prior to 2007, we elected to focus on analyzing trends in data over the past 15 years, from 2007 to 2021. We first analyzed trends in interventions tested over time. In 2007, drugs/biological compounds were the most represented intervention, with 37.8% of total interventions falling into this category (Figure 4A). However, over time there has been a gradual decrease in the proportion of interventions that are drugs; most recently in 2021, only 8.02% of all interventions were drugs. Figure 4B shows the breakdown of different subcategories of drugs comprising the "Drug" category. In most years, neuromodulatory, herbal/natural, and "Other" subcategories represent the greatest contribution to the Drug category. While most types of interventions have remained relatively stable over time, the Neuromodulation/electrical stimulation and Rehab/training/exercise categories have increased over time (Figure 4A). In 2021, Neuromodulation/electrical stimulation represented 27.8% of all interventions, and Rehab/training/exercise represented 25.3% of all interventions. In 2021 alone, 112 new clinical trials were initiated (Figure 2A); of these, 45 utilize Neuromodulation/electrical stimulation, and 41 utilize Rehab/training/exercise. In the past 5 years (2017–2021), 162 new clinical trials for Neuromodulation/electrical stimulation and 190 new trials for Rehab/training/exercise were initiated.

We did not detect many major shifts in the representation of primary and secondary outcome measures over time (Figures 4C,D). Some general trends emerged; for example, primary outcomes such as lower extremity motor function have stayed relatively steady over time, whereas upper extremity motor function has gradually increased (Figure 4C). Some primary outcome measures, such as autonomic dysreflexia, thermoregulation, and depression/anxiety, have remained consistently underrepresented compared to other outcome measures. For secondary outcome measures, some have remained consistently high over the past 15 years, such as pain, independence, and quality of life (Figure 4D). Overall, the representation of most secondary outcomes has remained relatively stable. Together, these data reveal that representation of primary and secondary outcomes has remained relatively stable over time.

Discussion

Emerging trends in SCI clinical trials

Of all the 1,149 clinical trials we reviewed, we observed that the majority of these enrolled <100 participants (Figure 2B). The number of participants enrolled in a clinical trial is uniquely based on the design of the trial, phase of the trial and therapeutic being tested. Note that higher recruitment will be needed to sufficiently power the study (Bracken et al., 1997; Fawcett et al., 2007). Enrollment of clinical trials specifically for SCI present challenges such as low incidence of injury, variable injury/severity among each participant, highly debatable approaches regarding therapeutic intervention and high cost of enrolled participants (Mulcahey et al., 2020). Several studies have examined these challenges of recruitment and the difficulties of maintaining recruitment in clinical trials and has opened the discussion for adaptive trial designs (Chow and Chang, 2008; Dragalin, 2011; Meurer et al., 2012; Meurer and Barsan, 2014; Bauer et al., 2016; Blight et al., 2019; Hubli et al., 2019; Kwon et al., 2019; Seif et al., 2019; Mulcahey et al., 2020).

Notably, we found that 72% of SCI clinical trials employed only one intervention (Figure 2I). It is a common consensus that to combat the complex nature of SCI, there will be no "magic bullet" single treatment; rather, effective therapies will likely be combinatorial in nature (Bunge, 2001; Ramer et al., 2005; Hawryluk et al., 2008; Olson, 2013; Griffin and Bradke, 2020). Of the 28% of trials using more than one intervention, almost 75% of these employed rehab/training/exercise as one of the interventions. Furthermore, only 5.1% of these combinatorial trials are either Phase 3 or Phase 4 studies. Hence, this data indicates a need to progress toward advancement of combinatorial clinical trials to combine the most promising therapies. Scientists and clinicians now face the challenge of figuring out how to incorporate rigor into study design while testing the greatest number of therapeutics in combination.

According to ClinicalTrials.gov, "*Primary and secondary outcomes are required by law to be analyzed and reported if any data was collected for the outcome. The primary and secondary endpoints should be pre-specified*". The primary outcome is the outcome measure of greatest importance and usually the one used in the power calculation during clinical trial design. The highest-ranked categories in primary outcome are motor (lower extremities/locomotion), safety, and pain while the lowest ranked are autonomic dysreflexia, thermoregulation, and sleep (Figure 3C). Similarly, the highest ranked categories of primary outcome also have the highest enrolled participant totals, while autonomic dysreflexia also has the lowest number of enrolled participants (Figure 3D). A natural question, therefore, is, "Does this reflect the priorities of the SCI community" (Anderson, 2004)? However, this is a difficult question to answer. It is clear that the expressed needs and priorities change from person to person, and are dependent on a variety of factors such as injury

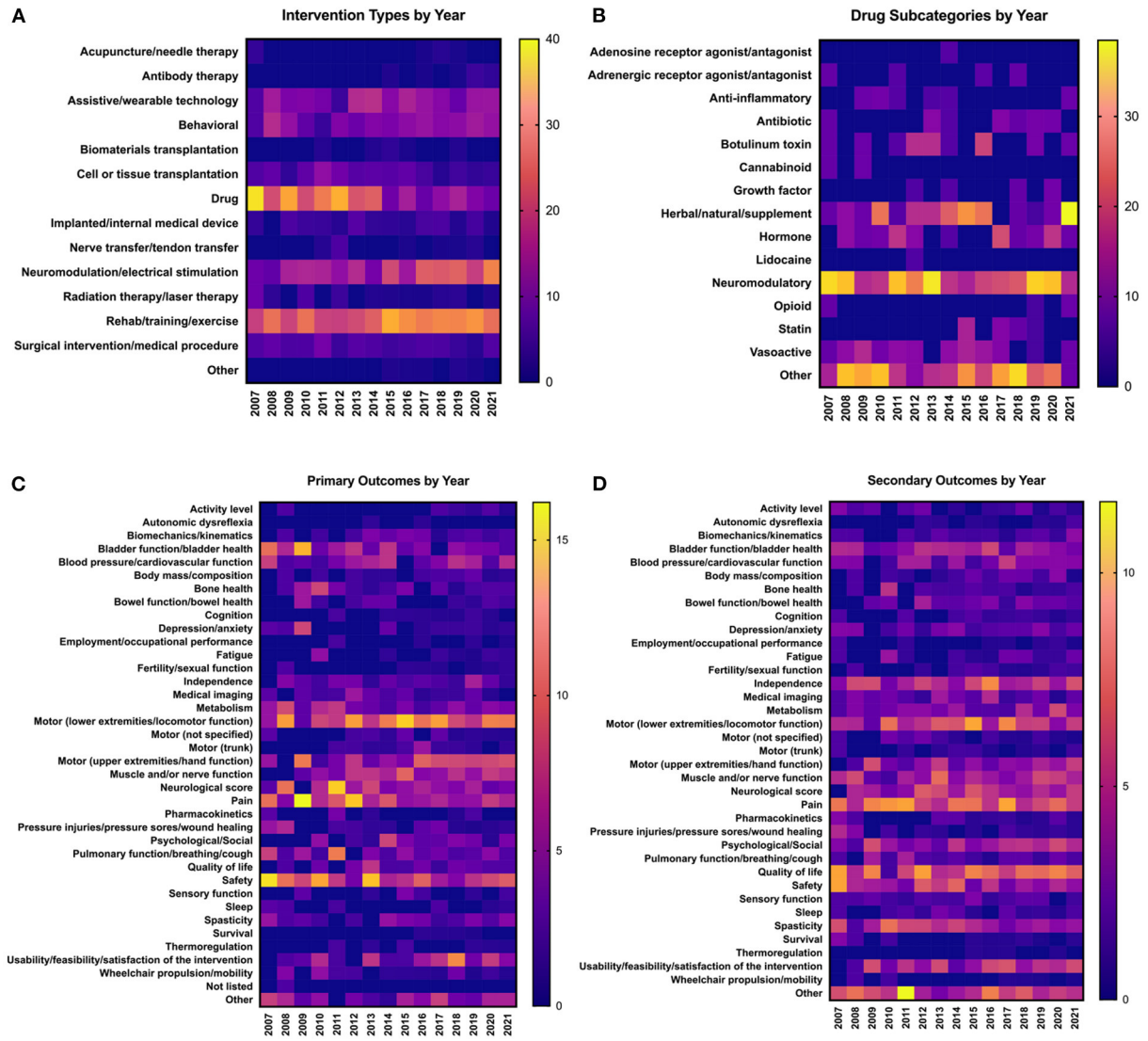

FIGURE 4
Trends in clinical trial interventions and outcomes over time. Data are from clinical trials initiated between 2007 and 2021. All data are represented as percentages of the trials in a given year that utilize each type of **(A,B)** intervention or **(C,D)** outcome; values in individual columns add up to 100%. **(A)** Frequency of types of interventions used in clinical trials each year. **(B)** Breakdown of the types of drugs that make up the "Drug" category in **(A)**. Values in individual columns add up to 100% of total drugs in a given year. **(C)** Frequency of types of primary outcome measures assessed each year. **(D)** Frequency of types of secondary outcome measures assessed each year.

level, severity, and time after injury (i.e., acute or chronic) (Glass et al., 1991; Anderson, 2004; Simpson et al., 2012; Trezzini and Phillips, 2014; Zanini et al., 2021).

Trends over time

Over the past 15 years, clinical trials have undergone some notable shifts in the representation of intervention and outcome types. It is important to note that clinical trial records may be incomplete prior to September 2007, when registration and submission of clinical trials and study results with ClinicalTrials.gov first became legally mandated through Section 801 of the Food and Drug Administration Amendments Act (FDAAA 801; clinicaltrials.gov/ct2/manage-recs/fdaaa), with the exception of phase 1 drug investigations, small clinical trials to determine feasibility, and certain clinical trials to test prototype devices (prsinfo.clinicaltrials.gov/ACT_Checklist.pdf). Hence, this could result in artificially low numbers prior to 2008, as there were likely more trials

being conducted than were registered to ClinicalTrials.gov. Another consideration is that beginning in 2004, the International Committee of Medical Journal Editors (ICMJE) have required any interventional human trials to be registered at ClinicalTrials.gov as a prerequisite for publication (clinicaltrials.gov/ct2/manage-recs/background).

Beginning in 2007, the most represented intervention category was "Drug," mainly comprised of neuromodulatory drugs; this may explain why most clinical trials in advanced phases are drug-related. As the representation of drug-based interventions has gradually decreased over time, there were concomitant increases in both rehab/training/exercise and neuromodulation/electrical stimulation (Figure 4A). This increase undoubtedly reflects advancements in technology allowing novel engineering of neuromodulation/electrical stimulation and a widely accepted consensus that rehabilitation is fundamental to improved outcomes (Whalley Hammell, 2007; Gomara-Toldra et al., 2014). An example of this is the combination of assistive technology (e.g., exoskeletons) with rehab/training/exercise. In 2014, the FDA approved the first robotic exoskeleton, ReWalk (ReWalk Robotics, Inc.) (Zeilig et al., 2012; Miller et al., 2016; Ahuja et al., 2017b). As noted above, hundreds of new clinical trials testing neuromodulation- and rehabilitation-based interventions have been initiated in the past few years alone. If this trend continues, the future of clinical SCI research will be overrepresented with these types of interventions.

Although some outcomes—for example, bladder function/health as a primary outcome—appear to be have decreased representation over time (Figure 4C), this is not due to a net reduction in bladder trials. For example, from 2007 to 2021 there has been an average of 4.2 $\pm$ 2.1 clinical trials measuring bladder function/health as a primary outcome per year, with 4 trials in 2007 and 4 trials in 2021 (Supplementary Table 1). In other words, the total numbers of trials measuring bladder function/health are not decreasing over time, but as the number of total clinical trials grow, bladder outcomes are not keeping up. This is also true for trials measuring pain as a primary outcome; representation of pain appears to decrease over time, but studies have actually increased from 4 trials in 2007 to 11 trials in 2021 (Supplementary Table 1). It is important to consider these trends in light of the challenges faced by the SCI community; for example, pain was ranked as the #1 most frequently cited challenged faced by those living with SCI according to a recent NASCIC survey (North American Spinal Cord Injury Consortium, 2019).

Gaps in clinical trial reporting

ClinicalTrials.gov was developed in an effort to make all ongoing trials accessible to clinicians and patients, combat publication bias, and enhance transparent reporting of clinical trials (Dickersin and Rennie, 2003). This website is a valuable data source, allowing users to track and evaluate the progression of clinical trials in a centralized repository with mandated regulations for reporting results (Zarin et al., 2011). This database also allows ease of systematic analyses elucidating trends in clinical trial design and in therapeutic interventions, as others have done previously in different fields (Hirsch et al., 2013; Jaffe et al., 2019; Wortzel et al., 2020). Our analyses clearly demonstrate that there are gaps in reporting including a lack of clarity with regard to categorizing trials as "interventional," reporting the specific characteristics of the SCI itself, or reporting of study results. More broadly, multiple studies have identified areas for potential improvement in reporting and usability for ClinicalTrials.gov (Wu et al., 2016; Chaturvedi et al., 2019; Warner et al., 2021). In 2021, Warner et al. conducted a systematic analysis on a subset of data extracted from spinal cord injury clinical trials; the authors identified key areas of improvement in reporting of these clinical trials (Warner et al., 2021). For instance, only 11.2% of trials correctly identified their study type, provided valid study status and provided sufficient detail about injury characteristics (Warner et al., 2021).

In our analysis, gaps in reporting became apparent during systematic review of clinical trial characteristics. One of the most noteworthy examples is that although almost half of clinical trials were marked as "Completed," 75.4% of completed trials have no results available on ClinicalTrials.gov (Figures 2D,E). This is similar to a previous finding that only 23.5% of 344 SCI trials with "Completed" status had results posted on ClinicalTrials.gov (Warner et al., 2021). However, we found that the absence of posted results did not necessarily mean that results from the study were not available elsewhere. We performed a PubMed search of 50 randomly selected trials that are listed as "Completed" with "No results available," and found that 27 of 50 (54%) of these trials had published results associated with the study outcomes. ClinicalTrials.gov denotes that "when results are not available for a study, the results tab is labeled "No Results Posted." Results of a study may not be posted for the following reasons: the study may not be subject to U.S Federal requirement to submit results, the deadline for results submission has not passed or the submission of results information has been delayed by the submission of a certification or a request to extend the results submission deadline" as per the FDAAA 801 Final Rule (clinicaltrials.gov/ct2/about-site/history). This issue of reporting is not new and has been observed by authors of other meta-analyses based on ClinicalTrials.gov data (Anderson et al., 2015; Warner et al., 2021). It is crucial that the public, scientific and clinical community be able to see results of clinical trials so that informed decisions can be made moving forward and integrated into the decision of participation, funding and approval of future clinical trials. Working with incomplete datasets leaves individuals unequipped to judge the novelty

or innovation of future trials and can directly contribute to redundancy of clinical trials. To remedy this, we join others in suggesting that reporting publications and trial results to ClinicalTrials.gov should be required as part of clinical trial reporting standards (publications.parliament.uk/pa/cm201719/cmselect/cmsctech/1480/148002.htm).

These gaps in reporting underscore a need for better reporting standards and more transparent data sharing. Several studies have demanded that clinical trial results be open access (Kramer et al., 2017) and have recommended that efforts be made to harmonize/standardize data elements so that comparisons between trials can be made (Landis et al., 2012; Steward et al., 2012; Lammertse, 2013; Lemmon et al., 2014; Ahuja et al., 2017b; Gensel and Orr, 2021). Several initiatives have been established to enhance data sharing such as the creation of Open Data Commons-SCI (ODC-SCI) enabling FAIR Sharing practices (Biering-Sorensen et al., 2015; Callahan et al., 2017; Mulcahey et al., 2017; Fouad et al., 2020a), the development of TRACK-SCI (Transforming Research and Clinical Knowledge in SCI) (Tsolinas et al., 2020), the North American Clinical Trials Network SCI Registry (Grossman et al., 2012), the International Spinal Cord Society SCI Data Sets (DeVivo et al., 2006) and the National Spinal Cord Injury Statistical Center Database (DeVivo et al., 2002).

Perspectives from the clinician-scientist

In most cases, the burden of reporting falls on the clinician-scientists at the institution conducting the clinical trial (Tse et al., 2009). Some institutions have supported the creation of administrative positions dedicated to clinical trials reporting to ease the burden of the primary investigator. However, in our experience, the greater challenge lies in the strict formatting of outcomes required by ClinicalTrials.gov. Whereas, an Institutional Review Board can manage a variety of formatting, allowing for investigators to use language directly from a grant application, this is not available in ClinicalTrials.gov. This may directly impact data analysis because results for the funding agency is the priority. Similarly, results for a manuscript may take precedence over the results requested by ClinicalTrials.gov. Another obstacle is that clinicians are often asked to fill out required information in such a way that meets the website's standard but does not necessarily require important information (for example, we observed that several registered clinical trials left fields as "not listed," "unknown status" or "blank," see Figure 2 and Supplementary Table 1). This lack of "policing" has contributed to this incomplete data set where several trials do not have results posted or have left important information as inaccurately listed. It has become apparent that there needs to be a call for standardizing and updating these reporting standards. It could be beneficial to link IRB permitting with the ClinicalTrials.gov website thereby allowing more accurate reporting of data while also easing the paperwork burden on clinicians. Additionally, having IRB mandate reporting of results with permit renewal to ClinicalTrials.gov could present an avenue to enhance reporting of results.

Perspectives from the SCI community

SCI research and clinical trials have been conducted for several decades, yet there remains no FDA approved, proven effective treatment for any outcomes associated with SCI; available treatment options are limited, and there is continuing debate about the standard level of care. There has been justifiable frustration and apathy expressed by individuals living with SCI in reaction to the promise of treatments being "just around the corner" fueled by media hype, as well as the slow pace of translation after decades of pre-clinical research (Kwon et al., 2010).

Individuals with SCI have made clear their desire to be involved in the research process from start to finish (Morse et al., 2021). In a 2019 study by the North American Spinal Cord Injury Consortium, community members ranked their highest priorities as receiving research information and serving as advisors to research teams (North American Spinal Cord Injury Consortium, 2019). This brings up two important topics of discussion: inclusion of lived experience consultants and accessibility of research to this population. As a direct result of this continuing call for inclusivity in research, some funding agencies such as the Department of Defense SCI Research Program and the Paralyzed Veterans of America Research Foundation have included individuals living with SCI as peer reviewers on their grant review panels and have required new grant submissions to include SCI consumer advocates or lived experience consultants to partner with research laboratories (Anderson, 2021). Additionally, several institutions strongly encourage the development of partnership between researchers and SCI community.

With regard to accessibility of research, many barriers remain present. One major example that this review brings to attention is that although 76.5% of SCI clinical trials do not have results posted to ClinicalTrials.gov, it is often the case that if and when published results are posted, they are still inaccessible to general public due to subscription requirements for journal access. This is a major issue because if results are posted on ClinicalTrials.gov they are primarily in tabular format and lack interpretation that is present in peer-reviewed publications. *It is critically important for SCI community members to be able to access and interpret clinical trial data*. They need to be able to understand what types of clinical trials are ongoing, be able to determine whether there are any they are eligible for, and access/look at results so they can interpret results for themselves. Resources such

as scitrials.org and scitrialsfinder.net are working toward this goal. It would be useful, for example, if the national clinical trial registry developed a systematic process for suggesting clinical trials tailored to individuals based on profile suitability rather than consumer demand. To date, "*ClinicalTrials.gov is designed to benefit the general public by expanding access to trial information*" (Zarin et al., 2011), yet we found that this dataset was incomplete and will likely be inaccessible to the general public.

Finally, we have identified some actionable items that, if implemented, could be useful for improving the usefulness of clinical trial data to the SCI community. First, a designation labeling interventional SCI trials as "therapeutic" vs. "not therapeutic" would be helpful; we found that 2.62% of SCI clinical trials labeled as "Interventional" were not actually testing a therapeutic intervention (Figure 1), and it would be useful for SCI community members to easily identify trials of therapeutics. Second, some clarification would be useful regarding future planned trials associated with a given intervention, and expectations for future clinical translation. We found that inconsistent or inaccurate application of FDA phase status, as well as the absence of sequential or graduated trial strategies, suggest that most trials do not appear to be designed to progress toward FDA approval. Additionally, it is unclear how much conceptual or programmatic overlap exists among clinical trials testing very similar interventions (e.g., neuromodulatory interventions for locomotor recovery), so some cross-referencing to indicate relationships between trials that are testing the same device, or trials that are otherwise linked in scope, would be useful. Finally, as a future goal, some integration of ClinicalTrials.gov with major data sharing initiatives would be a useful approach to recognize synergies between studies and improve clinical trial design moving forward into the future.

Conclusion

This systematic review provides a comprehensive view of SCI interventional clinical trials. The number of new SCI clinical trials initiated each year continues to climb. A large proportion of new trials are focusing on interventions such as neuromodulation, electrical stimulation, and rehabilitation. Over time, trials testing drug-based interventions have decreased in representation. These findings should be useful to scientists, clinical researchers, and the SCI community as a resource for understanding the trends in, and evolution of, interventional SCI clinical trials. However, gaps in reporting to ClinicalTrials.gov may present barriers that will limit the usefulness of this data to the public, scientific, and clinical communities. There is a need for improving reporting standards to ClinicalTrials.gov.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author/s.

Author contributions

VD and JD conceived of the study, performed study classification and data analysis, and wrote the manuscript. NR, KK, SM, and MP performed study classification. CB performed data analysis and contributed to manuscript writing. JC, SL, KN, PN, MR, CG, and AS contributed to study design and manuscript writing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fncel.2022.977679/full#supplementary-material

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Improving translatability of spinal cord injury research by including age as a demographic variable

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Pre-clinical and clinical spinal cord injury (SCI) studies differ in study design, particularly in the demographic characteristics of the chosen population. In clinical study design, criteria such as such as motor scores, neurological level, and severity of injury are often key determinants for participant inclusion. Further, demographic variables in clinical trials often include individuals from a wide age range and typically include both sexes, albeit historically most cases of SCI occur in males. In contrast, pre-clinical SCI models predominately utilize young adult rodents and typically use only females. While it is often not feasible to power SCI clinical trials to test multi-variable designs such as contrasting different ages, recent pre-clinical findings in SCI animal models have emphasized the importance of considering age as a biological variable prior to human experiments. Emerging pre-clinical data have identified case examples of treatments that diverge in efficacy across different demographic variables and have elucidated several age-dependent effects in SCI. The extent to which these differing or diverging treatment responses manifest clinically can not only complicate statistical findings and trial interpretations but also may be predictive of worse outcomes in select clinical populations. This review highlights recent literature including age as a biological variable in pre-clinical studies and articulates the results with respect to implications for clinical trials. Based on emerging unpredictable treatment outcomes in older rodents, we argue for the importance of including age as a biological variable in pre-clinical animal models prior to clinical testing. We believe that careful analyses of how age interacts with SCI treatments and pathophysiology will help guide clinical trial design and may improve both the safety and outcomes of such important efforts.

KEYWORDS

neurotrauma, pre-clinical research, secondary injury, translation, sex as a biological variable

Introduction

The average age at the time of spinal cord injury (SCI) has increased over time. In the 1970s, the average age at the time of SCI was 28 years old (yo) but as of 2015 has increased to 43 years old (NSCISC, 2022). Despite the typical clinical SCI demographic as that of a middle-aged male, pre-clinical animal models predominately utilize young adult female rodents (Stewart et al., 2020). Recently, we comprehensively reviewed the effect of sex differences in SCI modeling and the importance of including sex as a biological variable (Stewart et al., 2020). Collectively, it is important to consider the significant dichotomy between clinical populations and animal models in the interpretation and applicability of pre-clinical SCI findings in the use toward clinical translation.

In this review, we will discuss why older age at the time of SCI is associated with worse functional outcomes in animal models as well as the confounding variables that affect the interpretation of age-dependent effects clinically. We will review several biological underpinnings of secondary injury and recovery that are affected by the aging process. Specifically, we will cover known physiological aging adaptations that influence SCI responses, exacerbate secondary injury, and worsen functional outcomes. We will also discuss the somewhat unpredictable and unexpected results of animal studies focusing on interventions tailored to treat age-associated physiological differences. The conclusion of our comprehensive evaluation, namely that age can have profound effects on treatment approaches, supports the re-evaluation of pre-clinical therapeutic strategies as well as indicates that the minimal information necessary to translate preclinical results into clinical trials should be reconsidered. Articles in this review were chosen based on a comprehensive review of pre-clinical animal literature covering comparisons between animal models of young adult ages and older, as well as selected clinical reports offering contrasting findings about the role of age in the SCI population.

Abbreviations: 3-NT, 3-nitrotyrosine; 4-HNE, 4-hydroxy-non-enol; 5-HT, serotonin; BBB, Basso Beattie and Bresnahan scale of locomotor recovery; BMS, Basso mouse scale; DHE, dihydroethidium; DNP, 2,4-dinitrophenol; dpi, days post-injury; GCL, gamma glutamyl-cysteine ligase; GPx, glutathione peroxidase; GSH, glutathione; IgG, immunoglobulin G; KO, knockout; MO, months old; MPTP, mitochondrial permeability transition pore; NAC, *N*-acetylcysteine; NACA, *N*-acetylcysteine amide; NASCIC, North American Spinal Cord Injury Consortium; NnT, nicatinamide nucleotide transhydrogenase; NOX, NADPH oxidase; PTEN, phosphatase and tensin homologue protein; RBCs, red blood cells; ROS, reactive oxygen species; SCI, spinal cord injury; SDF-1, stromal derived factor 1; TBI, traumatic brain injury; TH, tyrosine hydroxylase; YO, years old.

Age at time of spinal cord injury and the clinical population

Determining how age at the time of injury affects clinical outcomes after SCI is challenging. Mortality after SCI increases with age creating a potential selection bias where more resilient, or less severely injured, older individuals are a larger representation within longitudinal clinical data (Furlan and Fehlings, 2009). This bias leads to caveats regarding directly comparing across age groups. Additionally, the causes and mechanisms of injury differ between young and older persons, presenting a further confound. While comparing injury responses across age groups in animal models can address some caveats present in clinical data, central cord syndrome (CCS) is a common mechanism of SCI with a higher representation in older persons and is difficult to model in animals. Displacement injuries produced by vertebral distraction can manifest in a pathology similar to CCS (Chen et al., 2016), however, this model of SCI has not been evaluated across different ages in rodents. Overall, CCS is not represented as a common model of SCI in animals. Additionally, animal models of SCI do not include comorbidities commonly found in an aging population such as cardiovascular disease, cancer, etc., which increases the frailty and worsens outcomes of older populations by increasing the frequency of adverse events and length of hospitalization (Velanovich et al., 2013; Banaszek et al., 2020; Dicpinigaitis et al., 2022). Correspondingly, frailty has been correlated as a predictor of mortality in elderly individuals (Carlile et al., 2022). Evidence from thoracoabdominal aortic aneurysm repair indicates that paraplegia risk may be correlated with frailty [using sarcopenia (core muscle loss) as a marker of frailty], however, the extent to which age and frailty interact to affect SCI outcomes remains understudied.

As previously noted, the average age of SCI in the US has increased to 43 years old. The causes, spinal levels, and severity of SCI have also changed over time with the most frequent category of neurological injury being incomplete tetraplegia (48.6% in the US since 2015) (NSCISC, 2022). In the US, the leading cause of SCI across all age populations between 2015 and 2021 was motor vehicle accidents (37.7%), with falls as the second leading cause (31.4%) (NSCISC, 2022). In persons greater than 45 years old, falls are the primary cause of SCI with similar findings in other countries (Toda et al., 2018; NSCISC, 2020; Sun and Zhang, 2021). Particularly in older persons, low speed/low impact falls (from standing) can result in the most common type of incomplete SCI, CCS. Although there is no clear, universally agreed-upon definition of CCS, the clinical presentation includes a greater loss of function in the arms and hands, relative to the lower extremities. Although CCS occurs in younger persons due to high energy impact injuries, in older persons this type of injury is caused by cervical hyperextension from a fall where pre-existing cervical stenosis is present (contributing to spinal cord compression) and is not

always associated with spine fracture or dislocation (Avila and Hurlbert, 2021; Ameer et al., 2022). CCS has traditionally been considered to have a higher rate of recovery than other types of SCI, however, a recent publication where individuals with CCS were matched with non-CCS incomplete SCI (by severity and neurological level of injury, age), demonstrated that CCS individuals had less recovery compared with incomplete SCI (Blasetti et al., 2020). This complicates our understanding of how age impacts recovery following SCI, as a high percentage of clinical SCI are cervical, incomplete, with a frequent presentation of CCS, for which there is no animal model. It is anticipated that the incidence of incomplete SCI will increase over time due to trends in age and cause of SCI (Devivo, 2012). This demographic shift will likely include a commensurate increase in CCS, in an active, aging population, presenting a potential confound in the translation of preclinical animal studies to human SCI.

The difficulty in directly comparing outcomes between age groups in humans likely contributes to conflicting reports across retrospective studies examining age at the time of SCI (Scivoletto et al., 2003; Furlan and Fehlings, 2009; Furlan, 2021). For example, Furlan (2021) identified no significant differences in function between older and younger populations 1-year post-SCI from a re-evaluation of data from the first North American Spinal Cord Injury Consortium (NASCIC) trials on methylprednisolone (Bracken et al., 1985; Furlan, 2021). In contrast, several other independent reports have identified worse motor and sensory outcomes in individuals injured later in life (Cifu et al., 1999a,b; Dai, 2001; Seel et al., 2001).

Findings from the Furlan (2021) NASCIC re-assessment examined data from 306 participants treated with methylprednisolone, including 39 females and 267 males with an average age of 31 years old at the time of SCI. The mean age of 31 at the time of SCI, published in 1985, pre-dates the progressive shift toward older age at the time of SCI, which is now approximately 43 years old (Bracken et al., 1985; NSCISC, 2022). In the retrospective assessment, older age was defined as 65 years old or older at the time of SCI. Functional outcomes were determined using the change in NASCIS motor scores [14 muscles assessed on a 6-grade scale ranging from 1 (normal function) to 6 (no contraction) (Bracken et al., 1985)] obtained at 1-year post-injury from scores obtained at the time of admission. Neurological recovery scores were adjusted for confounders of sex, injury mechanism, ethnicity, level of SCI, type of wound (open or closed), consciousness on admission, and dose of methylprednisolone using multiple regression analysis against age at the time of injury. Importantly, in this report, there were only 13 individuals out of 306 participants in the 65 years or older group (Furlan, 2021).

Furlan (2021) identified a significant positive correlation between older age at the time of SCI and improved motor scores at 1-year post-injury. While at first, this appears to contradict pre-clinical studies that find worse outcomes with older age (Gwak et al., 2004a; Genovese et al., 2006; Siegenthaler et al., 2008a,b; Fenn et al., 2014; Hooshmand et al., 2014; Zhang et al., 2015, 2016, 2019; Takano et al., 2017; von Leden et al., 2017; Martín-López et al., 2021; Stewart et al., 2021b), there are several important caveats to consider. First, it is interesting to note that the original publication reported a significant increase in mortality within 1-year of SCI among individuals 50 years or older at the time of injury (Bracken et al., 1985). This significantly increased mortality could have introduced a selection bias preferencing individuals with more robust recovery from injury since data on aged individuals who died was not included when determining motor improvements at 1 year. Next, a trend toward different injury mechanisms in older individuals may indicate less severe injuries at the time of SCI (Furlan et al., 2010). It should be noted, however, that in the retrospective study injury severity scores (Frankel Grade scores) were not significantly different at baseline between young and aged groups (Furlan, 2021). Finally, and what has the most potential relevance to the discussion below, is the potential for methylprednisolone to have exerted an age-dependent effect, conferring larger therapeutic benefit to older individuals. Due to ethical concerns regarding withholding methylprednisolone treatment during the NASCIC study, all individuals enrolled in the study received treatment, no placebo treatment was given (Bracken et al., 1985). Considering data were normalized to baseline, observing an increase in motor recovery relative to baseline with older age might be explained by age-dependent differences in treatment efficacy (Furlan, 2021).

In contrast to Furlan (2021), several other reports associate older age at the time of SCI with worse clinical outcomes as measured by the American Spinal Injury Association (ASIA) Impairment Scale (AIS), functional independence measures, and/or capacity for over-ground locomotion (Bravo et al., 1996; Cifu et al., 1999a,b; Dai, 2001; Seel et al., 2001; Coleman and Geisler, 2004; Wilson et al., 2014; Oleson et al., 2016; Brouwers et al., 2020; Engel-Haber et al., 2020). A more recent meta-analysis of clinical reports collectively identified age as a significant variable associated with worse neurological and functional recovery (Kirshblum et al., 2021). Previously, Seel et al. (2001) reported that rehabilitation performance measures were worse with older age, often requiring increased lengths of stay prior to hospital discharge. Part of the cause of this functional disparity between ages may be due to reduced muscular strength, independent of SCI, in the aging population; a consideration for functional disparities after SCI regarding both age and sex (Thomas and Grumbles, 2014). Age-associated functional outcomes are also strongest after incomplete injuries characterized as AIS B or C (Kirshblum et al., 2021), implicating injury severity as a potential age-dependent caveat. It is important to note that unlike Furlan (2021), not all studies control for confounding variables such as injury mechanism, baseline score, etc. (Dai, 2001; Furlan, 2021). Further, several publications, after adjusting for these confounding variables,

have not found age to be associated with worse outcomes or reported weak relationships with age at the time of SCI and functional recovery (Furlan et al., 2010; Wilson et al., 2014; Furlan, 2021). Collectively, age does appear to be associated with worse outcomes, but whether reduced neurological recovery is a product of changing biological responses with aging or differences in clinical scenarios cannot be extrapolated from clinical reports.

Aging in animal models

Locomotor and sensory outcome differences

Many caveats with interpreting clinical reports (i.e., mortality, injury type) can be addressed through controlled modeling in animals. Comparing between younger and older rodents in pre-clinical SCI models has elucidated several underlying differences occurring with advancing age in the pathology and recovery following SCI. Specifically, when injury severity, anatomical location, and injury type are controlled across age groups in rats and mice, older age is associated with worse functional outcomes, even when comparing young adults (3–4 months old) to middle-aged (12–14 month-old) rodents (Gwak et al., 2004a; Genovese et al., 2006; Siegenthaler et al., 2008a,b; Fenn et al., 2014; Hooshmand et al., 2014; Zhang et al., 2015, 2016, 2019; Takano et al., 2017; von Leden et al., 2017; Martín-López et al., 2021; Stewart et al., 2021b). The ability to provide immediate and sustained care to reduce age-associated mortality and limit selection bias in animals may be a potential factor in animal studies. Further, animal studies are not confounded by age-dependent differences in central cord syndrome, which is not examined in most rodent models. Indeed, the age-dependent recovery observed in animal models is inconsistent with some clinical reports (Furlan and Fehlings, 2009; Furlan et al., 2010; Furlan, 2021).

An age-associated decrease in locomotor outcomes has been replicated across labs and in both mice and rats. In this review, we will not discuss differences between neonatal/juvenile/pediatric and adult ages but will limit discussion to differences between young-adult, middle-aged, and elderly groups. The majority of reports from rodent studies examining the effects of age at the time of SCI reproducibly demonstrate that older age results in worse functional outcomes. In rats, age-associated impairments in functional recovery have been demonstrated using the Basso, Beattie, Bresnahan scale of locomotor recovery (BBB) (Basso et al., 1995; Gwak et al., 2004a,b; Genovese et al., 2006; Siegenthaler et al., 2008a,b; Hooshmand et al., 2014; Roozbehi et al., 2015; von Leden et al., 2017; Martín-López et al., 2021). Similar results are observed in mice utilizing the Basso Mouse Scale (BMS) (Basso et al., 2006; Kumamaru et al., 2012; Fenn et al., 2014; Zhang et al., 2015, 2019; Takano et al., 2017; Stewart et al., 2021b). To date, only three reports failed to detect differences across older age (Nishi et al., 2020; Hook et al., 2022; Stewart et al., 2022b), one of which utilized immunodeficient Rag2gamma(C) knockout mice in cervical SCI, the implications for which will be discussed in more detail below.

In addition to locomotor recovery, older age at the time of SCI is associated with differences in sensory function in rodents (Gwak et al., 2004b; Gaudet et al., 2021; Stewart et al., 2022b). Use of the Hargreave's test for thermal hypersensitivity revealed that absolute values for paw withdrawal latency do not differ between ages after SCI, however, there is a pre-existing hypersensitivity with between older mice between 2- and 20-months of age, making the change from uninjured conditions larger in younger mice; importantly this finding was observed in a second independent report between mice of 4- and 14-month of age (Gaudet et al., 2021; Stewart et al., 2022b). In contrast to thermal allodynia, mechanical hypersensitivity is not different between 2- and 20-month mice at baseline but younger mice exhibit a greater sensitivity at 1-week post-SCI which then resolves and plateaus at approximately the same sensitivity as 20-month SCI-mice (Gaudet et al., 2021). While both evoked thermal and mechanical hypersensitivity appear to indicate that younger age is associated with larger changes in hypersensitivity responses after SCI, Gaudet et al. (2021) also reported that 20-month mice exhibit a greater frequency of behaviors associated with spontaneous pain development. Self-severing, or autotomy, occurred at a significantly greater frequency in 20-month, compared to 2-month, mice (Gaudet et al., 2021). Collective results from both mechanical and thermal sensitivity tests suggest that younger, rather than older, mice experience larger changes in hypersensitivity after SCI related to exogenously evoked stimuli, while older mice exhibit a greater frequency of behaviors associated with spontaneous pain (Gaudet et al., 2021). Importantly, an earlier report identified that younger adult mice, but not middle-aged mice, develop mechanical hypersensitivity following spinal hemisection, validating that spinal mechanisms persisting in younger mice may result in a greater chance of developing SCI-induced pain (Gwak et al., 2004b). These findings may be consistent with age-dependent differences in the capacity for axon growth, plasticity, and maladaptive plasticity, which may favor a larger response at younger ages.

Plasticity and regeneration

Several studies identify that functional recovery is indeed reduced in older rodents after SCI, however, the biological mechanisms that underlie decreased recovery continue to be explored. The aging central nervous system is well-known to possess a decreased capacity for plasticity and regeneration, particularly in the context of hippocampal memory formation

(Isaev et al., 2019). Hippocampal neurogenesis declines with age (Ahlenius et al., 2009). A decreased growth potential of mature neurons has also been identified after peripheral nerve axotomy with the speed of axon growth and the total abundance of regenerating axons declining with advanced age (Pestronk et al., 1980; Verdú et al., 2000; Kaneko et al., 2021; Wagstaff et al., 2021). A reduction in trophic factor and cytokine secretion, as well as mitigated intrinsic growth responses contribute to a decreased regenerative potential of peripheral nerves with aging (Stratton et al., 2020; Kaneko et al., 2021; Wagstaff et al., 2021). The influence of aging on axonal repair in the central nervous system has been recently reviewed (Sutherland and Geoffroy, 2020) and will only be discussed briefly below.

To date, there have only been three published reports evaluating the effects of advanced age on regeneration after SCI, with all three reports providing corroborating evidence for observations in other neurological conditions (Jaerve et al., 2011; Roozbehi et al., 2015; Geoffroy et al., 2016). Specifically, all three studies found a decrease in axon growth with older age (Jaerve et al., 2011; Roozbehi et al., 2015; Geoffroy et al., 2016). Results after controlled thoracic transection provided the first evidence that age-dependent decreases in plasticity play a major role in functional recovery after SCI (Gwak et al., 2004a). Axon plasticity and growth after SCI can include both shorter-distance sprouting and long-distance regeneration (Cafferty et al., 2008). There is an emerging understanding that different mechanisms mediate long-distance regeneration relative to short-distance sprouting (Geoffroy and Zheng, 2014). Age-dependent declines in axonal growth have since been observed in SCI conditions with and without interventions aiming to enhance regeneration and sprouting. Both sprouting below the lesion and regeneration of damaged axons are reduced with older age (Jaerve et al., 2011; Roozbehi et al., 2015; Geoffroy et al., 2016).

Exploring the age-dependent effects on different mechanisms of axon plasticity provides insight into the role of age on SCI responses. For example, converging literature identifies intracellular signaling through the mTor pathway as essential for inducing long-distance axon regeneration (Park et al., 2008; Liu et al., 2010; Danilov and Steward, 2015; Du et al., 2015; Geoffroy et al., 2015). One strategy aimed at enhancing mTor activity is through administering the chemokine stromal-derived factor-1 (SDF-1), which acts on g-protein-coupled receptors, CXCR4 and CXCR7 (Opatz et al., 2009). SDF-1 activates the PI3K/AKT pathway and leads to mTOR activation (Dillenburg-Pilla et al., 2015). SDF-1 causes potent axon growth *in vivo* after both optic nerve crush and SCI (Jaerve et al., 2011, 2012a; Heskamp et al., 2013; Negro et al., 2017; Stewart et al., 2017; Li et al., 2021). Jaerve et al. (2011) used SDF-1 to examine how aging differentially affects the potential for sprouting and regeneration within the spinal cord after injury (Jaerve et al., 2011). SDF-1 was infused into spinal cords after a dorsal hemisection in young (9–14 weeks old) and older (22–28 months) rats using osmotic pumps (Jaerve et al., 2011). Without treatment, older rats had reduced sprouting of spared serotonergic (5-HT), tyrosine hydroxylase (TH), and CGRP fibers below SCI lesions. Similarly, although treatment with SDF-1 resulted in more sprouting below the lesions of younger rats, there was little effect of SDF-1 on axon sprouting in older rats. In contrast to short-distance sprouting below the lesion, SDF-1 did induce growth and regeneration of damaged 5-HT and TH fibers into the lesion with no detectable differences between ages. Corticospinal tract (CST) fibers did not grow into or beyond the lesion but were found to sprout more in younger rats rostral to the lesion in response to treatment (Jaerve et al., 2011). Collectively, these data indicate that axons sustain a comparable ability to regenerate, but not sprout, with older age.

Contrasting findings were reported by Geoffroy et al. (2016) utilizing a PTEN knockout model to increase mTor activity and induce axon growth of the CST (Geoffroy et al., 2016). Geoffroy et al. (2016) knocked out PTEN from corticospinal tract neurons at either P1, 4–6-week, 10–week, or 12–18 months of age. They then performed T8 dorsal hemi-sections 4–6 weeks later and evaluated CST regeneration at 6-week post-injury. Increased age at the time of injury blunted axon regeneration caudal to the lesion with 12–18-month mice exhibiting no significant regeneration beyond the injury site. In contrast, younger mice receiving PTEN KO at P1 or at 4–6-week of age, then subsequently injured 4–6 weeks later, had growth and regeneration caudal to the lesion. Geoffroy et al. (2016) replicated an age-dependent decrease in regeneration by evaluating effects of PTEN KO on a second spinal tract, the rubrospinal tract, which is believed to have greater regenerative potential (Geoffroy et al., 2016). Specifically, in this experiment, 6-week-old and 8-month-old mice demonstrated some regeneration caudal to the lesion after PTEN KO, with 6-week-old mice having significantly more regenerating fibers caudal to the lesion and at further distances away from glial boundaries (Geoffroy et al., 2016).

While both SDF-1 and PTEN knockout act to enhance the intracellular mTor signaling pathway, mechanisms of action differ between the two manipulations in the timing, duration, and intensity of the effect. Specifically, a permanent PTEN knockout likely induces a more sustained and intense growth response, evidenced by the magnitude of regeneration across the lesion, and may be more sensitive at detecting an age-dependent decline in axon growth. Further, knocking out PTEN exhibits an effect at the level of the soma which might exhibit a stronger transcriptional effect compared to local infusion of SDF-1 near the lesion. Alternatively, the discrepancy regarding an age-dependent decline in regeneration between studies could be explained either by species differences or in the assessment of different fiber tracts. While the exact extent of how species differences affect regenerative potential is unknown, mice do exhibit a dense collagenous glial scar within the lesion center compared to rats which form cystic cavitation and leave empty

fluid filled spaces that are barriers to axon growth. Differences in the scaring response suggests there may be critical differences in the microenvironment affecting the potential for axon growth and regeneration in a species-dependent manner.

The two studies also differed in the methods and fiber tracks analyzed. Specifically, Jaerve et al. (2011) evaluated fibers using immunohistochemical labeling, specifically being 5-HT, TH, and CGRP axons, while Geoffroy et al. (2016) evaluated motor neuron tracts requiring tract tracing, specifically the rubrospinal and corticospinal tracts. While Jaerve et al. (2011) did trace for corticospinal tract growth they were unable to detect a significant regenerative effect into or beyond the lesions, prohibiting analysis of regeneration of this fiber tract. Regardless, both experiments provide evidence that axon growth and plasticity are diminished with advancing age and are less receptive to treatment approaches. Finally, it should be noted that axotomized motor neurons within the cortex of young and aged rats also display diverse transcriptional profiles after SCI which likely plays a role in the different growth responses to injury and intervention (Jaerve et al., 2012b).

Injury and inflammation

Spinal cord injury causes a robust intraspinal inflammatory response consisting primarily of neutrophils, microglia, and macrophages within the first week of injury. At later timepoints, adaptive immune cells, e.g., b- and t-cells, infiltrate the injured spinal cord. Fenn and colleagues were among the first to provide evidence that older age at the time of injury (3-month vs. 18-month) leads to an exacerbated intraspinal inflammatory response. Specifically, we observed a loss of IL-4 receptor (IL-4R) on microglia and macrophages in 18-month-old mice after SCI (Fenn et al., 2014). IL-4R signaling induces an alternative, anti-inflammatory, macrophage phenotype that enhances tissue repair and regeneration *in vivo* after SCI (Kigerl et al., 2009; Gensel and Zhang, 2015). We observed an age-associated shift of microglia and macrophages toward a more pro-inflammatory phenotype with advancing age that contributed to an exacerbated secondary injury response (Fenn et al., 2014). Subsequently, we observed that older mice (4- vs. 14-month) have an imbalance in inflammatory cytokines surrounding the lesion, favoring a more pro-inflammatory (vs. reparative) environment with advanced age (Zhang et al., 2015). The pro-inflammatory cytokine, IL-12, and anti-inflammatory cytokine, IL-10, are expressed in relatively equal proportions intraspinally in older (14-month-old) SCI mice. In contrast, in young mice (4-month-old), IL-10 expression levels significantly increase over time and protein levels of IL-10 significantly increase more in young vs. aged mice by 7 dpi within the lesion (Zhang et al., 2015).

Older age at the time of injury is also associated with increased recruitment of macrophages into the lesion in both rats and mice (Hooshmand et al., 2014; Zhang et al., 2019; Stewart et al., 2021a). We also observed that intraspinal macrophages in 14-month mice produce significantly less anti-inflammatory IL-10 and significantly more reactive oxygen species (ROS) during the sub-acute stages of SCI relative to macrophages in 4-month animals (Zhang et al., 2015, 2016, 2019). Indeed, older mice have larger lesions and accumulate more oxidative stress by 7-days following T9 contusion SCI (Zhang et al., 2015, 2016). Age-dependent increases in ROS production are attributed to phagocytic cells from older animals expressing higher abundances of NADPH Oxidase 2 (Nox2), which generates the reactive oxygen species, superoxide, in macrophages and microglia (Zhang et al., 2016; Stewart et al., 2021a). Increases in Nox2 with age occurs in both mice and rats and in both traumatic and non-traumatic SCI (Zhang et al., 2016; von Leden et al., 2017; Michaels et al., 2020; Stewart et al., 2021a). An accumulation of ROS end products indicative of oxidative damage, 4-hydroxynonenal (4-HNE), and 3-Nitrotyrosine (3-NT), are increased in older mice at 7-days after SCI (Zhang et al., 2016, 2019; Stewart et al., 2021b), consistent with age-dependent changes in macrophage activation and phenotype. When we targeted the age-dependent increase in Nox2 using apocynin, a Nox inhibitor, we detected a larger therapeutic response in middle-aged (14-month) compared to adolescent (4-month) mice after SCI (Zhang et al., 2019). Specifically, apocynin decreased oxidative stress and intraspinal inflammation in an age-dependent manner and improved locomotor outcomes only in 14-month mice. These age-dependent inflammatory responses have therapeutic implications for SCI and further demonstrate that both the underlying biology of SCI as well as treatment efficacy change with age.

Two other recently published manuscripts provide more evidence highlighting the importance of inflammation in age-dependent effects after SCI. First, Nishi et al. (2020) evaluated how age and mouse strain affect functional outcomes after SCI (Nishi et al., 2020). While the same lab had previously reported an age-dependent decline in recovery in 18-month compared to 3-month rats after cervical SCI (Hooshmand et al., 2014), recovery was not affected after cervical SCI in 4- vs. 16-month Rag2gamma(C) knockout mice (Nishi et al., 2020). Rag2gamma(C) knockout mice are immunodeficient in elements of adaptive immunity, specifically having a loss of T-, B-, and natural killer cells (Nishi et al., 2020), as well as reduced serum IgG (Lee et al., 2018). While the role of infiltrating IgG in the spinal cord acutely after SCI is not thoroughly understood, it likely plays a role in binding to cellular debris to encourage phagocytosis from macrophages (Kopper and Gensel, 2018). Of interest, IgG increases within the spinal cords of wild-type mice in an age- and sex-dependent manner after SCI (Stewart et al., 2022a). Accordingly, Nishi and colleagues mention that the lack of adaptive immune cells may have masked the age-dependent pathophysiology and advocate for examinations of immune

cell-age interactions in SCI (Nishi et al., 2020). The lack of age-associated effects after SCI in immunodeficient mice further implicates inflammation as a key regulator of age-associated SCI pathophysiology.

Targeting inflammation by knockout out micro-RNA-155 (miR-155) has also been used as a strategy to determine the effects of inflammatory signaling on pain development in older age (2- vs. 20-month) animals after SCI (Gaudet et al., 2021). miR-155 has been shown to regulate both neuron growth and extension *in vivo* after SCI, as well as attenuate pro-inflammatory signaling in macrophages and mitigate macrophage accumulation within SCI lesions (Gaudet et al., 2016). miR-155 knockout (KO) mice injured at 2 months of age demonstrate an alleviation of hypersensitivity within the first 2 weeks of SCI, whereas miR-155 KO mice injured at 20 months of age do not differ from wild-type controls. In contrast, miR-155 KO did mitigate an age-dependent increase in mortality after SCI as well as an age-dependent development of spontaneous pain. Because miR-155 KO also reduces lesion sizes in adolescent mice (Gaudet et al., 2016), mitigation of pain development might not be exclusively associated with inflammatory modulation and could be attributed to sparing of axons that control pain perception (Gaudet et al., 2021). Regardless, miR-155 KO demonstrates that immune-associated strategies aimed at mitigating pain can also display age-dependent effects (Gaudet et al., 2021). The collective evidence from several reports now implicates age-related changes occurring throughout the inflammatory axis as maladaptive and exacerbate the pathophysiology of SCI.

Mitochondrial function

Redox metabolism is known to change with advanced age and resembles a shift toward a stronger reliance on glycolysis for energy production, a term coined the Warburg effect which was originally identified in cancer (Samudio et al., 2009; Burns and Manda, 2017) and is emerging as a hallmark of age-related differences in SCI (von Leden et al., 2019). Mitochondria utilize proton gradients built up within the inner membranous space to drive ATP production. Mitochondria are said to be coupled when the electron transport chain produces a proton gradient in the inner membranous space and when that gradient increase necessitates an increase in ATP production. When the mitochondrial membranes are permeable to protons, the gradient dissipates and electron transport fails to increase ATP production, which is termed an uncoupled response (Berry et al., 2018). In an uncoupled mitochondrion, protons produced from the electron transport chain do not contribute to ATP production. Mitochondria regulate this proton gradient through changing cellular respiratory rates and/or activating mitochondrial uncoupling proteins which allow protons to flow back into the mitochondrial matrix from the inner membrane space (De Simone et al., 2015; Zhao et al., 2019).

While at first it may seem maladaptive to intentionally uncouple mitochondria due to the suppressive effects on ATP production, a proton gradient that is too strong induces resistance to electron flow through the respiratory membrane complexes and generates ROS in the form of superoxide (Berry et al., 2018). When the resistance of electron transfer increases, electrons are captured by electrophilic soluble oxygen to form superoxide instead of reducing their energy state through the electron transport chain to form CO_2 (Berry et al., 2018).

Physiological coupling of mitochondria is regulated on a spectrum to mitigate and control free-radical formation at the balance of maintaining cellular energy demands. Older mitochondria exist in a more uncoupled state relative to younger mice, and consequently, produce less ATP for cellular energy (Conley et al., 2007; Chistiakov et al., 2014; Stefanatos and Sanz, 2018). Paradoxically, despite observing mild uncoupling with older age at rest, older mitochondria generate more ROS, which likely underlies the sustained mild uncoupling effect (Stefanatos and Sanz, 2018). Observing an increase in ROS production despite being more uncoupled suggests that underlying dysfunction accrues in mitochondria with advanced age. Indeed, older mitochondria are less capable of buffering cytosolic calcium before opening the mitochondrial permeability transition pore (MPTP) which is known to activate caspase cascades and initiate apoptosis (Mather and Rottenberg, 2000; Paradies et al., 2013). These findings suggest that older mice are more susceptible to both an increase in mitochondrial ROS production and exhibit decreased capacity for buffering cytosolic calcium before opening the MPTP, of which increased intracellular calcium is known to participate in excitotoxicity and secondary injury after SCI.

In vivo delivery of small doses of the pharmacological uncoupler, 2,4-dinitrophenol (DNP) can induce a mild-uncoupling response that aids in reducing ROS produced by mitochondria (Jin et al., 2004; Pandya et al., 2007; Patel et al., 2009; da Costa et al., 2010; Geisler et al., 2016; Stewart et al., 2021b; **Figure 1F**). Mitochondrial ROS production is believed to be a major source of free-radical damage after neurotrauma and treating mice and rats with DNP to mildly uncouple mitochondria is neuroprotective in rats with SCI and TBI as well as mice with TBI (Jin et al., 2004; Pandya et al., 2007; Patel et al., 2009). Our first report using DNP to treat SCI in mice, rather than rats, indicated toxic effects on younger mice and therapeutic effects on middle-aged mice (Stewart et al., 2021b). Specifically, three separate experiments identified that a very mild dose of DNP (1.0 mg/kg/day) exerted reciprocal effects between younger (4-month) and older (14-month) mice after SCI in several outcomes including locomotor abilities, tissue sparing, and mitochondrial function (Stewart et al., 2021b). Because DNP not only mitigates ROS production, but also lowers calcium buffering by mitochondria (Tsai et al., 1997; Korde et al., 2005), there are several potential reasons that could account for an age-divergent response to uncoupling (Stewart et al., 2021b). Regardless, the important take-home

message is that a similar dose of the same drug, DNP in this experiment, had opposite effects on outcomes that were dependent upon age at the time of SCI (Stewart et al., 2021b). This study has provided a profound example of why including age as a biological variable either prior to clinical trials or in analyzing findings from clinical trials is important for defining populations that may be sensitive to treatment effects.

Redox metabolism

Mitigating free-radical damage has been an emphasis for treating acute SCI for several decades (Braughler and Hall, 1982, 1983; Anderson et al., 1985; Bracken et al., 1997). Indeed, the anti-inflammatory and antioxidant effects of methylprednisolone established a rich history of improving outcomes in animal models of SCI, and to a more controversial extent, in humans (Anderson et al., 1985; Bracken et al., 1997; Hall, 2016). Numerous studies identify increases in ROS production and subsequent damage with older age at the time of SCI in rodents (Genovese et al., 2006; Zhang et al., 2016, 2019; von Leden et al., 2017; Stewart et al., 2021b, 2022b). Several techniques have been used to better understand ROS accumulation with older age. First, immunological labels against downstream products of ROS damage, 4-HNE and 3-NT, are upregulated with age at 7-dpi in mouse spinal cord sections and homogenates (Zhang et al., 2016; Stewart et al., 2021b) and up to 30-dpi in rat spinal cord homogenates (von Leden et al., 2017). While evidence of an age-dependent ROS accrual is found at 7-dpi in mice, neither 4-HNE or 3-NT are upregulated in an age-dependent manner at 3-dpi (Zhang et al., 2016; Stewart et al., 2022b). The observation of delayed age-dependent oxidative damage between 3- and 7-dpi implicates infiltrating macrophages as a facilitator of ROS damage with older age (Zhang et al., 2019). After SCI, macrophages emerge at 3-dpi, peak between 7- and 14-dpi, and persist chronically after injury (Beck et al., 2010).

In addition to immunological labeling to detect oxidative damage, systemic delivery of dihydroethidium (DHE), a dye sensitive to superoxide formation, reveals an age-dependent increase in active ROS production at both 3- and 7-dpi (Zhang et al., 2016). DHE is a fluorescent dye that undergoes a red spectral shift upon reacting with superoxide and differs from immunological labeling by being an indicator of active, ongoing, ROS production rather than an accumulation of end products (Peshavariya et al., 2007). Using DHE, we revealed that the largest percentage of cells in the spinal cord oxidizing DHE at 3-dpi were macrophages or microglia (Zhang et al., 2016). This observation is consistent with our subsequent observations of an age-dependent increase in Nox2 within macrophages and microglia (Zhang et al., 2016, 2019; Stewart et al., 2021a) and strengthens the argument that an age-dependent increase in ROS damage is caused by altered macrophage activation. Reports as to whether macrophages infiltrate in greater number after SCI at older ages are inconsistent (Hooshmand et al., 2014; von Leden et al., 2017; Zhang et al., 2019; Furlan et al., 2020; Stewart et al., 2021a; Li et al., 2022), however, all studies evaluating differences in macrophage physiology with age identify that older macrophages present with phenotype characteristics of greater ROS production after SCI (Hooshmand et al., 2014; von Leden et al., 2017; Zhang et al., 2019; Stewart et al., 2021a; Li et al., 2022). Taken together, collective data has identified ROS as an age-dependent contributor to SCI pathophysiology.

While the capacity to defend against free radicals is quite complex, one major cellular pathway, the glutathione (GSH) system, has been investigated for its known changes occurring with age (Jones et al., 2002; Liu, 2002; Ghosh et al., 2014; Ferguson and Bridge, 2016). Cellular GSH regulation utilizes a series of GSH peroxidases (Gpx) to sequester different types of free radicals. GSH is a re-usable tripeptide antioxidant that is used as a substrate by Gpx to reduce radicals and is recycled back into a usable form by GSH reductase at the expense of NADPH. Advanced age diminishes cellular levels of both GSH as well as the availability of its amino-acid constituent, cysteine, within the plasma (Jones et al., 2002; Liu, 2002; Ghosh et al., 2014; Ferguson and Bridge, 2016). The ability to produce and maintain adequate cellular GSH levels is believed to be bottlenecked by either the availability of its cysteine substrate or the availability of the enzyme responsible for the first of two ligation reactions: glutamate-cysteine ligase (GCL) (Griffith, 1999; Lu, 2009). Both GSH and GCL abundance decrease with age in other organ systems (Liu, 2002; Ghosh et al., 2014; Ferguson and Bridge, 2016). Further, both GSH and GCL activity diminishes following SCI. We recently reported that GSH does indeed diminish with older age (4- vs. 14-month mice) within the spinal cord independent of injury, identifying a reduced capacity for older rodents to defend against oxidative stress after SCI (Stewart et al., 2022b).

Glutathione depletion after SCI occurs as early as 24-h post-SCI and remains depleted for up to 72-h, and likely longer, with greater decreases at 72- compared to 24-h post-injury (Patel et al., 2014; Stewart et al., 2022b). Interestingly, however, 14-month mice did not have a consistent decrease in GSH after SCI, likely a consequence of having pre-depleted levels of GSH in uninjured conditions (Stewart et al., 2022b). Gpx activity levels in 4-, but not 14-month, mice is significantly increased in response to SCI (Stewart et al., 2022b). In contrast, 14-month mice have an increase in Gpx activity independent of SCI (Stewart et al., 2022b), likely as a compensatory mechanism responding to increased basal levels of ROS production with advanced age within the spinal cord (von Leden et al., 2017). Taken together, an age-dependent decrease in GSH likely sensitizes older mice to oxidative stress acutely after spinal trauma (Stewart et al., 2022b).

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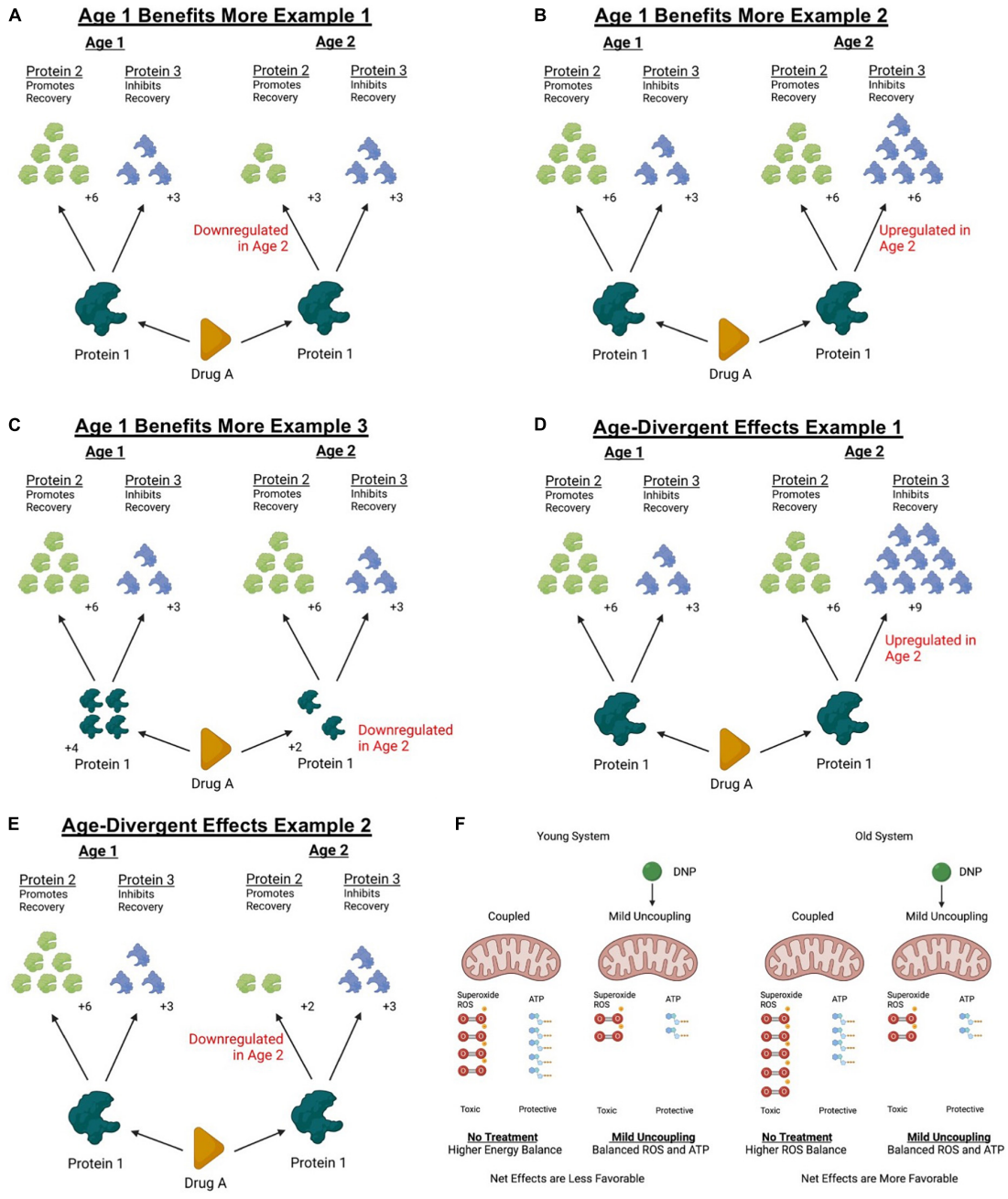

FIGURE 1

Theoretical models of how age can affect response to treatment. Treatments targeting molecular mechanisms often exert downstream influence on several biological targets. Downstream effectors of a therapeutic intervention can exert both permissive and detrimental effects that can compete to determine the net outcome of a treatment. Aging can change protein abundances of diverse molecular pathways as well as affect cellular responses to injury. Panels **(A–F)** represent some simplified theoretical models of how changes occurring with age can affect net responses to treatment. **(A)** For example, if a reparative downstream effector of an interventions biological target is downregulated with age, older systems might not respond with as large of a therapeutic response. **(B)** Similarly, if a detrimental effector of the intervention is upregulated with age, the net effects might appear smaller in an older animal or disappear altogether. **(C)** Further, the abundance of the biological target itself could be differentially regulated with age and affect treatment efficacy. **(D)** It could also be possible for the net balance of a treatment to change from a beneficial effect to a toxic effect if detrimental downstream regulators are upregulated beyond the effectors that might confer a treatment benefit. **(E)** Or finally, the abundance of the beneficial biological target could be downregulated with age to a point where detrimental effects outweigh a therapeutic effect. In the case of a discussed example above (see Section "Mitochondrial function"), treating 4- and 14-month mice with a mild mitochondrial uncoupler exerted age-divergent effects. **(F)** While it is unknown exactly why the age divergent response was observed, it could be possible that the duality of the treatment response shifted toward conferring a beneficial outcome in older mice. Knowing that older mitochondria produce more ROS and less ATP at rest, it remains possible that older mice exhibited a greater decrease in ROS sufficient to outweigh the detrimental effects of reduced ATP production while uncoupled. This figure was created with BioRender.com.

Targeting GSH dysfunction after SCI has been performed by providing cysteine analogs such as n-acetylcysteine (NAC) or a more recently developed *n*-acetylcysteine amide (NACA) which has better bioavailability within the spinal cord (Kamencic et al., 2001; Hanci et al., 2010; Karalija et al., 2012, 2014; Patel et al., 2014; Guo et al., 2015; Olakowska et al., 2017; Stewart et al., 2022b). Treating SCI in rats with NAC or NACA restores cellular levels of GSH, protects against oxidative stress, and improves mitochondrial, behavioral, and histopathological outcomes (Kamencic et al., 2001; Hanci et al., 2010; Karalija et al., 2012, 2014; Patel et al., 2014; Guo et al., 2015; Olakowska et al., 2017). Owing to a pre-existing decrease in GSH with older age, as well as evidence of more ROS damage accumulating in older SCI-mice (Zhang et al., 2016, 2019; Stewart et al., 2021b), we predicted that NACA treatment would have a robust protective effect in 14- compared to 4-month mice. Contrary to this hypothesis, 14-month mice treated with NACA trended toward worse functional and histopathological outcomes, despite observing a significant increase in GSH and an improved redox ratio (GSH/GSSG) (Stewart et al., 2022b). While the mechanisms underlying these trends remain unknown, this work provides yet another example of how treatment responses can be unpredictable at different ages, even when the biological underpinnings change with age in a manner that point to a seemingly obvious outcome.

Mortality and health differences

While the effects of aging on inflammation, mitochondrial function, and redox metabolism have been well-characterized in many physiological systems, there are other consequential interactions between age and SCI not directly related to central pathology. Similar to clinical findings, older mice experience greater mortality after SCI (Genovese et al., 2006; Takano et al., 2017; Stewart et al., 2020; Gaudet et al., 2021; Martín-López et al., 2021). Findings from several independent laboratories indicate that older mice die more frequently compared to younger mice within weeks following injury (Genovese et al., 2006; Takano et al., 2017; Stewart et al., 2020; Gaudet et al., 2021; Martín-López et al., 2021). More intriguingly, older male mice experience a higher mortality compared to older females at 14-months of age (Stewart et al., 2020). Clinical reports are similar, finding that either advanced age or being male is associated with increased mortality within a year post-SCI (Furlan and Fehlings, 2009; Furlan, 2021). The reasons for increased mortality with age in humans are likely multifaceted and difficult to model in rodents (as discussed in the next paragraph). Even in rodent models where comorbidities can be controlled, the reason for this age- and sex-dependent mortality remains unknown.

The consequences of being older at the time of injury are not just associated with an increase in unexpected death but are also associated with other measures of morbidity such as weight loss. Older rats and mice lose more weight after SCI as a percentage of body weight compared to younger rats and mice (Siegenthaler et al., 2008b; Stewart et al., 2020). Notably, weight changes in animal models follow a different trajectory compared to clinical populations and may be a better metric of morbidity in animal models. Both weight loss and gain are observed in clinical populations depending on several factors potentially affected by mobility and rehabilitation (Powell et al., 2017). Where-as total weight loss may not significantly differ between ages after SCI in humans (Powell et al., 2017), older age has been associated with a shift toward less lean muscle mass and more body fat distribution (Spungen et al., 2003).

The cause of increased mortality in older male mice remains unknown, but our observations point to a potential hematologic contributor. We recently reported that 14-month male mice appeared colder to the touch in the days following SCI and that 14-month male mice had noticeable less red blood cells (RBCs) in the blood after spinning down plasma compared to 4-month mice (Stewart et al., 2020). SCI induced a sex-by-age interaction in the RBC/Plasma ratio when normalized to sham-injured controls with ratios significantly decreased in older 14-month male mice by 28-dpi relative to younger 4-month male mice after injury. When evaluating the effects of aging alone on hematopoiesis a few studies have corroborated these findings. Male mice are reported to have a significant decrease in hematopoiesis during middle age whereas females do not (So et al., 2020). Anemia is reported in middle-aged male mice independent of SCI with the effects profound enough that the authors proposed using the aged C57BL/6 male mice as a model for anemia (Guo et al., 2014). Whether this age- and sex-dependent decrease in RBCs is relevant to the observed mortality remains unknown, but does point to a potential systemic contribution to outcome differences that change with age, and most importantly in a sex-dependent manner after SCI.

Finally, a recent study by Hook et al. (2022) identified that bone volumes decrease in an age-dependent manner. Both male and female mice experience a loss of bone volume with aging alone (2–3 vs. 20–23 months), however, only younger mice have a compounding loss of bone volume after SCI. While SCI did not compound an age-dependent loss in bone volumes, Hook et al. (2022) emphasize that the significant loss of bone volumes independent of injury in older mice could already be approaching a floor effect, thus prohibiting the identification of an SCI-induced decrease. An important emphasis in the study by Hook et al. (2022) is that older mice did not present with worse functional outcomes. While only a few reports have not identified an effect of aging on motor outcomes (Nishi et al., 2020; Hook et al., 2022; Stewart et al., 2022b), in the study by Hook et al. (2022), a lack of functional differences presented a unique opportunity to understand that systemic effects of SCI, rather than functional ability/activity or weight supporting, might underly the bone loss at younger ages (Hook et al., 2022).

Male and females do not age the same

While work including age or sex as a biological variable is amassing, it remains important to recognize that males and females do not biologically age the same. The influence of sex hormones and how they change with age can give rise to reciprocal effects on several biological processes. For example, estradiol, which is believed to exert neuroprotective effects in SCI, sharply declines during menopause in humans (Kachadroka et al., 2010; Koebele and Bimonte-Nelson, 2016; Stewart et al., 2020). Not only is this hormonal trajectory unique to females, but it also does not occur to the same extent in rodents. In contrast to humans, mice, and rats experience only a small decrease in estradiol relative to the average plasma estradiol in younger rodents (approximately 75% of average) (Lu et al., 1979; Dubal et al., 2012), but maintain a chronic retention of estradiol after reproductive senescence (Koebele and Bimonte-Nelson, 2016; Stewart et al., 2020). Young female rodents undergo normal cycling of the estrus cycle and experience a corresponding cycling of plasma estradiol levels (Lu et al., 1979). In contrast, reproductive senescent rodents sustain a chronic estrous phase and corresponds to a sustained maintenance of estradiol in the blood (Lu et al., 1979). Depending on the cycle stage in younger rodents and the age of the older comparison, the sustained estradiol levels in reproductive senescent rodents may be higher or lower compared to younger rodents which increases the challenges with comparing across ages (Lu et al., 1979). It is therefore important to acknowledge that animal models focused on elucidating the effects of aging can be difficult to extrapolate to the human condition, particularly if only female rodents are utilized.

Similar to humans, testosterone levels in rodents decreases with advancing age (Machida et al., 1981). While the role of testosterone on SCI injury and recovery is poorly understood, there is evidence that testosterone may play a neurotoxic role (Hauben et al., 2002). Specifically, castration of mice and rats prior to SCI or delivery of a testosterone antagonist both resulted in improvements in motor recovery in males after SCI. It therefore may be possible for an age-related decline in testosterone to be mildly neuroprotective within the injured spinal cord. In contrast, however, a decline in testosterone with advancing age is associated with reduced erythropoiesis which leads to an increase in anemia in older mice (Guo et al., 2014), and may account for an increased mortality found at older ages in rodents. Ultimately the effects of decreased testosterone with age on the SCI central or peripheral pathology is not well-understood.

Thus far, we have identified several sex-by-age interactions which validate that some age-dependent injury responses are sex-specific. Specifically, the example provided above that identified a sex-by-age interaction of RBC/Plasma ratio is indicative of how aging can differentially affect outcomes in a sex-specific manner (Stewart et al., 2020). Further, we and others have previously reported that male mice have an early acute proliferation of microglia within and surrounding both SCI and TBI lesions (Doran et al., 2019; Stewart et al., 2021a), however, this sex difference disappears in middle-age (Stewart et al., 2021a). Further, IgG which infiltrates the spinal cord following SCI follows an age- and sex-dependency with 14-month female mice having a significantly larger increase with older age compared to male mice (Stewart et al., 2022a). Although additional studies are needed to evaluate sex-by-age interactions in animal models of SCI, these three examples highlight that the effects of aging may not be generalizable across sexes.

Complications with investigating age as a biological variable in animal models

While a consensus across several labs has concluded that older age reduces functional recovery after SCI in rodents, it remains important to highlight a few potential caveats that exist in animal modeling. First, it is impossible to test the same cohort of mice at two different ages at the same time, resulting in the use of different cohorts to represent differences in age. In some cases, the challenge associated with obtaining older mice has resulted in experimental strategies which utilize either retired breeders from animal colonies or the use of mixed populations of young and old mice from different colonies such as those purchased from the Jackson laboratories, Charles River, and the National Institute of Aging (NIA) aged animal repository. Utilizing animals from different colonies has an inherent potential to influence the aging process and SCI pathophysiology in unanticipated ways. For example, the C57BL/6 mouse lines have developed different but known mutations within the Charles River and Jackson Laboratory colonies. C57BL/6N mice from Charles River carry a recessive mutation in the Crb1 gene known to induce ocular lesions that impair visual perception in homozygous mice from the breeding colony (Mattapallil et al., 2012). In contrast, C57BL/6J mice from the Jackson Laboratory carry a mutation in the nicotinamide (NAD) nucleotide transhydrogenase (NnT) gene which induces impaired glucose metabolism (Ripoll et al., 2012; Gameiro et al., 2013), and affects inflammatory macrophage phenotypes (Ripoll et al., 2012; Fontaine and Davis, 2016). Importantly, however, while the C57BL/6 mouse colony from NIA is maintained by Charles River, the colony is separate from the Charles River mice and originated from the Jackson Laboratory C57BL/6J strain. This provides one example of how matching cohorts appropriately can be challenging in aging studies. Regardless, aging has been a predictor of worse recovery across all age-matching strategies.

Another complication with including different ages is the role of anatomical growth both of the animal as a whole, as well as the spinal cord in particular. Mice and rats gain weight with age, which can confound the interpretation or analysis of results. Of significant interest is an increase in spinal cord size and diameter with aging (Hooshmand et al., 2014; Stewart et al., 2021a,b). Fourteen months mice have a spinal cord of up to 1.4x the diameter of a 4-month mouse which adds challenges to interpreting outcomes. For example, 14-month mice have significantly larger lesions compared to 4-month mice after SCI, but when data is normalized to a percent of total tissue the differences between ages is reduced (Hooshmand et al., 2014). In this situation, the same injury force manifests in a larger total area of lesioned tissue in older mice, but the magnitude of an age-dependent effect is reduced when considering proportional differences in size. The size difference of the spinal cord has the potential to affect other outcomes as well, such as evaluating for inflammatory cells within or surrounding the lesion (Li et al., 2022). For example, event counts obtained from flow cytometry might represent more total tissue obtained from older mice even if the length of tissue obtained was held consistent. Evaluation of cells using stereological approaches to obtain a cell count density per-cubic area would normalize outcomes but could also be compromised by how data is normalized. Normalizing to lesion area might not provide the most meaningful outcomes, particularly if one would not expect cell densities to differ within the immediate lesioned environment. Normalizing cell counts to total section area might provide a better comparative assessment between two age groups as opposed to limiting assessments within the lesion or absolute counts. Regardless of the details, studying age as a biological variable is complicated by nuanced differences that are not readily apparent on a first-glance analysis of data, such as an age-dependent accumulation of auto-fluorescent lipofuscin in macrophages that can affect immunohistochemical analyses (Vida et al., 2017).

Baseline differences are not simply limited to anatomical discrepancies that develop with aging. Baseline differences in functional outcomes, such as sensory thresholds and measures of forelimb strength, are reported to decline with older ages in uninjured rodents (Gaudet et al., 2021; Stewart et al., 2022b). In three separate studies, two evaluating thermal sensitivity and one forelimb strength, older mice had lower baseline values prior to SCI but identical absolute values compared to younger mice after injury (Nishi et al., 2020; Gaudet et al., 2021; Stewart et al., 2022b). If interpreting data as an absolute value, there is no difference between ages in thermal sensitivity of the hind paws after thoracic SCI, or strength of the ipsilateral forepaw after cervical hemi-section. If these values were interpreted as a percent change from baseline, it would appear that younger mice had a larger proportional loss. It becomes difficult to extrapolate these outcomes to clinical relevance when this age-dependent effect was driven by differences at baseline. Specifically, it is difficult to answer whether absolute values or percent change from baseline are the most meaningful outcome to consider.

Finally, while mortality can be mitigated to a degree in animal models, as reported above, several reports have detected an increased early death of mice and rats after SCI at older ages (Genovese et al., 2006; Takano et al., 2017; Stewart et al., 2020; Gaudet et al., 2021; Martín-López et al., 2021). Observing differences in mortality does potentially introduce a selection bias by allowing successful data collection only from the most robust older animals. Overall, while it is possible to control and account for many confounding variables introduced by including age as a biological variable in pre-clinical SCI studies, there are still important considerations for evaluating age and appropriately interpreting obtained data.

Discussion

To summarize key findings from this literature review, age as a biological variable effects SCI injury and recovery processes as well as responses to treatments in often unpredictable ways. The average age at time of injury has increased to a mean age of 43 which creates a need to better understand the role of age in the SCI pathophysiology. There are several differences in the etiology of SCI between younger and older clinical demographics, the most pronounced of which is the primary mode of injury is caused by a higher prevalence of slip-and-fall accidents at older age. Slip-and-fall accidents are hallmarked by less severe conditions, often manifesting in central cord syndrome-like pathology. Differences in the primary mechanism of injury and/or severity of the initial insult makes comparing outcomes throughout the spectrum of age challenging. Indeed, several clinical reports implicate older age at the time of SCI as a variable which negatively impacts recovery, however after accounting for confounding variables, the impacts of age are less clear. The use of animal models to better understand the recovery potential throughout the spectrum of age has reproducibly determined that older age limits the recovery of motor functions after SCI. Mechanisms underlying a diminished functional recovery after SCI at older ages have implicated a reduced capacity for axon growth and regeneration in both non-intervened and intervened conditions, as well as more severe secondary injury cascades. Aging affects the acute pathophysiology of SCI through changes occurring at the level of inflammation as well as subcellular microenvironments. Macrophages which infiltrate the lesion after SCI display more aggressive and pro-inflammatory phenotypes that generate more reactive oxygen species at older ages. Within the cell, mitochondria accumulate age-related dysfunctions even prior to SCI that result in the generation of more oxidative stress as well as a reduced capacity to buffer cytosolic calcium. Oxidative damage is increased in the sub-acute stages of SCI at older ages, which can be explained

likely by a combination of both an increase in the production of reactive oxygen species and a decrease in antioxidant defense. Most intriguingly, treating older mice after SCI with several antioxidant-based strategies has resulted in outcomes which would either be predictive based on the underlying biology, or completely counterintuitive to the logical hypothesis. The examples provided in this review emphasize an emerging pre-clinical notion that age at time of SCI can unpredictably affect treatment responses.

Conclusion

Using animal models of SCI, we have identified a precedence for considering age as a biological variable in both pre-clinical and clinical research studies aiming to develop treatment approaches. The underlying pathophysiology of SCI changes with age in meaningful ways and treating those biological maladaptation's yields unpredictable results. We have, thus far, identified treatment approaches that display larger and smaller treatment effects in older animals, as well as opposite effects to those observed in younger rodent counterparts. While we elucidate how aging affects different elements of the pathophysiology of SCI, we are finding that treatment approaches might not act the same throughout the spectrum of age. Most interventions acting on biological targets often have multiple effects, some beneficial to recovery and others detrimental, and changing the balance of these nuanced effects can influence the outcomes of treatment efforts. It is not difficult to imagine models in which a single molecular target can both promote recovery and damage, and to identify how changes in a system with older age can alter the net response to treatment (**Figure 1**). Following this review, we propose that therapeutic treatments should be examined across the spectrum of age in pre-clinical models prior to investing time and resources into human investigations. Results from animal testing will re-enforce clinical trial design by providing insights into which age ranges are most susceptible to experiencing a benefit from treatment, while simultaneously helping to avoid potential adverse outcomes that can hurt both the clinical trial success as well as individuals. The pre-clinical findings reviewed above make an argument for a need to include age as a biological variable in clinical research design and interpretation, at the very least by using animal models to guide the age inclusivity. Beyond the implications to clinical research, emerging evidence in basic science is supporting the idea that treatment efforts deemed marginal or insignificant in young rodent models may potentially exert a more significant effect at older ages, and therefore can implicate a re-evaluation of treatment efforts in older animal models. Collectively, work highlighted above emphasizes the need to include age as a biological variable for emerging treatment approaches to help more accurately predict safety and efficacy of therapeutic advances toward clinical trials.

Author contributions

AS and JG: conceptualization and funding acquisition. JG: supervision. LJ: writing of the clinical aspects. All authors: writing and approving the submitted version.

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Conflict of interest

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.

Publisher's note

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Consequences of spinal cord injury on the sympathetic nervous system

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Spinal cord injury (SCI) damages multiple structures at the lesion site, including ascending, descending, and propriospinal axons; interrupting the conduction of information up and down the spinal cord. Additionally, axons associated with the autonomic nervous system that control involuntary physiological functions course through the spinal cord. Moreover, sympathetic, and parasympathetic preganglionic neurons reside in the spinal cord. Thus, depending on the level of an SCI, autonomic function can be greatly impacted by the trauma resulting in dysfunction of various organs. For example, SCI can lead to dysregulation of a variety of organs, such as the pineal gland, the heart and vasculature, lungs, spleen, kidneys, and bladder. Indeed, it is becoming more apparent that many disorders that negatively affect quality-of-life for SCI individuals have a basis in dysregulation of the sympathetic nervous system. Here, we will review how SCI impacts the sympathetic nervous system and how that negatively impacts target organs that receive sympathetic innervation. A deeper understanding of this may offer potential therapeutic insight into how to improve health and quality-of-life for those living with SCI.

KEYWORDS

spinal cord injury, sympathetic nervous system, dysregulation, sympathetic innervation, preganglionic neurons, therapeutics

1. Introduction

Trauma to the central nervous system (CNS) results in debilitating damage to multiple motor, sensory, and autonomic circuits (Norton and Kobusingye, 2013). Spinal cord injury (SCI) is a devastating form of neurotrauma that is often associated with paralysis but can also result in permanent dysfunction of organs within multiple physiological systems. The pathophysiology of SCI contains two discrete stages: a primary injury and a secondary injury. The primary injury refers to the actual mechanical, tissue damage immediately following injury (reviewed in Alizadeh et al., 2019). There are a variety of different types of primary injuries, e.g., a contusion, a compression, a long-term compression, and a partial or complete transection. Different types of SCI can produce a more severe injury. For example, a complete transection of the spinal cord severs all of the axons within the cord at the level of the injury, leaving no spared tissue. Alternatively, a contusion or a blow to spinal cord often leaves some spared tissue that may be a substrate for functional recovery post-SCI. Therefore, the level and type of injury can greatly influence the amount of dysfunction produced from a SCI. The secondary injury, initiated within minutes of the primary injury event, refers to a

multitude of biochemical, cellular, and molecular changes that continue to ensue for weeks to months and further damage the lesion site, and surrounding tissue within the cord, and affected peripheral organs (Alizadeh et al., 2019).

The loss of function that results after SCI is determined by the severity and at which level of spinal segments the injury happened. The higher and more severe the SCI, a larger region of spinal cord is impacted and is no longer connected to the brain. For instance, in the context of sympathetic regulation, a complete SCI above thoracic segment 1 (T1), results in complete loss of modulatory, descending inhibitory supraspinal control over the sympathetic preganglionic neurons (SPNs) located in thoracolumbar spinal cord below the level of injury. This loss of inhibitory control biases the sympathetic system to a more excitatory state. Moreover, intraspinal plasticity of propriospinal and sensory neurons caudal to the injury also influences increased activity of spinal sympathetic neurons and therefore loss of descending inhibition from supraspinal regions along with the intraspinal plasticity both contribute to heightened sympathetic reflexes that culminate in the dysfunction of peripheral organs receiving sympathetic input. On the other hand, a SCI at a lower level (i.e., T11) would disrupt any organ systems receiving sympathetic input below the injury site, but less of the sympathetic system would be "disconnected" from the brain, resulting in fewer organ systems directly impacted by a lower-level SCI than a higher one. Here, we will review the anatomy of the autonomic nervous system, with a focus on the sympathetic nervous system, and how a SCI impacts function of effector organs that are innervated by the sympathetic nervous system.

2. Organization of the autonomic nervous system

The autonomic nervous system plays an important role in maintaining homeostasis and modulates the involuntary activity of most peripheral organ systems within the body (McCorry, 2007). The sympathetic and the parasympathetic nervous systems are subdivisions of the autonomic nervous system that provide control over multiple peripheral organ systems (**Figure 1**). The sympathetic nervous system mainly functions during "fight or flight" situations to increase arousal states. The parasympathetic system is involved in "rest and digest" situations to conserve energy for later use.

The SPNs reside in the intermediolateral cell column (IML) of thoracolumbar spinal cord segments T1-L2 (McLachlan and Oldfield, 1981; Oldfield and McLachlan, 1981; Anderson et al., 1989). SPN projections exit the spinal cord *via* the ventral root and release acetylcholine onto sympathetic postganglionic neurons located in ganglia within the sympathetic trunk (also known as the sympathetic chain). From here, sympathetic postganglionic neurons send adrenergic projections to a variety of target organs. Catecholaminergic neurotransmitters, like norepinephrine and epinephrine, are responsible for the "fight or flight" response.

Parasympathetic preganglionic neurons originate within the brainstem and the sacral spinal cord segments (S2–S4). The preganglionic neurons within the brainstem project out *via* cranial nerves III (oculomotor), VII (facial), IX (glossopharyngeal), and X (vagus) (McCorry, 2007; Gibbins, 2013). These parasympathetic preganglionic nerves innervate eye muscles, lacrimal and salivary glands, nasal cavity, and organs in the thorax and abdomen such as the heart, lungs, and digestive system. The parasympathetic preganglionic neurons within the sacral spinal cord exit *via* the pelvic splanchnic nerves to innervate the organs in the lower portion of the body, such as the lower portion of the large intestine, urinary organs, and reproductive organs (McCorry, 2007). Axons of the preganglionic neurons of the parasympathetic system tend to be longer than the axons of the SPNs and project out of the cord to synapse onto parasympathetic ganglia located closer to the target organs compared to the ganglia of the sympathetic nervous system. Pre- and postganglionic neurons of the parasympathetic nervous system release acetylcholine onto effector organs.

Both sympathetic and parasympathetic nervous systems are modulated *via* descending supraspinal control from the brain. Specifically, the paraventricular nucleus of the hypothalamus project directly onto both SPNs and parasympathetic preganglionic neurons. The locus coeruleus, medulla, and raphe nucleus are extra-hypothalamic areas that regulate the sympathetic nervous system. Extra-hypothalamic control over the parasympathetic nervous system includes the amygdala, nucleus ambiguous, raphe nucleus, and the periaqueductal gray. Limbic areas such as the amygdala, cingulate gyrus, and insula also provide descending control by projecting to the hypothalamus that directly innervates both the sympathetic and parasympathetic preganglionic neurons.

3. Plasticity of the spinal sympathetic circuit following spinal cord injury

Damage to the spinal cord severs the connections from supraspinal centers to the spinal sympathetic reflex circuit (**Figure 2**). Because of this, SCI eliminates the feedback regulation of autonomic reflexes and disrupts function of multiple organs and systems that receive sympathetic and parasympathetic input. This is described in more detail below. Not only does SCI sever supraspinal control over the SPNs of the spinal sympathetic circuit, but it also results in neuroplasticity, i.e., changes that affect function of the circuit caudal to the injury site. Following SCI, plasticity occurs at the synapse, cellular, and circuit levels. This intraspinal plasticity within the spinal sympathetic reflex circuit has been shown to contribute to the development of sympathetic hyperreflexia and consequent damage to peripheral organs which increases the risk of mortality in individuals living with SCI.

3.1. Primary afferent sprouting

Following SCI, there is sprouting of nociceptive, primary afferent fibers caudal to the injury site, as seen with increased $CGRP^+$ (a marker for nociceptive fibers) fibers in the dorsal horn as well as around laminae VII/X around the central canal (Krenz and Weaver, 1998; Hou et al., 2009; Mironets et al., 2018, 2020). This sprouting can be detected as early as 2-weeks post SCI and is thought to be one reason behind increased activation of propriospinal neurons by sensory stimuli described above (Krenz and Weaver, 1998; Hou et al., 2009; Mironets et al., 2018, 2020). In rodent models, this $CGRP^+$ fiber sprouting has been shown

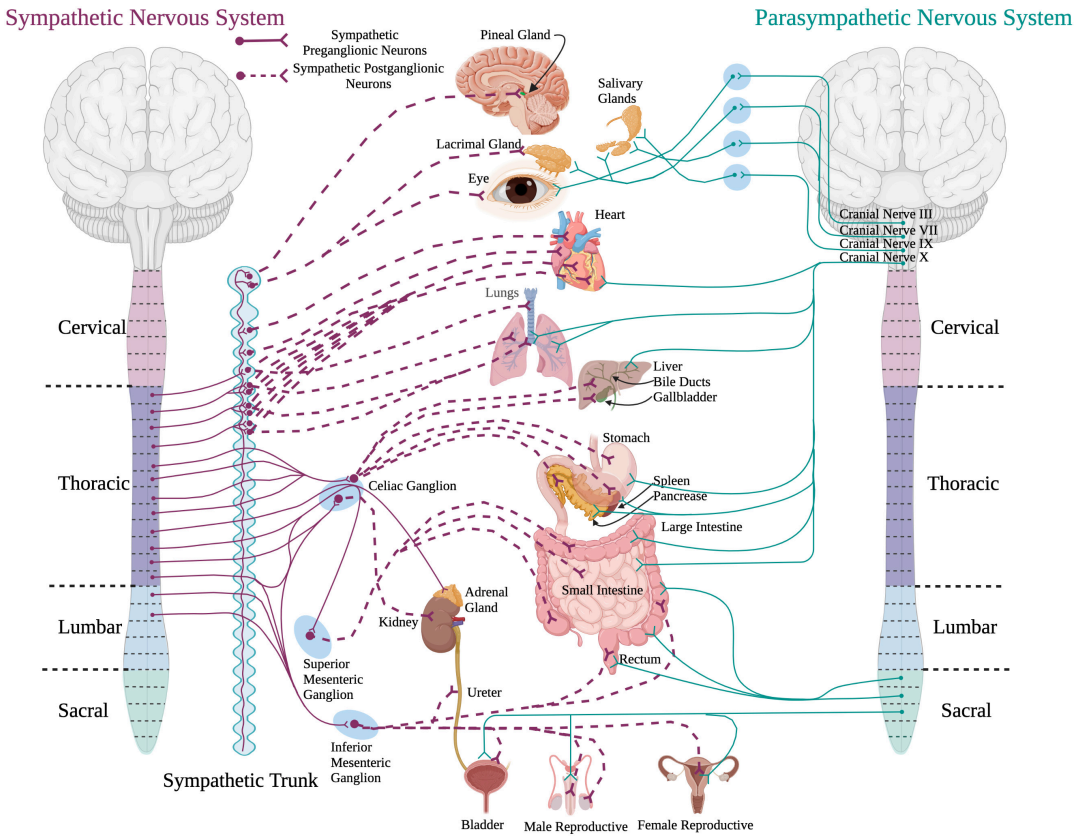

FIGURE 1
A schematic of the sympathetic and parasympathetic innervation to peripheral organs. Sympathetic preganglionic neurons reside in the intermediolateral cell column in the thoracolumbar spinal cord and project out to and synapse upon sympathetic postganglionic neurons within the sympathetic chain. The sympathetic postganglionic neurons innervate target organs in the periphery. For the parasympathetic system, preganglionic neurons residing in the brainstem and sacral spinal cord send projections directly to target organs in the periphery. (Created using www.biorender.com).

to be increased within the laminae VII/X around the central canal 8-weeks post injury but not increased within the dorsal horn (Mironets et al., 2020). However, within the human SCI population, $CGRP^{+}$ fiber sprouting has been shown to be present in the chronic stages after SCI (Ackery et al., 2007). Interestingly, increased afferent sprouting has been shown to be dependent on nerve growth factor (NGF) signaling which is also increased following SCI (Bakhit et al., 1991; Brown et al., 2004; Donnelly and Popovich, 2008). This is further supported by the data that shows blocking NGF *via* intrathecal administration of a NGF neutralizing antibody following SCI attenuates this SCI-increased sprouting (Krenz et al., 1999), demonstrating the role of these fibers in sympathetic dysfunction.

3.2. Propriospinal and interneuron plasticity

Plasticity of both long-distance propriospinal interneurons as well as local interneurons of the spinal sympathetic circuit has also been implicated in sympathetic dysregulation after SCI. For instance, following SCI, there is sprouting of the primary nociceptive afferents onto long-distance propriospinal neurons, spinal interneurons that projecting across multiple spinal segments (Krenz et al., 1999; Cameron et al., 2006; Hou et al., 2008), including those that project from lumbosacral spinal cord rostrally toward the IML in thoracic spinal cord. As a result, a sensory stimulus below the level of injury could robustly activate more propriospinal interneurons. This increased activation of propriospinal neurons leads to (1) activation of SPNs directly or (2) activation of many local sympathetically correlated interneurons residing in the IML that in turn activate SPNs (Krassioukov et al., 2002; Landrum et al., 2002; Hou et al., 2008; Ueno et al., 2016; Mironets et al., 2018). Local spinal interneurons in the IML in close proximity to SPNs largely contribute most of the input to SPNs. After SCI, it has been shown that a sensory stimulus below the level of injury results in increased activation of this neuron population (Ueno et al., 2016; Mironets et al., 2018). Inhibiting these local excitatory interneurons in the thoracic spinal cord near SPNs effectively diminishes the increased excitability of the spinal sympathetic circuit following injury as well as diminishing SCI-immunosuppression (Ueno et al., 2016; Noble et al., 2022). Moreover, silencing these interneurons delays the onset of SCI-induced plasticity of the sympathetic circuit (Noble et al., 2022). Interestingly, activating excitatory interneurons in uninjured mice partially replicates the anatomical and molecular changes that are seen within the sympathetic circuit following SCI (Noble et al., 2022). Others have reported a decrease of glutamatergic (excitatory) spinal interneurons and an

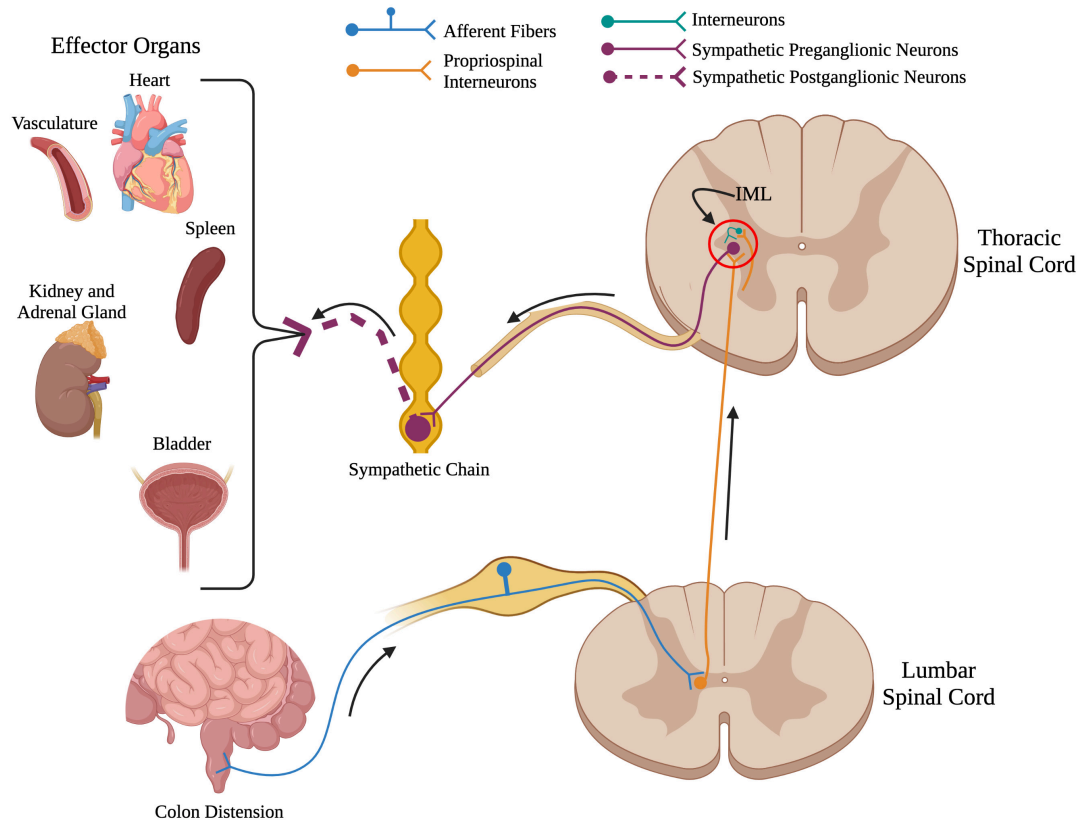

FIGURE 2
Overview of the spinal sympathetic circuit. When there is a stimuli below the level of injury such as colon distension in the above example, primary afferent fibers from that organ travel into the spinal cord and synapse onto propriospinal interneurons located near the central canal. From here, propriospinal interneurons travel rostrally across many spinal segments to synapse upon either (1) sympathetic preganglionic neurons located in the intermediolateral cell column, or (2) interneurons located near sympathetic preganglionic neurons. Sympathetic preganglionic neurons project out to the sympathetic chain to synapse onto sympathetic postganglionic neurons that innervate target organs in the periphery. (Created using www.biorender.com).

increase of GABAergic (inhibitory) spinal interneuron inputs onto SPNs (Llewellyn-Smith and Weaver, 2001). However, this increase in GABAergic inputs has been suggested to diverge from their canonical, inhibitory function and assume an excitatory role after SCI (Huang et al., 2016). It is apparent that interneuron plasticity is complex and much remains to be understood.

3.3. SPN plasticity

Sympathetic preganglionic neurons project out of the spinal cord through the ventral root and depolarize postganglionic neurons that then directly synapse onto target organs. SCI severs the supraspinal descending inhibitory control over SPNs leaving them solely under control of spinal circuits such as spinal interneurons of the sympathetic circuit. This loss of descending inhibition over SPNs leads to decreased inhibition of the spinal sympathetic circuit and, therefore, an exaggerated sympathetic reflex response, i.e., sympathetic hyperreflexia (Ueno et al., 2016; Mironets et al., 2020). SPNs also show profound morphological changes following SCI. For instance, within 3 days post-injury, there is atrophy of SPNs (e.g., decreased soma size and dendrite length) caudal to the injury site (Weaver et al., 1997; Krassioukov et al., 1999; Klimaschewski, 2001; Llewellyn-Smith and Weaver, 2001). However, this atrophy appear to be short-lived and SPNs appear normal morphologically by 2-weeks following SCI (Krassioukov and Weaver, 1996).

3.4. Mechanisms that contribute to plasticity of the spinal sympathetic circuit

While elucidating mechanisms that underlie the aforementioned plasticity is still an area of active research, several have been identified. The neuroimmune system is one mechanism that has been proposed to play a role in plasticity in the sympathetic system. Following SCI, there is persistent activation of both central and peripheral neuroimmune and inflammatory processes (Chen et al., 2018). The local inflammatory response post-SCI consists of activation of microglia that reside within the spinal cord and a peripheral immune response consists of infiltrating neutrophils, monocytes, and lymphocytes (T- and B-cells) into the spinal cord. These activated immune cells express factors, such as cytokines [e.g., tumor necrosis factor-alpha (TNFα), IL-1β, and IL-6] and neurotrophic factors (e.g., BDNF, NGF, and BDNF), that have been implicated in driving cellular and

anatomical plasticity within the CNS, including within the spinal cord (Donnelly and Popovich, 2008; O'Reilly and Tom, 2020). As mentioned above, sprouting of $CGRP^+$ fibers nociceptive primary afferent fibers influences activation of the spinal sympathetic circuit and contributes to sympathetic dysregulation after SCI (Krenz et al., 1999; Cameron et al., 2006; Hou et al., 2008; Mantyh et al., 2011; Mironets et al., 2018). Increased levels of NGF after SCI is associated with sprouting of these fibers that express the NGF-responsive receptor TrkA (Averill et al., 1995; Hou et al., 2008; Mantyh et al., 2011).

Chronically elevated levels of TNFα after SCI have also been shown to contribute to plasticity of the spinal sympathetic circuit. Levels of TNFα are chronically elevated after SCI has also been implicated in sprouting of $CGRP^+$ afferent fibers (Mironets et al., 2018, 2020). Inhibiting TNFα signaling *via* the biologic XPro1595 decreases injury-induced sprouting of $CGRP^+$ afferent fibers and diminished recruitment of interneurons into the spinal sympathetic circuit. This was associated with diminished autonomic dysreflexia and immune dysfunction associated with sympathetic hyperreflexia (Mironets et al., 2018, 2020).

Another mechanism that was recently identified is SCI-induced expression of thrombospondins (TSP), proteins known to stimulate synapse formation (Liauw et al., 2008; Eroglu et al., 2009; Tyzack et al., 2014; Risher et al., 2018), specifically excitatory synapses, *via* binding to neuronal α2δ-1 calcium channel subunits (Christopherson et al., 2005; Risher and Eroglu, 2012). Following SCI, astrocytes and macrophages increase their secretion of within hours following the initial injury and remain elevated weeks after the injury (Wang et al., 2009; Boroujerdi et al., 2011; Zeng et al., 2013; Brennan et al., 2021). Blocking TSP binding to neuronal α2δ channel subunits after SCI *via* administration of Gabapentin, an anti-epileptic an analgesic drug that also binds to α2δ results in decreased excitatory synaptogenesis and reduces excitability of the sympathetic circuit (Eroglu et al., 2009; Risher and Eroglu, 2012; Risher et al., 2018), demonstrating that TSP plays a role in plasticity within the spinal sympathetic circuit.

Although maladaptive plasticity following SCI contributes to the development of sympathetic hyperreflexia and damage to peripheral organs, it is important to note that plasticity itself is not always detrimental. For example, the spinal bladder reflex circuit and its ability to partially recover following SCI (discussed in more detail below) is a prime example of a potential beneficial effect of SCI-induced plasticity on sympathetic function.

4. Dysfunction of organ systems following spinal cord injury

4.1. Cardiovascular system

The cardiovascular system delivers oxygen, nutrients, blood, and hormones to sustain peripheral organ systems. In order to do this, the heart pumps blood containing these substances through a complex and vast network of blood vessels present in every type of tissue. Normal cardiovascular function requires a balance of activity between the sympathetic and parasympathetic systems (**Figure 3A**). Specifically, SPNs from thoracic spinal segment 1 to 4 (T1–T4) send projections to postganglionic neurons in the sympathetic chain that then directly innervate the heart. Moreover, parasympathetic neurons in the brainstem, specifically the dorsal motor nucleus of the vagus and the nucleus ambiguous, send projections out of the cord *via* cranial nerves IX and X and directly innervate the heart (Ciriello and Calaresu, 1982; Hopkins, 1987). While the heart is innervated by both sympathetic and parasympathetic nervous systems, the vasculature is innervated solely by the sympathetic nervous system.

Normally, in uninjured individuals, the sympathetic nervous system releases epinephrine and norepinephrine to accelerate the heart rate and blood pressure while the parasympathetic nervous system reduces heart rate and blood pressure. In times of stress or fearful situations, this increased heart rate *via* sympathetic activation further increases blood flow to the muscles and increases the alertness of the individual. Additionally, when baroreceptors in blood vessels detect a drop in arterial pressure, the sympathetic nervous system is activated and increases vasoconstriction of blood vessels in the periphery. Moreover, increased sympathetic activity has been linked to high blood pressure (Mark, 1996; Schlaich et al., 2004; Grassi, 2009).

Spinal cord injury can disrupt supraspinal regulatory control to the SPNs, producing cardiovascular system dysfunction (**Figure 3B**). High-level SCI, i.e., at or above thoracic spinal segment 6 (T6), produces hypotension or low resting blood pressure (Mathias et al., 1979; Krassioukov and Claydon, 2006). Orthostatic hypotension occurs when there is a decrease of the systolic blood pressure by 20 mmHg or more, or a decrease in the diastolic blood pressure by 10 mmHg or more (Freeman et al., 2011). Although spinal shock initially following the injury results in hypotension, this hypotensive response can persist for years following SCI (Krassioukov and Claydon, 2006). Cardiac dysrhythmias can also occur after SCI, depending on the level and severity of injury (Krassioukov et al., 2007). These irregular beats of the heart are more serious initially following high-level SCI but seem to diminish as the injury progresses (Krassioukov et al., 2007). Similarly, individuals with high level injuries commonly have cardiac arrhythmias, specifically bradycardia or slowed heartbeat (Mathias, 1976; Winslow et al., 1986; Lehmann et al., 1987). Bradycardia is problematic mainly in the acute phase of SCI (within the first week), which is thought to be due to the initial spinal shock of sudden loss of descending supraspinal input leaving the spinal sympathetic reflex circuit unchecked and an autonomic unbalance between the sympathetic and parasympathetic systems (Frankel et al., 1975; Mathias, 1976; Lehmann et al., 1987; Grigorean et al., 2009; Shaikh et al., 2016).

Autonomic dysreflexia is a life-threatening disorder that is extremely common in the SCI population (Curt et al., 1997). A noxious stimulus below the level of injury, like a full bladder or fecal impaction, causes a massive sympathetic response which leads to vasoconstriction and ultimately hypertension and a concomitant decrease in heart rate (Curt et al., 1997; West et al., 2015; Partida et al., 2016; Mironets et al., 2018, 2020). Loss of descending supraspinal control as well as plasticity within the spinal sympathetic reflex circuit following SCI results in abnormal, heightened sympathetic responses known as sympathetic hyperreflexia. autonomic dysreflexia is a measure used as a readout of sympathetic hyperreflexia as autonomic dysreflexia hallmarks as sudden hypertension and a concomitant decrease in heart rate (Curt et al., 1997; Krassioukov et al., 2003;

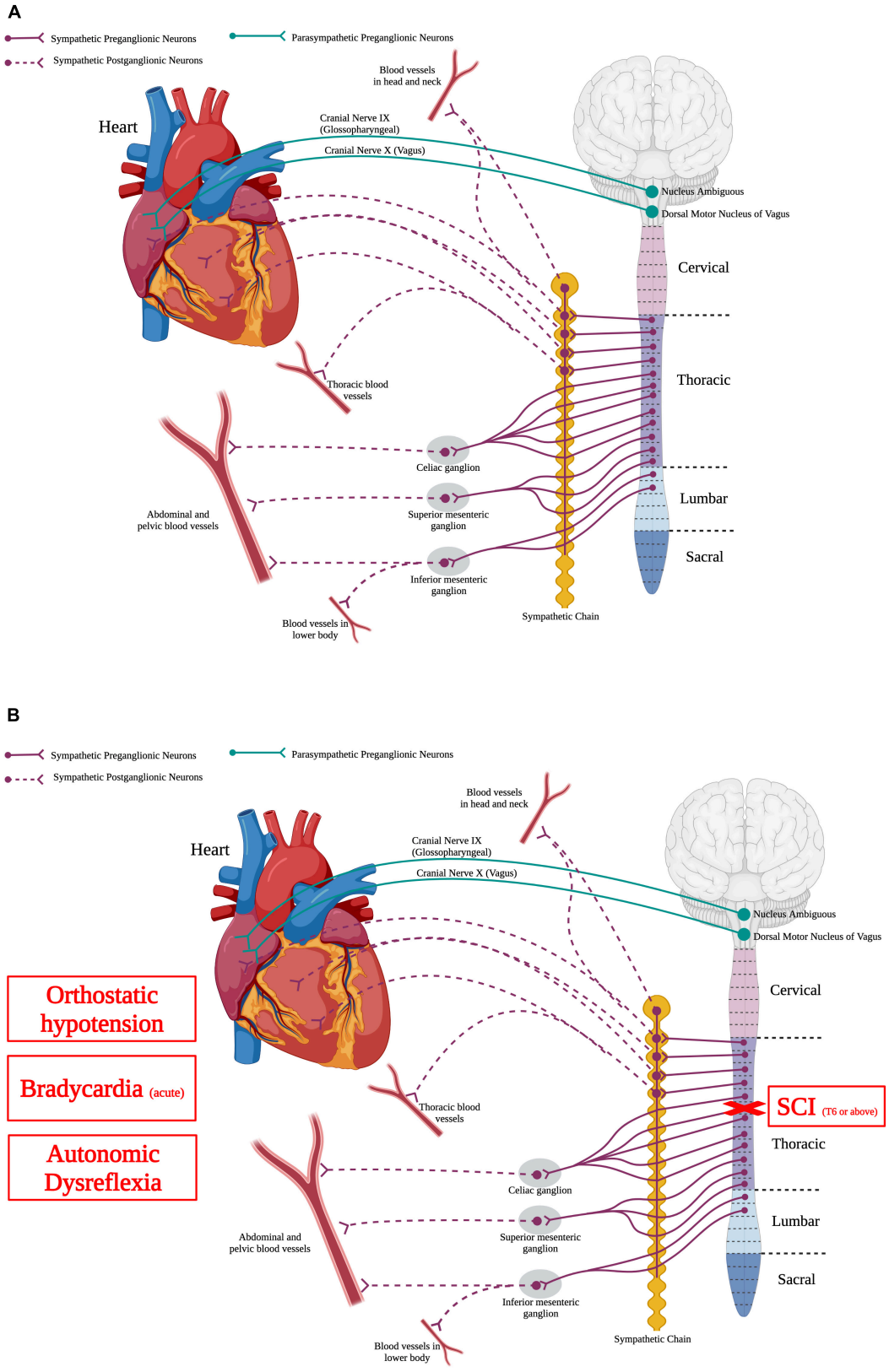

FIGURE 3
Autonomic control of the cardiovascular system and how SCI impacts the cardiovascular system. **(A)** The heart is innervated by sympathetic postganglionic neurons that receive input from sympathetic preganglionic neurons in thoracic segments 1–4. Parasympathetic regulation of the heart originates from the nucleus ambiguous and dorsal motor nucleus of vagus within the brainstem. Autonomic control over the vasculature is solely from the sympathetic nervous system. **(B)** SCI at or above T6 results in deranged sympathetic activity that results in orthostatic hypotension, bradycardia in the acute phase, and autonomic dysreflexia. (Created using www.biorender.com).

Zhang et al., 2013). Moreover, such sympathetic hyperreflexia detrimentally impacts other peripheral effector organs. Over time, the number of autonomic dysreflexia events increase in frequency and severity which ultimately increase the individuals' risk of stroke and myocardial infarction, cardiovascular disease, infection, and death (Krassioukov and Weaver, 1995; Mayorov et al., 2001; Rabchevsky et al., 2012; Zhang et al., 2013; West et al., 2015). Acutely, within the week following SCI, there is early onset of autonomic dysreflexia that is thought to be due to the sudden loss of descending supraspinal input to the spinal sympathetic reflex circuit (Krassioukov et al., 2003; Zhang et al., 2013). However, approximately two weeks post SCI, a second phase of autonomic dysreflexia occurs in which the number of events per day dramatically increase, and the change in blood pressure becomes much more severe (Zhang et al., 2013). This second phase of autonomic dysreflexia is thought to be due to intraspinal plasticity such as sprouting of sensory afferents and propriospinal axons within the spinal sympathetic circuit, leading to sympathetic hyperexcitability as described previously (Cameron et al., 2006; Hou et al., 2008).

Intensification of autonomic dysreflexia over time after SCI has also been associated with detrimental remodeling of the peripheral vasculature (Alan et al., 2010; Rummery et al., 2010; Mironets et al., 2018). After high-level SCI, there is upregulation of adrenergic receptor expression, with results in the vasculature becoming hyperresponsive to vasopressors, e.g., norepinephrine or phenylephrine (Arnold et al., 1995; Landrum et al., 1998; Lee et al., 2016). This remodeling is thought to be a consequence of and also a contributor to the development of autonomic dysreflexia. Furthermore, driving the sympathetic system in rodents with a high-thoracic SCI by repeatedly triggering sympathetic hyperreflexia with colorectal distension results in vasculature that is even more responsive to vasopressors (Alan et al., 2010; Mironets et al., 2018). These data suggest that repeated bouts of sympathetic hyperreflexia exacerbate peripheral cardiovascular dysfunction. Moreover, one could surmise that decreasing the frequency and severity of sympathetic hyperreflexia would diminish detrimental remodeling of the peripheral vasculature. Indeed, treatments that decrease the frequency and severity of sympathetic hyperreflexia after high-level SCI, such as the TNFα inhibitor XPro1595, also attenuate the hyperresponsiveness of peripheral vasculature normally observed after such injuries; arteries from SCI animals treated with XPro1595 have more normal sensitivity to vasopressors than vehicle-treated SCI animals (Mironets et al., 2018).

4.2. Pineal gland

The pineal gland is an endocrine gland located deep within the brain that is one of the many organs innervated by the sympathetic nervous system. While melatonin is produced by a variety of organs, the pineal gland is a major source. Melatonin secretion is significantly elevated during the dark phases of circadian rhythms and plays a role in regulating circadian cycles (Lynch, 1971; Yingli Jing and Yan, 2018).

Normally, the amount of light is sensed by the retina, which sends that information to the suprachiasmatic nucleus, which relays that to the paraventricular nucleus (Moore, 1973; Munch et al., 2002). This information then travels down the fibers of the paraventricular nucleus that synapse on SPNs in the IML of the upper thoracic spinal cord segments. The SPNs from the IML project out to the postganglionic neurons located within the cervical sympathetic chain (Bowers et al., 1984). The postganglionic sympathetic neurons directly innervate the pineal gland and release norepinephrine during dark cycles of the circadian rhythm to stimulate melatonin synthesis (Huang and Lin, 1984; Lumsden et al., 2020).

Spinal cord injury can cause significant dysfunction of the pineal gland, depending on the level of injury. An injury in the location of upper thoracic segments or higher would sever the descending fibers of the paraventricular nucleus to the SPNs that regulate function of the pineal gland. An injury in mid-thoracic or lower levels of the spinal cord would not directly impact the fibers descending from the paraventricular nucleus to the SPNs, thus regulation of the pineal gland would remain intact. Accordingly, melatonin levels of individuals with a cervical spinal injury do not increase during the dark cycles of the circadian rhythm, like they would in most able-bodies individuals or individuals with a lower-level SCI (Kneisley et al., 1978; Fatima et al., 2016). These findings support that SCIs below the SPNs that are responsible for projecting out to the postganglionic neurons that innervate the pineal gland result in intact melatonin and pineal gland functioning. This decreased nighttime melatonin levels in SCI individuals may explain dysfunctions of sleep patterns and proper sleep in this population. Increasing levels of melatonin has been explored as a means to treat able-bodied individuals with perturbed sleep patterns. Interestingly, it has been found that following SCI, the pineal gland is able to produce melatonin again with electrical stimulation of the sympathetic pathway responsible for innervating the pineal gland (Lumsden et al., 2020), identifying a possible means to treat sleep disorders after SCI (Scheer et al., 2006).

4.3. Visual system

Many components of the visual system are also innervated and controlled by the sympathetic nervous system; eye musculature, arteries and nerves around the eyes, and glands associated with the eyes and is ultimately impacted by SCI (**Figure 4**). The sympathetic pathway innervating the visual system is similar to the one innervating the pineal gland that was previously discussed. SPNs located in the IML of the upper thoracic spinal segments project out to the superior cervical ganglion in the sympathetic trunk, where they synapse on postganglionic neurons (Miller et al., 2005; McDougal and Gamlin, 2015). Those postganglionic neurons project axons to the ciliary ganglion and innervate the ipsilateral eye and surrounding structures, like the lacrimal glands (Tóth et al., 1999; Ruskell, 2003).

The pupil is a structure of the eye responsible for regulating the amount of light that is allowed to hit the retina. The size of the pupil directly correlates with the amount of light that is able to pass through to the retina. Because of this function, the pupil must be able respond to changing levels of light quickly by contracting or relaxing. This pupillary light reflex is

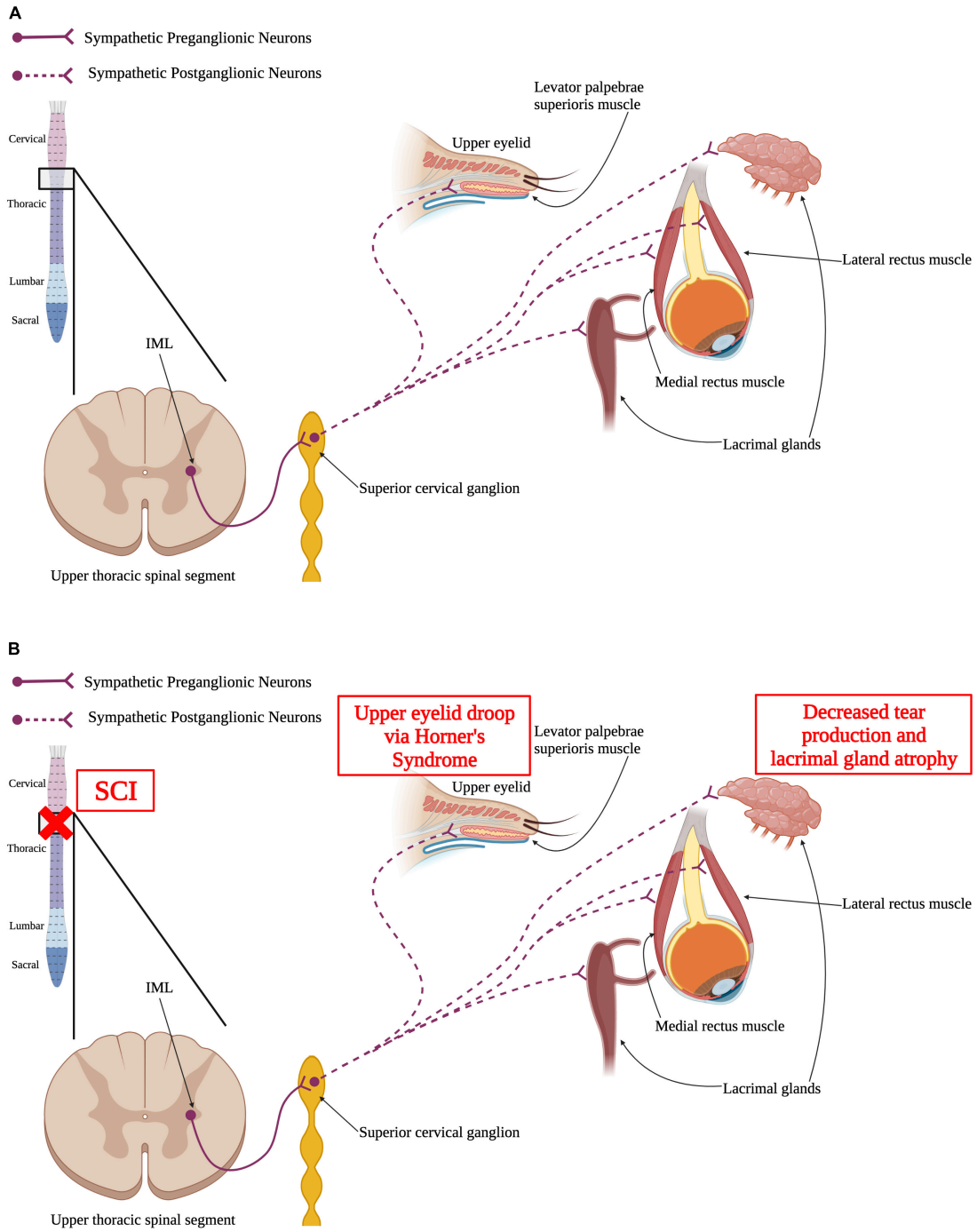

FIGURE 4
Autonomic control of the visual system and how SCI impacts the visual system. **(A)** Sympathetic preganglionic neurons in upper thoracic spinal segments extend out of the spinal cord *via* the ventral horn to synapse upon sympathetic postganglionic neurons in the superior cervical ganglion. These neurons then directly innervate the levator palpebrae superioris muscle within the upper eyelid, the lacrimal glands, and the medial and lateral rectus muscles. **(B)** SCI may result in Horner's syndrome where the upper eyelid begins to droop. Similarly, SCI may cause the lacrimal glands to have reduced tear production and atrophy that can ultimately result in dry eye. (Created using www.biorender.com).

mediated by two muscles, the sphincter pupillae and the dilator pupillae (McDougal and Gamlin, 2015) and is tightly regulated by the autonomic nervous system (**Figure 5A**). The sphincter pupillae muscle is innervated by the parasympathetic nervous system and the dilatory pupillae is innervated by the sympathetic nervous system (McDougal and Gamlin, 2015). Thus, a SCI in the upper thoracic spinal segments or higher could result in an impaired pupillary light reflex (**Figure 5B**). Damage to SPNs in the upper thoracic spinal segments can produce Horner's syndrome, a condition where the pupil is contracted, the upper portion of the eyelid begins to droop, and there is an inability to sweat on one side of the face (Zeitzer et al., 2005; Lee et al., 2007).

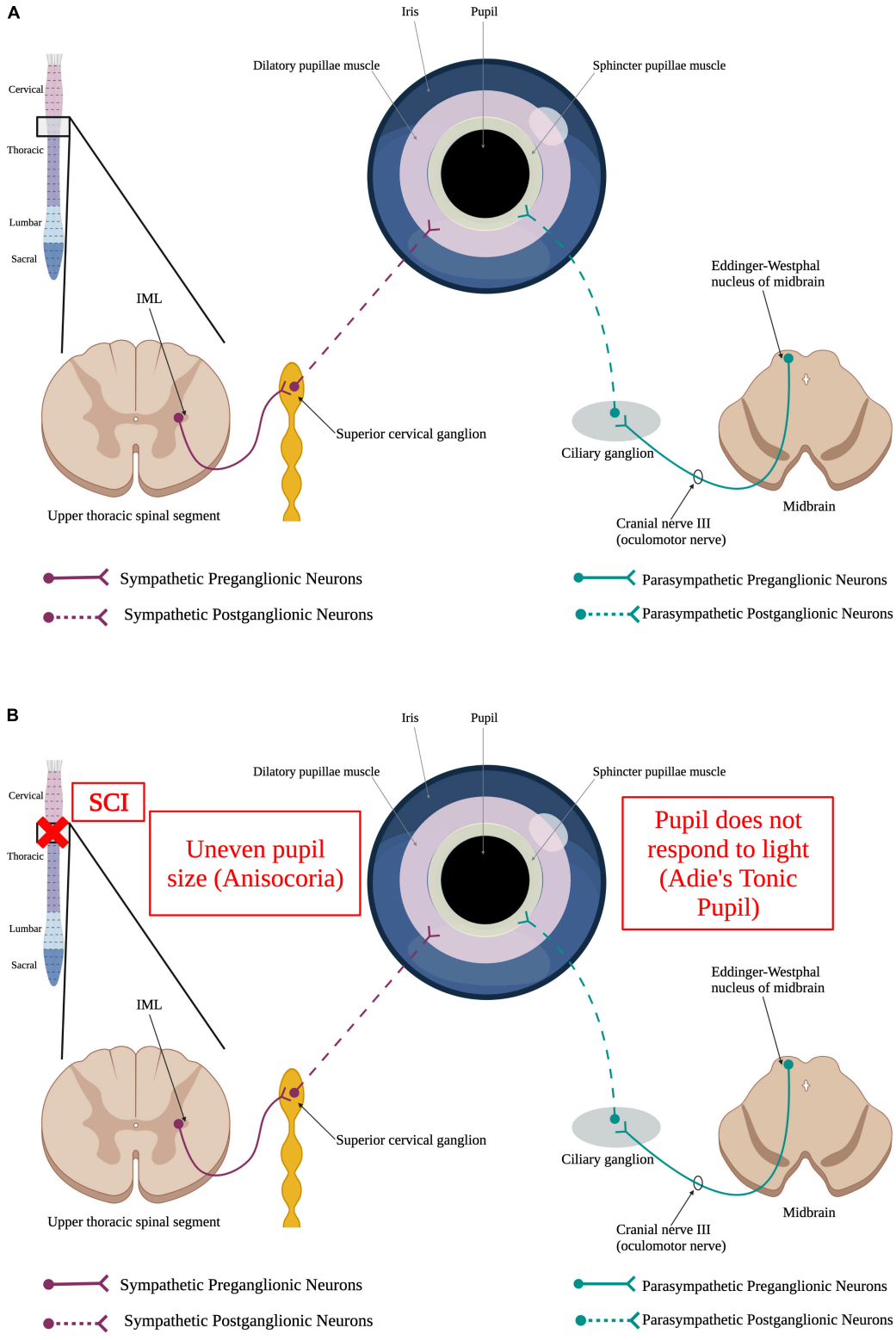

FIGURE 5
Autonomic control of the pupillary light reflex and how SCI impacts it. **(A)** When the retina detects a dim light, it sends a stimulus to the brain *via* the optic nerve. This signal, in turn, results in the activation of sympathetic preganglionic neurons in the upper thoracic spinal cord that synapse onto sympathetic postganglionic neurons within the superior cervical ganglion. Some of these sympathetic postganglionic neurons innervate the dilatory pupillae muscle (pink). Release of norepinephrine from the sympathetic postganglionic neuron terminals cause the pupil to dilate to allow more light to reach the retina. Alternatively, perception of a bright light by the eye results in the activation of the parasympathetic nervous system. Parasympathetic neurons within Edinger–Westphal nucleus of the midbrain extend their axons out *via* the oculomotor nerve to synapse onto parasympathetic postganglionic neurons within the ciliary ganglion. These postganglionic neurons innervate the sphincter pupillae muscle (yellow) to cause the pupil to constrict, limiting the amount of light reaching the retina. **(B)** SCI causes irregular dilation of pupils resulting in uneven pupil size, a condition known as anisocoria, and Adie's Tonic Pupil where the pupil does not properly respond to light. (Created using www.biorender.com).

Lacrimal glands are located around each eye and are responsible for tear production to supply fluid across the eye to prevent dry eye. Control of lacrimal glands and the production of tears has been shown to be regulated by the sympathetic nervous system (Dartt, 2009; Jin et al., 2020). Following upper thoracic or higher SCI, there is a decrease in tear production and lacrimal gland atrophy (Jin et al., 2020). A decrease in tear production can result in dry eye syndrome that can produce things like ocular surface damage (Dartt, 2004; Stern et al., 2004). Decreases in visual acuity have been shown post-SCI and is thought to be due to the increased risk of hyponatremia within the SCI population compared to able-bodied individuals (Barletta et al., 1994; Giordano et al., 2000; Karlsson and Krassioukov, 2004).

4.4. Respiratory system

The respiratory system is made up of the trachea, bronchi, diaphragm lungs, and pulmonary plexus. The main function of the respiratory system is to exchange oxygen for carbon dioxide. As an individual breathes in, oxygen comes into the body and gets transported to peripheral organs in exchange for exporting waste gases, like carbon dioxide. Normal functioning of the respiratory system requires the somatic nervous system to control the muscles involved in breathing. Thus, SCI damage to the somatic nervous system can result in paralysis of the muscles responsible for breathing (e.g., the diaphragm and intercostal muscles), causing respiratory dysfunction. Normal respiratory function also requires autonomic nervous system control over smooth muscles and secretory glands within the respiratory system. The autonomic control requires a balance of both the parasympathetic and sympathetic nervous systems.

Sympathetic preganglionic neurons in the IML of T1–T5 synapse on postganglionic cells in the inferior cervical ganglion that directly innervate the bronchial vasculature and lungs to modulate secretion from submucosal glands (Barnes, 1986; Kummer et al., 1992). The level of injury highly determines the amount of respiratory dysfunction following SCI. An SCI below T5 leaves the sympathetic and parasympathetic innervation of the respiratory tract undamaged. Respiratory complications following high-level SCI is thought to be due to a disrupted balance of the sympathetic and parasympathetic pathways (Ditunno et al., 2004; Berlly and Shem, 2007; Garstang and Miller-Smith, 2007). SCI at or above T5 will sever supraspinal control over the sympathetic system regulating respiratory function but will not impact the parasympathetic system (Krassioukov, 2009).

The parasympathetic and sympathetic nervous systems play opposite roles in control of the smooth muscles within the airways. Specifically, the parasympathetic pathway facilitates contractions of these smooth muscles whereas the sympathetic nervous system facilitates the relaxation of the airway smooth muscles (Grimm et al., 2000; Canning, 2006). Because SCI leads to sympathetic nervous system damage while leaving the parasympathetic system intact, SCI has been shown to disrupt this balance and lead to hyperactivity of the airway smooth muscles which have been associated with increased incidence of asthma within SCI individuals (Grimm et al., 2000; Canning, 2006).

Spinal cord injury patients who experience autonomic dysreflexia have increased risk of pulmonary embolism and edema (Kiker et al., 1982; Aito et al., 2002; Christie et al., 2011; Davison et al., 2012). Pulmonary embolism is when arteries within the respiratory system get blocked *via* blood clots (Aito et al., 2002; Christie et al., 2011). SCI often results in weakness and spasticity of the muscles within the chest, impairing the ability to properly cough to expel foreign objects within the lungs (Roth et al., 1997; Wang et al., 1997). Abnormal autonomic input to the smooth muscles of the airways and increased pulmonary edema have been shown to increase the risk of sleep disordered breathing and sleep-wake disorders within the SCI population (Sankari et al., 2019a,b). Decreased sympathetic regulation to the airways of the respiratory system produces bronchoconstriction and increased nasal resistance which contributes to increased pressure of the upper airway and sleep disordered breathing (Krassioukov, 2009; Wijesuriya et al., 2017). Additionally, as mentioned previously, SCI disruption to the sympathetic nervous system results in impaired melatonin secretion from the pineal gland. This data shows that disruption of circadian rhythms in the SCI population influences the sleep-wake cycles. These findings support that disruptions to the airways as well as to melatonin secretion from the pineal gland can play a role in disrupted sleep patterns and behaviors, preventing individuals from getting necessary sleep.

4.5. Liver

The liver is an essential organ that works to metabolize macronutrients, filter blood, and regulate iron storage and release (Goodus and McTigue, 2020). The sympathetic nervous system innervates the liver and plays a role in osmotic homeostasis, metabolism, blood and bile flow, and responses to injury within the liver (Hsu, 1992; Jensen et al., 2013; Kandilis et al., 2015; Mizuno and Ueno, 2017). SPNs located in the IML of T7–12 project out to the celiac ganglion to synapse on postganglionic sympathetic neurons where they form the greater splanchnic nerve and innervate the liver (Hsu, 1992; Yi et al., 2010; Jensen et al., 2013; Mizuno and Ueno, 2017). Therefore, SCI at T7–12 or higher would result in dysfunction of proper hepatic functioning. This overall liver following SCI increases circulating glucose and lipids, as well as insulin resistance which leads to an increased risk of cardiovascular disease, diabetes, and stroke (Bauman and Spungen, 1994, 2001; Farkas and Gater, 2018; Goodus and McTigue, 2020).

Inflammatory responses are the immune system's way of responding to harmful stimuli and can serve as sort of a defense mechanism for the body. While an acute inflammatory response can be beneficial, if this activated inflammatory response goes unchecked and becomes chronic, such as after SCI, it can start becoming detrimental to peripheral organs such as the liver. The liver is an organ that has been shown to play a strong role in the initiation as well as the progression of the systemic inflammatory response produced by SCI. Immediately following injury, activated macrophages and microglia at the injury site secrete pro-inflammatory cytokines that then enter the blood stream (Hundt et al., 2011; Fleming et al., 2012). The liver detects this increase in proinflammatory cytokines in the blood and begins to produce acute phase response proteins, such as C-reactive protein (CRP), serum amyloid A (SAA), and mannose-binding protein, that work to further increase the inflammatory response in

an attempt to minimize the detrimental effects of the injury (Hundt et al., 2011; Fleming et al., 2012). Immediately following SCI and well into the chronic stages, SCI has been shown to increase hepatic leukocyte recruitment as well as increased hepatic proinflammatory cytokines levels (Fleming et al., 2012; Sauerbeck et al., 2015). This data supports that the liver plays a role in the development as well as intensification of the systemic immune response post SCI.

One of the other main functions of the liver is to metabolize macronutrients like fats, proteins, and carbohydrates. When the sympathetic control of the liver is damaged by SCI, altered metabolic activity of the liver ensues. Following SCI, there is increased lipid and ceramide accumulation within the liver the is induced almost immediately following SCI but persists into the chronic phases (Sauerbeck et al., 2015). Ceramides are lipids within the liver that play a role in inflammation and insulin resistance. The SCI-induced increase in ceramides results in inhibition of insulin signaling which further promotes resistance to insulin (Sauerbeck et al., 2015). This increased insulin resistance following SCI is a prominent driver of type 2 diabetes, which is known to be more prevalent within the SCI population (Bauman and Spungen, 1994; Bauman et al., 1999; New et al., 2002; Cragg et al., 2013). Similarly, increased hepatic insulin resistance is also strongly correlated with non-alcoholic fatty liver disease. Therefore, the liver following SCI has shown a similar phenotype to fatty liver disease of non-alcoholic steatohepatitis (Sauerbeck et al., 2015).

With metabolism of nutrients being one of the main functions of the liver, it makes sense that the daily diet of an individual could play a role in attenuating some of the dysfunction of the liver following SCI. Green tea extract has been shown to be hepatoprotective and attenuate hepatic steatosis (Bruno et al., 2008; Park et al., 2011; Masterjohn and Bruno, 2012). Administering a diet rich in green tea extract prior to SCI significantly reduced the amount of iron accumulation associated with the chronic phase of liver disfunction following SCI. However, it did not attenuate liver macrophage activation nor lipid and fatty acid accumulation following SCI (Goodus et al., 2018).

Spinal cord injury-induced damage to the liver has also been associated with diabetes and cardiovascular disease (Bauman and Spungen, 2001; Lee et al., 2005). Low-density cholesterol is directly transported to the arteries and its build up there increases the risk for cardiovascular disease. On the other hand, high-density cholesterol gets transported to the liver, where it can be removed from the blood. SCI has been shown to increase the amount of low-density cholesterol and decrease the amount of high-density cholesterol. High-sensitivity C-reactive protein (hsCRP), one of the acute phase proteins produced by the liver in response to a systemic inflammatory response and has been used as a marker for increased risk of cardiovascular disease, is elevated in SCI patients, indicating that liver dysfunction following SCI is likely to contribute to other metabolic disorders (Lee et al., 2005).

4.6. Gallbladder

The gallbladder sits right underneath the liver and is responsible for storing the bile produced by the liver. Bile, a mixture of cholesterol, bilirubin, and bile salts, is released when food is consumed to aid in digestion of fats. The gallbladder is connected to the liver and duodenum of the small intestine through the biliary tract. When uninjured individuals consume food, the parasympathetic nervous system, along with the hormone cholecystokinin produced in the upper regions of the intestine, signals the gallbladder to contract, and release the stored bile into the small intestine to assist in digestion.

The sympathetic nervous system is involved in inhibiting gallbladder contraction (Persson, 1972, 1973; Banfield, 1975). SPNs residing in the IML of T7–10 project axons out *via* the greater splanchnic nerve and synapse upon postganglionic sympathetic neurons in celiac ganglion that innervate the gallbladder and bile ducts (Tandon et al., 1997). SCI at or above T7–T10 severs supraspinal, descending inhibition of the sympathetic nervous system responsible for innervating the gallbladder, thereby reducing the motility of the gallbladder and resulting in stasis (Nino-Murcia et al., 1990).

This reduced motility after SCI increases the likelihood for the development of gallstones, or cholelithiasis, which are hardened stones of bile that are mostly made up of cholesterol that can get lodged within the biliary ducts (Apstein and Dalecki-Chipperfield, 1987; Fong et al., 2003; Baltas et al., 2009). Gallstones can block the cystic duct and cause inflammation of the gallbladder, known as cholecystitis. Consequently, gallstone disease is significantly more common in patients with SCI compared to uninjured patients which greatly increases the risk of acute pancreatitis or inflammation of the pancreas (Apstein and Dalecki-Chipperfield, 1987). The leading mechanism for increased risk of gallstone disease within the SCI population is decreased gallbladder motility that causes a state of inactivity of the gallbladder therefore increasing the risk of gallstone development (Apstein and Dalecki-Chipperfield, 1987; Fong et al., 2003). This less frequent contraction of the gallbladder results in impaired filling and ejection of bile (Nino-Murcia et al., 1990; Fong et al., 2003). This can increase the susceptibility of solidification of bile, also known as bile sludge. With decreased gallbladder motility in SCI individuals, it increases the development of biliary sludge compared to individuals with SCI below T10 (Tandon et al., 1997; Baltas et al., 2009).

4.7. Pancreas

The pancreas is an organ that is closely related to the gallbladder. While the gallbladder stores bile produced by the liver, the pancreas produces and stores its own enzymes that assist in digestion and breakdown of food. The pancreas can be broken down into two parts: the exocrine pancreas and the endocrine pancreas. Most of the pancreas is considered the exocrine pancreas that is responsible for producing the pancreatic enzymes to aid in digestion. The endocrine pancreas is comprised of clusters of cells called islet beta cells that work to release hormones, like insulin and glucagon, and works to maintain glucose homeostasis. Both the parasympathetic and sympathetic nervous systems innervate the pancreas. Parasympathetic activity stimulates islet beta cells to secrete insulin whereas sympathetic activity inhibits insulin secretion from the pancreas (Kiba, 2004). Both the parasympathetic and sympathetic nervous systems innervate the endocrine pancreas by directly innervating the islet cells in order to adjust glucose homeostasis with food intake (Teff, 2011;

Thorens, 2011; Rodriguez-Diaz et al., 2012). It is thought that this parasympathetic-sympathetic balance works to properly regulate the pancreas for normal functioning.

Parasympathetic preganglionic neurons involved in innervating the pancreas reside in the dorsal vagal nucleus within the brainstem. From here, parasympathetic preganglionic neuron axons travel out of the CNS to synapse upon parasympathetic postganglionic neurons located in the pancreatic ganglia. Parasympathetic postganglionic neurons directly innervate the pancreas (Rodriguez-Diaz et al., 2012).

The sympathetic circuit responsible for controlling the pancreas is similar to that for the liver, gallbladder, and bile ducts. SPNs located in the T6-L2 IML send axons out through the splanchnic nerve to the celiac ganglion. The sympathetic postganglionic neurons within the celiac ganglia innervate the pancreas (Sharkey and Williams, 1983; Renehan et al., 1995; Won et al., 1998).

Spinal cord injury at or above T6-L2 would disrupt the neural control of the pancreas, causing dysfunction of the organ. One such consequence is increased occurrence of pancreatitis (Nobel et al., 2002; Pirolla et al., 2014; Ho et al., 2021). Pancreatitis, a life-threatening disease that needs to be assessed immediately, is an increased inflammatory response with increased serum levels of pancreatic enzymes, such as p-amylase and lipase, which can cause corrosion of the pancreatic parenchyma (Nobel et al., 2002; Pirolla et al., 2014). This corrosion can result in devastating damage inflammation and/or necrosis that can then spread to other organs, causing their failure (Pirolla et al., 2014; Schepers et al., 2019).

It has been suggested that pancreatitis could also be caused be a disrupted balance of the sympathetic-parasympathetic control of the sphincter of Oddi, a smooth muscle that is located at the end of the gallbladder and pancreatic ducts to allow for bile and pancreatic juice to flow into the duodenum of the small intestine (Carey et al., 1977). The sphincter of Oddi receives innervation from both the sympathetic nervous system *via* the superior mesenteric ganglion and the parasympathetic nervous system *via* the vagus nerve. Increased cholinergic activity produces spasms of the sphincter of Oddi (Carey et al., 1977; Novaes et al., 1982). These spasms result in retention of pancreatic enzymes, increasing the risk of pancreatitis (Carey et al., 1977; Novaes et al., 1982; Chen et al., 2000). Following SCI, increased sympathetic nervous system activity produces spasms of the sphincter of Oddi and stasis of pancreatic secretions.

Inside the pancreas are clusters of beta cells called islets that are responsible for production of the hormones insulin and glucagon (Rodriguez-Diaz and Caicedo, 2014). These hormones are delivered to the liver to maintain glucose homeostasis (Rodriguez-Diaz and Caicedo, 2014). Pancreatic islets are directly innervated by the sympathetic nervous system. While the islet cells can function without any autonomic innervation (Brodows et al., 1976; Corrall and Frier, 1981; Rodriguez-Diaz and Caicedo, 2014), it is clear that sympathetic innervation plays a role in normal pancreatic function. Loss of sympathetic input to the islet cells is associated with impaired glucagon response and the onset of diabetes (Mei et al., 2002; Taborsky et al., 2009). Thus, it makes sense that a SCI that perturbs sympathetic regulation would disrupt glucose homeostasis. Thus, SCI can increase risk of diabetes through dysfunction of multiple organs, including the liver, as described above (Bauman and Spungen, 2001; Lee et al., 2005), and the pancreas (Cheng et al., 2020).

4.8. Gastrointestinal tract

The gastrointestinal (GI) tract is comprised of all the major organs of the digestive system. After food is swallowed, it goes through the throat and down the esophagus to the stomach. The stomach serves as both a reservoir for food as well as a digestion point. Once food reaches the stomach, the stomach contracts and produces digestive enzymes to help break down the food prior to passing to the small intestine and large intestines. The lower GI tract consists of the colon and the rectum which are the final two digestive processes where reabsorption, storage, and elimination happens (Holmes and Blanke, 2019).

Some parts of the GI tract, such as the intestines, can mostly control itself with the enteric nervous system (Browning and Travagli, 2014). The enteric nervous system is embedded in the wall of the GI tract. This nervous system allows for the GI system to control itself independently of the CNS. The enteric nervous system is made up of the myenteric plexus and submucosal plexus and includes sensory, motor, and interneurons to work to coordinate gut motility and secretions (Chung and Emmanuel, 2006). The enteric nervous system contains complete reflex circuits consisting of sensory neurons that sense changes within the digestive system, a local circuit of neurons that is responsible for integrating this information, and motor neurons that regulate activity of the muscles within the GI tract that mediate gut movement, secretions of digestive enzymes, mucus, and stomach acid.

Although the enteric nervous system has been shown to be able to work independently of the CNS, it still communicates heavily with the two autonomic nervous system branches to regulate GI functions (Bornstein et al., 1988; Chung and Emmanuel, 2006; Browning and Travagli, 2014). Both the parasympathetic and sympathetic postganglionic neurons synapse directly onto neurons within the enteric nervous system (*via* the vagus nerve and the pelvic nerves) to regulate their activity. The two branches of the autonomic nervous system provide a connection between the enteric nervous system of the gut and the CNS. As mentioned previously, the parasympathetic system is referred to the "rest and digest" system. Therefore, it makes sense that the parasympathetic system activates the GI tract for digestion by stimulating GI secretion and motor activity of GI muscles. Oppositely, the sympathetic nervous system works to inhibit release of digestive secretions, inhibit contraction of GI sphincters, and regulates blood flow to the GI tract *via* vasoconstriction (Bornstein et al., 1988; Browning and Travagli, 2014).

Normal function of the stomach relies more heavily on autonomic control in addition to the enteric nervous system. Sympathetic innervation of the stomach is similar to the liver, gallbladder, and pancreas. SPNs in T6 to T9 project axons out *via* the greater splanchnic nerve and synapse on the postganglionic cells in the celiac ganglion. Those sympathetic postganglionic neurons innervate the stomach (Chung and Emmanuel, 2006; Browning and Travagli, 2014).

Sympathetic function is also important for function of the ascending colon and the small and large intestine. Axons from SPNs

in T9 to T12 travel out of the cord *via* the lesser splanchnic nerve to the and synapse onto postganglionic neurons in the superior mesenteric ganglion. These neurons innervate the ascending colon and the large and small intestine (Chung and Emmanuel, 2006; Browning and Travagli, 2014). The descending colon and the rectum receive input from the postganglionic neurons in the inferior mesenteric ganglion. These neurons receive input *via* the lumbar splanchnic nerve upon from SPNs in T12 to L3 (Chung and Emmanuel, 2006; Browning and Travagli, 2014). Thus, SCI at or above L3 could result in dysfunction of the GI tract.

Gastrointestinal tract complications have been shown to be more frequent in individuals with SCI compared to uninjured individuals (Gore et al., 1981; Chen et al., 2004; Holmes and Blanke, 2019). SCI individuals are more prone to experience heart burn, chest pain, and hiatus hernias where the upper stomach pushes through the muscle in between the abdomen and diaphragm creating chest pain, and inflammation of the esophagus (esophagitis), which could all be due to an increase of gastroesophageal reflux disease (GERD) following SCI (Stinneford et al., 1993; Singh and Triadafilopoulos, 2000; Silva et al., 2008). Gastroesophageal reflux is back flow of stomach acid or bile flowing back through the esophagus, which can irritate the esophagus and producing heartburn and/or acid reflux. The increased prevalence of GERD within the SCI population increases the risk of esophagitis following SCI.

Neurogenic bowel is a common colonic dysfunction following a SCI at or above L3 SCI. This is associated with symptoms like constipation, abnormal elimination reflexes, and reduced contractions of the colon (Lynch et al., 2001; Coggrave et al., 2014). This decreased intestinal movement can result in lack of blood flow and tissue death of the intestines, gastric dilation or twisted stomach, ulcers of the stomach lining or small intestines have all been increased acutely following SCI (Gore et al., 1981). Other symptoms include incontinence, abdominal pain, abnormal gastric activity, vomiting, nausea, peptic ulcers, diarrhea, rectal bleeding, hemorrhoids, and fecal impactions have all been reported to be higher in individuals suffering from SCI (Gore et al., 1981; Han et al., 1998; Vallès et al., 2006; Ebert, 2012).

Individuals with a mid-thoracic or higher SCI have increased gastrin section and decreased gastrointestinal emptying and motility (Bowen et al., 1974; Kewalramani, 1979; Berlly and Wilmot, 1984; Fynne et al., 2012). This slower GI emptying and movement and increased gastrin secretion could lead to gastroduodenal bleeding or Cushing's ulcer which has shown to be increased in individuals with an SCI at T5 or higher (Cushing, 1932; Bowen et al., 1974; Kewalramani, 1979; Berlly and Wilmot, 1984).

As previously mentioned, the autonomic nervous system is a link between the bidirectional gut-brain axis. Recently, the gut microbiota, microorganisms residing within digestive tracts, have been shown to influence gut-brain axis (Carabotti et al., 2015). The gut microbiota has been of increasing interest to the SCI field since the sympathetic nervous system provides a link between the gut and the brain and has been shown to be influenced by the gut microbiota. Moreover, the gut microbiota have been shown to play a critical role in the maintenance of homeostatic processes (Gilbert et al., 2018). The development of neurogenic bowel post-SCI is associated with increased gut dysbiosis, a disrupted balance of the microbiota residing within the GI tract (Carding et al., 2015), with an increase in pro-inflammatory bacteria (Gungor et al., 2016; Kigerl et al., 2018; Bazzocchi et al., 2021). This increased prevalence of gut dysbiosis within the SCI population has been associated with GI disorders like irritable bowel syndrome (IBS) and inflammatory bowel diseases in the SCI community (Han et al., 2009; Tseng et al., 2017). Preclinically, gut dysbiosis has been shown to impair recovery following SCI and a diet rich in probiotics could serve as a potential therapeutic for sympathetic induced gut dysbiosis following SCI (Kigerl et al., 2016). As it is becoming more apparent that the microbiome shapes many aspects of overall health, it is very important to increase understanding of the implications of SCI-induced gut dysbiosis.

4.9. Spleen

The spleen is a part of the lymphatic system that is located between the diaphragm and the stomach within the abdomen. The spleen serves many necessary functions. It removes damaged red blood cells and controls the white to red blood cell balance. The spleen also plays a key role in the body's immune defense by fighting invading bacteria *via* antibody production (Bronte and Pittet, 2013; Noble et al., 2018). The spleen stores leukocytes, immune cells that work to fight infections within the body, including neutrophils, eosinophils, and basophils, monocytes, and T and B cells. When the body is fighting off infections, the number of leukocytes increase and is referred to leukocytosis. Because of its imperative role in immune function, people without a spleen, or with a malfunctioning one, are at greater risk for infections (Madden et al., 1997; Davidson and Wall, 2001). Innervation of the spleen *via* the sympathetic chain ganglia increases throughout development, suggesting the need for this vital organ throughout life (Madden et al., 1997).

Sympathetic preganglionic neurons that play a role in regulating spleen function are located in the IML of T4–T12 (Anderson et al., 1989; Nance and Burns, 1989; Strack and Loewy, 1990; Cano et al., 2001). They project out of the spinal cord *via* the greater splanchnic nerve to the celiac ganglion, where the SPNs form synapses on the postganglionic neurons that then directly innervate the spleen (Felten et al., 1985; Straub, 2004; Nance and Sanders, 2007; Bratton et al., 2012). The parasympathetic nervous system does not innervate the spleen (Bellinger et al., 1993; Bratton et al., 2012).

Damage to the sympathetic nervous system *via* SCI impact's splenic function. Immune suppression following SCI is commonly reported and is likely due to disrupted modulation of sympathetic innervation of the spleen (reviewed by Noble et al., 2018). Specifically, individuals with an SCI above sympathetic innervation of the spleen (T3 or higher) have increased risk of infections due to an immune suppressive response (Brommer et al., 2016). Preclinical SCI models suggest that increased activation of the sympathetic nervous system after high-level SCI results in splenic atrophy and leukopenia, low white blood cell count within the spleen, to contribute to immunodeficiency (Zhang et al., 2013; Ueno et al., 2016; Mironets et al., 2018, 2020). As a result, high-level SCI results in impaired ability to fight off bacterial and viral infections, leading to increased mortality within the SCI community (Bracchi-Ricard et al., 2016; Mironets et al., 2020). Preclinical data shows that this dysfunction has been associated

with impaired T-cell function (Zha et al., 2014; Bracchi-Ricard et al., 2016). Interestingly, treatment prior to SCI with memory T cells were shown to be effective in the weeks following SCI for protection against infections (Norden et al., 2019).

Splenic dysfunction after SCI has been linked to accumulations of norepinephrine and glucocorticoids (Popovich et al., 2001; Lucin et al., 2007; Zhang et al., 2013). The hypothalamic-pituitary-adrenal axis (HPA axis) is responsible for glucocorticoid production while the sympathetic nervous system produces norepinephrine. Normally, the HPA axis and the sympathetic nervous system work in concert to facilitate proper immune functioning. Following SCI, the HPA axis is activated and the control over the sympathetic system is impaired further supporting the accumulations of norepinephrine and glucocorticoids (Lucin et al., 2007; Zhang et al., 2013). After SCI at T3, levels of both cortisol and norepinephrine are elevated and there is impaired antibody synthesis and splenocyte cell death (Lucin et al., 2007; Zhang et al., 2013; Prüss et al., 2017). This is not the case after a lower thoracic injury (T9) that spares more descending input to the spinal sympathetic circuit; levels of cortisol are increased but levels of norepinephrine and antibody synthesis are similar compared to uninjured controls (Lucin et al., 2007; Zhang et al., 2013; Prüss et al., 2017).

Interestingly, treatments that decrease plasticity of the sympathetic nervous system after SCI, such as gabapentin and the TNFα inhibitor XPro1595 and attenuate sympathetic hyperreflexia have also been shown to reduce SCI-associated splenic atrophy and loss of leukocytes (Mironets et al., 2018, 2020; Brennan et al., 2021).

4.10. Adrenal gland

The adrenal gland secretes hormones to serve in many essential functions like responding to stress, regulation of metabolism, blood pressure, and immune function. An adrenal gland sits on top of each kidney and the internal medulla is surrounded by the outer cortex. Although these two components seemingly serve two distinct functions, they are largely interconnected (Bornstein et al., 1991). The adrenal cortex produces steroid hormones like androgens and estrogens to regulate sexual function and aldosterone to regulate blood pressure by regulating the amount of salt and water within the body. The adrenal cortex also produces cortisol *via* the HPA axis in response to stress (Gavrilovic and Dronjak, 2005). The adrenal medulla contains Chromaffin cells that secrete catecholamines, like epinephrine and norepinephrine, needed for the sympathetic fight-or-flight response (Bornstein et al., 1991). Because of this, the adrenal medulla is highly interconnected with the sympathetic nervous system and relies on it for proper functioning (Goldstein and Kopin, 2008).

Sympathetic innervation of the adrenal gland is slightly different compared to the previously discussed organ systems. SPNs innervating the adrenal gland reside in the IML in the spinal cord at T9–T10. However, instead of leaving the spinal cord and entering a ganglion to synapse on the postganglionic neurons, SPNs travel out of the spinal cord *via* the lesser splanchnic nerve and synapse directly onto the adrenal gland (Kesse et al., 1988; Parker et al., 1993). It has been suggested that the adrenal medulla serves as a modified sympathetic ganglion and Chromaffin cells could serve as the postganglionic sympathetic neurons in this system.

Abnormal functioning of the HPA axis is thought to result in adrenal insufficiency and dysfunction of the adrenal gland, causing the body to improperly deal with stressors, such as a SCI itself (Lecamwasam et al., 2004). Adrenal insufficiency has been shown to be increased in SCI individuals compared to non-injured individuals (Garcia-Zozaya, 2006; Pastrana et al., 2012). Adrenal insufficiency can surface as hypotension, hyperkalemia, and hyponatremia (Lecamwasam et al., 2004; Garcia-Zozaya, 2006; Pastrana et al., 2012). While disruption of the HPA axis and the sympathetic nervous system plays a role in SCI-induced suppression of the immune system, as discussed above, SCI may also directly increase activation of the adrenal gland to increase cortisol levels, independent of the HPA axis, as an additional mechanism for SCI-induced suppression of the immune system (Prüss et al., 2017).

4.11. Urinary system

The urinary system (kidneys, ureters, bladder, and the urethra) serves many essential functions. It works to remove waste and regulate vitamins and minerals within the body. The urinary system is also involved in blood pressure regulation. The urinary system requires the involuntary control of the autonomic nervous system. Because of this, damage to the sympathetic nervous system disrupts supraspinal control of the urinary system resulting in organ dysfunction. The upper urinary tract consists of the kidneys and the ureters while the lower urinary tract consists of the bladder and the urethra. The kidneys are connected to the bladder by tubes called ureters. Once the kidneys filter out the fluids, urine flows through the ureters to the bladder where it is stored until voiding. Usually, the ureters serve as a one-way system and urine is unable to back flow into the kidneys. However, if the bladder becomes too full or there are problems within this system, such as abnormal sympathetic nervous system function, urine can backflow and cause both bladder and kidney problems.

4.11.1. Kidney

The kidneys function to filter blood and discard the waste and regulate the homeostasis of chemicals and minerals within the body. The kidneys also release hormones that help regulate blood pressure, produce red blood cells, and create healthy bones. They require input and regulation from the sympathetic nervous system to function properly. SPNs regulating kidney function are in the IML of T11-L1 (Ferguson et al., 1986; Huang et al., 2002). Axons from these SPNs leave the spinal cord and travel to the celiac ganglion, where they synapse with postganglionic sympathetic neurons that directly innervate each of the kidneys. Thus, SCI that results in dysfunction of the sympathetic nervous system can impact the kidneys.

Kidney problems are prevalent following SCI and are closely related to bladder and lower urinary tract problems. SCI at or above T11-L1 results in loss of descending inhibitory control over the sympathetic nervous system that innervates the kidneys. It has been found that individuals with SCI have increased prevalence of renal calculi or kidney stones (Welk et al., 2017). This increased risk of kidney stone development is thought to be due to the dysfunction of the mineral homeostasis function of the kidneys,

therefore increasing calcium levels that then crystalize and form kidney stones (Naftchi et al., 1980; Shunmugavel et al., 2010). If left untreated, kidney stones can increase the risk of kidney failure and kidney atrophy (Constantinople et al., 1979). Similarly, loss of sympathetic regulation of the kidneys disrupts the function of regulating chemicals and minerals within the body. Because of this, hyponatremia, an abnormal decrease in blood sodium level, has been shown to be increased in patients with high level SCI (Furlan and Fehlings, 2009). Hyponatremia can also increase the risk of hypotension (Furlan and Fehlings, 2009). Similarly, oxidative stress following injury produces renal inflammation which has been shown to be a large contributor to renal crystal formation (Khan, 2005).

We recently found that high-level SCI dramatically alters kidney function. Because kidneys are highly vascularized, there is a need to protect this critical organ from fluctuations in systemic blood pressure. Renal autoregulation is a homeostatic mechanism that regulates the amount of blood flowing through the kidney. As systemic blood pressure increases, resistance of the renal vasculature increases to maintain a constant renal blood flow. Surprisingly, after chronic high-level SCI in rodents, renal autoregulation is completely abolished; there was virtually no change in renal vascular resistance in response to an increase in blood pressure, allowing more blood to flow unimpeded into the kidney. This loss of renal autoregulation was associated with injury to the kidney, as indicated by higher levels of neutrophil gelatinase-associated lipocalin, fibrosis, and inflammatory signaling than in animals with low-level SCI (Osei-Owusu et al., 2022).

4.11.2. Bladder

The bladder is responsible for storage of urine and periodically voiding urine, also known as micturition, when the bladder becomes too full, and a threshold is reached. Normal urinary storage and voiding requires the integration of autonomic and somatic controls. The two continuous activities of filling and voiding are coordinated through contraction and relaxations of the bladder detrusor, urethra, and urethral sphincter muscles which are under high autonomic control (**Figure 6**). The external urethral sphincter is a striated muscle that is controlled by the somatic nervous system.

Storage and voiding of urine require both sympathetic and parasympathetic pathways. Supraspinal control from Barrington's nucleus and the periaqueductal gray region in the brainstem sends projection down to both the sympathetic circuit in T11-L2 and the parasympathetic circuit in S2–S4. SPNs in T11-L2 extend out of the ventral root of the spinal cord and form the hypogastric nerve to synapse onto sympathetic postganglionic neurons in the inferior mesenteric ganglion that directly innervate the bladder detrusor and urethra muscles (Hou and Rabchevsky, 2014). Parasympathetic preganglionic neurons reside in the sacral spinal cord (S2–S4) and project out to synapse on parasympathetic postganglionic neurons. Therefore, SCI at or above the lower thoracic segments would affect the sympathetic nervous system and disrupt urinary function (**Figure 7**).

In infants, control to void or store urine is regulated by the involuntary autonomic nervous system. This is seen when infants are unable to control the storage of their urine and void spontaneously. However, as humans mature, they obtain conscious control over the bladder, deciding whether to void or continue storing urine. Control of the bladder works somewhat like a switch. As the bladder fills, the external urethral sphincter is contracted while the detrusor muscle remains relaxed. Once the bladder becomes full, the switch is flipped from storage to voiding where the sphincter relaxes while the detrusor contracts, allowing for micturition. Both functions of storing urine and micturition require its unique sets of neural controls, such as autonomic and somatic circuits within the spinal cord that communicate with supraspinal areas for further regulation (Fowler et al., 2008). Within the autonomic nervous system control, the sympathetic nervous system works to inhibit detrusor contraction to facilitate the ability to store urine. As the bladder fills and reaches a particular threshold, the parasympathetic pathway then takes over to contract the detrusor muscle and expel urine from the bladder.

Normally, the detrusor muscle contracts as the sphincter muscle relaxes to void the bladder. However, after SCI, the detrusor and sphincter have been shown to contract simultaneously resulting in detrusor-sphincter dyssynergia causing insufficient voiding (Chang et al., 2000; Fowler et al., 2008). These urinary problems, also known as neurogenic bladder, refers to the abnormal ability for the bladder to fill, store, and void urine efficiently, is very common following SCI (Ruutu and Lehtonen, 1984; Chao et al., 1993; Waites et al., 1993; Chen et al., 1999; Lawrenson et al., 2001). Because of this, urine can sit in the urinary tract longer than necessary and exposes the urinary system to bacteria, which increases the chances of infection within SCI individuals in both the upper and lower urinary tracts and can ultimately lead to renal failure (Ruutu and Lehtonen, 1984; Chao et al., 1993; Lawrenson et al., 2001; Welk et al., 2017). Thus, SCI patients have higher rates of urinary tract infections that, if left untreated, could be life threatening (Waites et al., 1993; Welk et al., 2017).

Neuroplasticity after SCI is thought to contribute to aberrant bladder function. Specifically, increased levels of NGF after SCI causes neurons to undergo morphological and physiological changes such as bladder enlargement, increased CGRP sprouting, and tyrosine hydroxylase-positive $(TH)^+$ sympathetic fibers (Kruse et al., 1995; Yoshimura and de Groat, 1997; Schnegelsberg et al., 2010). These changes have been shown to lead to bladder hyperactivity, detrusor overactivity, and increased detrusor-sphincter dyssynergia (Seki et al., 2002, 2004; Vizzard, 2006). Treatment with anti-NGF antibodies to prevent some of this maladaptive plasticity in SCI models has been shown as a possible treatment to decrease detrusor overactivity and detrusor-sphincter dyssynergia (Seki et al., 2002, 2004; Vizzard, 2006).

Following SCI, the bladder is initially areflexic. However, over time, it regains partial functional recovery due to plasticity. In an intact system, small myelinated (Aδ) afferents relay information directly from the bladder to the spinal cord and are responsible for mediating micturition. However, following SCI, unmyelinated (C) fibers have been shown to sprout and contribute more to micturition (Kruse et al., 1995; de Groat and Yoshimura, 2006, 2012). Specifically, C-fiber soma size increases after SCI (Kruse et al., 1995; Yoshimura and de Groat, 1997), and they also have a lower activation threshold (Yoshimura and de Groat, 1997). Similarly, it has been found that TH^+ neurons reside in the lumbosacral spinal cord further supporting their involvement in the spinal bladder reflex. Interestingly, following SCI, there is an increase in TH^+ fibers which regulate the partial recovering of micturition (Hou et al., 2016; Qiao et al., 2021). This is

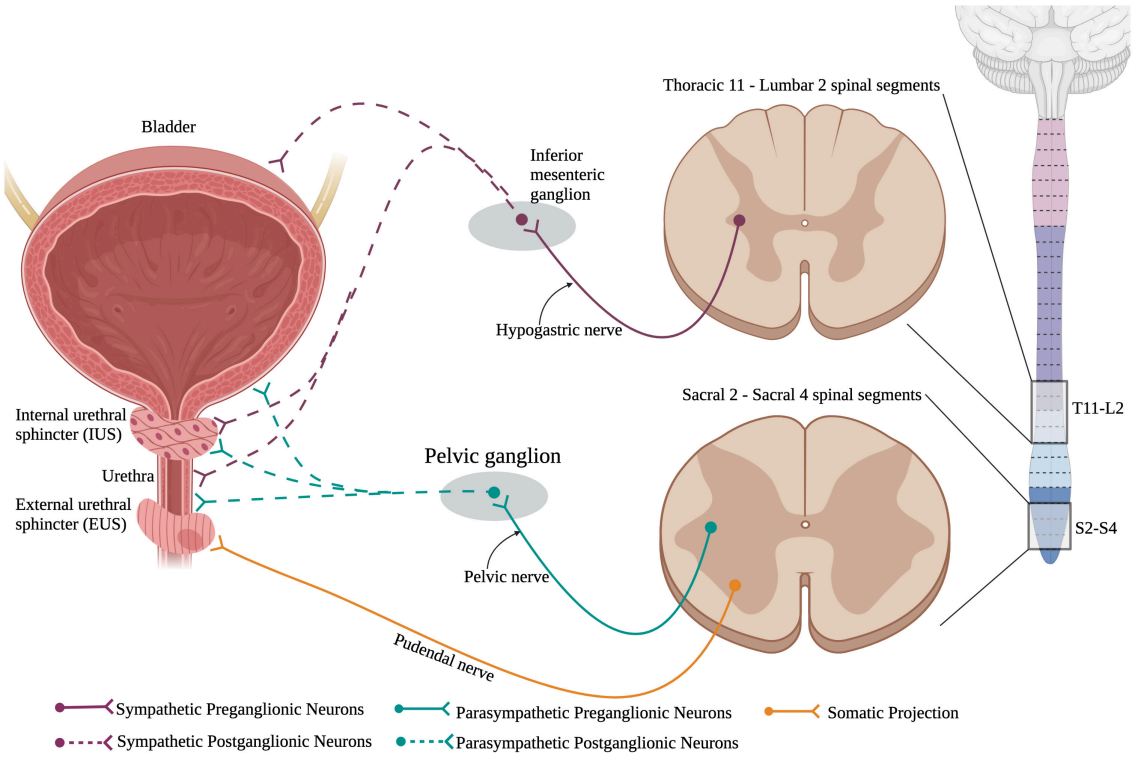

FIGURE 6
Neuronal control of the lower urinary tract (LUT). The lower urinary tract receives neuronal control from both branches of the autonomic nervous system and the somatic nervous system. Sympathetic control arises from sympathetic preganglionic neurons in thoracic segments 11 to lumbar segment 2 that extend axons out the hypogastric nerve and synapse onto sympathetic postganglionic neurons within the inferior mesenteric ganglion. These sympathetic postganglionic neurons innervate the bladder, IUS, and urethra. Parasympathetic preganglionic neurons in sacral spinal cord segments 2–4 project axons out of the cord *via* the pelvic nerve and synapse onto parasympathetic postganglionic neurons within the pelvic ganglion. These postganglionic neurons directly innervate the bladder, IUS, and urethra. Interestingly, the external urethral sphincter is the only muscle in the LUT that is controlled *via* the somatic nervous system. This allows for voluntary control over urinary functions. (Created using www.biorender.com).

further supported by work that shows depleting TH^+ fibers in the lumbosacral spinal cord results in a decrease of bladder contractions and the ability to void urine (Hou et al., 2016). The partial recovery of micturition with the spinal bladder reflex, depicts an example of the benefits of intraspinal plasticity following SCI.

Interestingly, neuromodulation *via* electrical stimulation is a promising means to improve bladder function after SCI. Epidural stimulation of the lumbosacral spinal cord was shown to improve bladder function (e.g., voiding) after SCI (Herrity et al., 2018; Walter et al., 2018). It is thought that epidural stimulation increases activation of severed spinal circuits below the level of injury to work to restore function following SCI (Herrity et al., 2018; Walter et al., 2018). Specifically, epidural stimulation in rodents with SCI elicits voiding within seconds of onset of stimulation (Gad et al., 2014; Hoey et al., 2021). These preclinical data showed promising translational effects for epidural stimulation to be examined in humans. Epidural stimulation was shown to attenuate neurogenic bladder in humans (Walter et al., 2018) and also improve voiding function *via* modulating detrusor contraction and subsequent external urethral sphincter relaxation in SCI people (Herrity et al., 2018; Walter et al., 2018).

Stimulation of the spinal cord is not the only means to improve bladder function. Stimulation of the pudendal nerve in the pelvic region also improves voiding function and increased bladder contractions in chronic SCI cats (Tai et al., 2007). Specifically, this work showed that during bladder filling, pudendal nerve stimulation inhibited reflexive voiding and increased bladder capacity (Tai et al., 2007). Furthermore, pudendal nerve stimulation also improved voiding efficiency following SCI (Naderi and Safarinejad, 2003). Overall, neuromodulation *via* epidural or pudendal nerve stimulation is a promising therapeutic treatment to improve bladder function for people living with SCI.

4.12. Reproductive system

Males and females have their own unique reproductive organs responsible for producing sperm or egg cells, transporting these cells, and facilitate proper fertilization to produce offspring. The male reproductive organs consist of the testicles, the vas deferens, prostate gland, and the penis. The female reproductive organs consist of ovaries, fallopian tubes, and the vagina. The reproductive organs not only serve for producing offspring, but they are also involved in sexual function of both males and females. Interestingly, individuals who sustained a SCI list regaining sexual function as a higher priority over restoring fertility (Anderson et al., 2007a), indicating how much sexual function impacts quality of life.

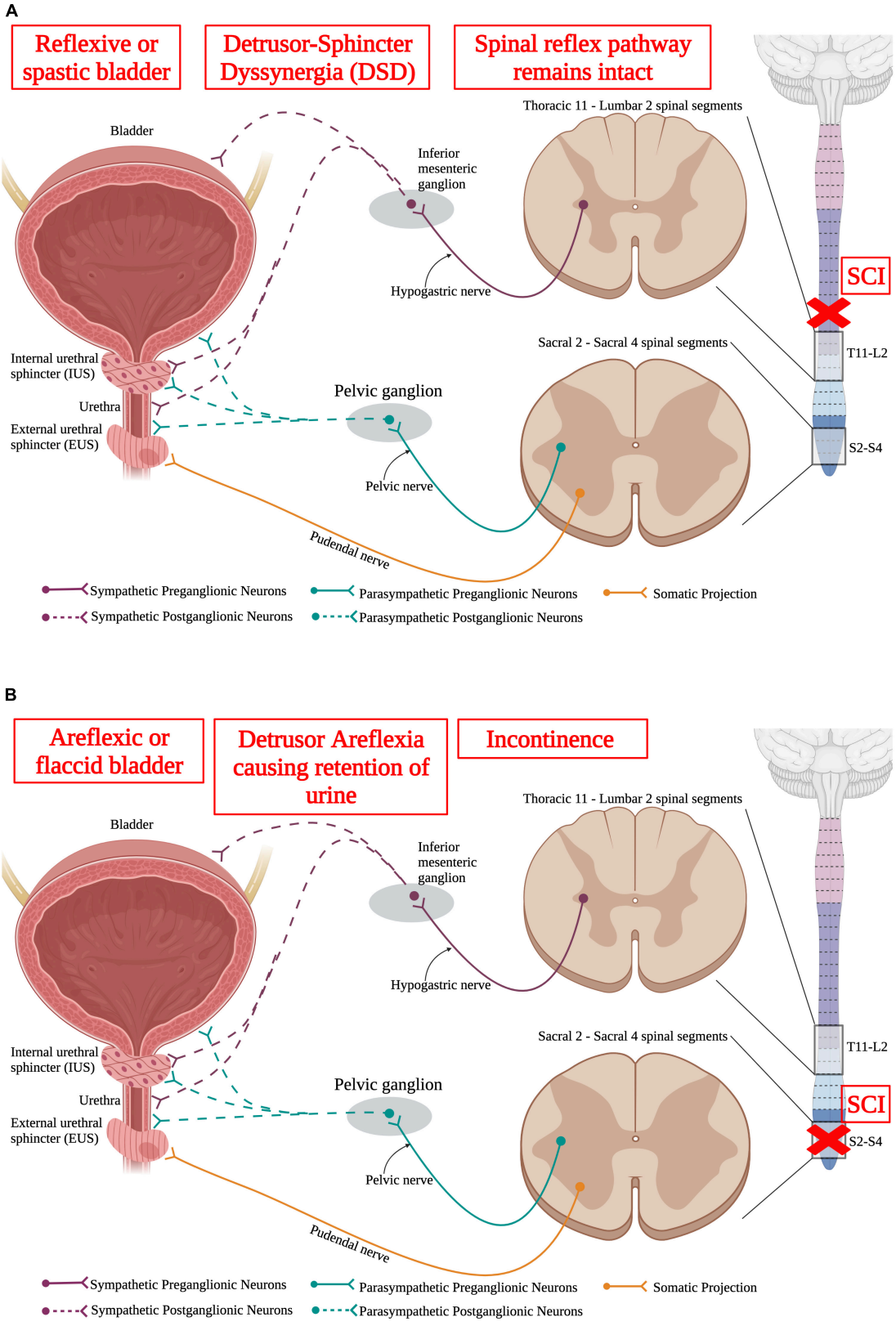

FIGURE 7
Spinal cord injury results in lower urinary tract (LUT) dysfunction. **(A)** An upper motor neuron injury is when there is no direct damage of the neurons that innervate the bladder, leaving the spinal reflex pathway intact. An upper motor neuron injury results in a reflexive or spastic bladder and detrusor-sphincter dyssynergia (DSD), where the detrusor and sphincter muscles abnormally contract simultaneously, resulting in inefficient voiding. **(B)** Lower motor neuron injury results in damage to the neurons that directly innervate the bladder. A lower motor neuron injury produces an areflexic or flaccid bladder where the detrusor muscle does not contract. This results in retention of urine in the bladder that makes the bladder become over-stretched. (Created using www.biorender.com).

Control over the reproductive system and sexual function heavily involves both branches of the involuntary autonomic nervous system and the somatic nervous system. Although there are distinct differences between male and female reproductive and sexual functions, there are some similarities. For example, the autonomic nervous system regulates vascular dilation for penile or clitoral erection, regulates prostatic or vaginal secretions, and regulates smooth muscle contractions in both the penis and vagina during erection and orgasm.

For the reproductive system, SPNs located in the IML of T12-L2 project out of the spinal cord to form the hypogastric nerve and synapse onto sympathetic postganglionic neurons in the prevertebral ganglia. These neurons directly innervate the reproductive organs in both males and females (Hancock and Peveto, 1979; Jänig and McLachlan, 1987). Therefore, SCI at or above this level cause abnormalities of reproductive and sexual function.

4.12.1. Male

Penile erection requires a balance of the parasympathetic and sympathetic systems. Although the role of the sympathetic nervous system on penile erection is largely debated, generally the parasympathetic system serves a "pro-erectile" function whereas the sympathetic system serves a "anti-erectile" function (Diederichs et al., 1991; Giuliano and Rampin, 2000; Steers, 2000). However, following SCI, the parasympathetic system and local spinal reflex pathways that can elicit short-lived reflexive erections remain intact (Bodner et al., 1987; Courtois et al., 1993). There are two types of erections, reflex and psychogenic. A reflex erection involves the spinal parasympathetic reflex pathway residing in the sacral spinal cord. Upon physical contact of the penis, sensory information is relayed to the sacral spinal cord that results in motor output and penile erection. In SCI individuals where the sacral spinal cord is not damaged, reflex erections can still happen. The sympathetic nervous system, on the other hand, has been shown to modulate psychogenic erections that are independent of genital stimulation but are instead produced by thoughts, sights, sounds, and fantasies (Courtois et al., 1993; Biering-Sørensen and Sønksen, 2001). After stimulation, supraspinal centers send messages to the SPNs in the spinal cord causing the penis to become erect. Thus, SCI at or above T11 disrupts supraspinal control and decreases psychogenic erections (Anderson et al., 2007b). Injury at this level spares the lumbosacral reflex circuit, however, and a reflexogenic erection can still occur. However, this type of erection is often not rigid enough for intercourse.

Ejaculation also requires a balance of the parasympathetic and sympathetic nervous systems. Ejaculation can be broken down into two different components, emission and expulsion (Newman et al., 1982). The emission phase requires high input from the sympathetic nervous system where it causes the vas deferens and bladder to contract to allow for semen to enter the urethra (Giuliano and Clement, 2005). The sympathetic nervous system is implicated in preventing backflow of semen into the bladder (Shafik and El-Sibai, 2000). The parasympathetic nervous system is involved in the expulsion phase where it causes the urethra to contract and expel semen to the environment (Carro-Juárez et al., 2003; Motofei and Rowland, 2005). SCI individuals have significantly lower rates of ejaculation compared to non-injured individuals, and sometimes even no ejaculation during orgasm, also known as anejaculation (Chéhensse et al., 2013). Additionally, ejaculation in males with an injury at or above T6, has been shown to induce an autonomic dysreflexia event previously mentioned in the cardiovascular section (Sheel et al., 2005).

Spinal cord injury produces problems with penile erectile dysfunction and abnormal ejaculation, which can contribute to low fertility rates in SCI individuals. However, SCI also can result in necrospermia, where the sperm is not viable, hypogonadism, where the testes are dysfunctional, or hypospermatogenesis, where there is a decrease in the amount of sperm within the semen (Mallidis et al., 1994; Elliott et al., 2000; Naderi and Safarinejad, 2003; Brown et al., 2006; Patki et al., 2008). One possible mechanism for this decreased sperm motility in SCI males could be due to increased levels of leukocytes and inflammatory cytokines within the seminal plasma following SCI (Trabulsi et al., 2002; Basu et al., 2004).

Erectile dysfunction after SCI often results in relationship concerns, increased stress, anxiety, and depression, and decreased self-esteem and confidence. Along with the many changes that come with SCI, erectile dysfunctions can further worsen the psychological effects many suffer after SCI.

4.12.2. Female

Female sexual arousal and clitoral erection also requires input from both the parasympathetic and sympathetic nervous systems. As with males, female sexual arousal can be broken down into reflexive arousal, which involves the parasympathetic pathway, and psychogenic arousal, which involves the sympathetic pathway (Sipski et al., 1995). The parasympathetic pathway *via* the pelvic nerves in S2–S4 controls things like clitoral erection, vaginal lubrication, and vulvar swelling (Park et al., 1997; Giuliano et al., 2001). The SPNs of the sympathetic system in T10-L2 eventually forming the hypogastric nerve innervates the cervix and uterus and initiate rhythmic contractions of these muscles and facilitates sexual arousal.

Individuals with a SCI that disrupts the supraspinal control over the sympathetic nervous system may still display reflexive sexual arousal signs if the parasympathetic reflexive control remains intact (Meston and Gorzalka, 1995; Lorenz et al., 2012).

Spinal cord injury individuals have reported difficulty becoming both psychologically as well as physically aroused (Anderson et al., 2007c; Othman and Engkasan, 2011; Hajiaghababaei et al., 2014). This is partly due to sympathetic nervous system dysfunction and impaired psychogenetic sexual arousal after SCI. Additionally, SCI damage to the cord at T12 or higher has been shown to decrease vaginal lubrication due to interruption of sympathetic innervation and ensuing disruption of vaginal blood flow (Bérard, 1989; Forsythe and Horsewell, 2006; Anderson et al., 2007c; Hajiaghababaei et al., 2014). However, reflexive lubrication in women with SCI at or above T12 is still functional due to the sacral parasympathetic nervous system being preserved (Bérard, 1989).

Nevertheless, the ability to achieve orgasm does not appear to be impacted by SCI, regardless of level or completeness of injury but that the latency to complete orgasm is longer in women with SCI compared to uninjured individuals (Sipski et al., 2001). Moreover, SCI women have been shown to have normal menstrual cycles within the year of injury onset and are able to conceive and carry infants to full term (Cross et al., 1992; Reame, 1992; Atterbury and Groome, 1998).

5. Considerations for the future

Depending on the level of injury and the severity of the injury, SCI can detrimentally impact descending inhibitory supraspinal control over the sympathetic nervous system. This, along with the intraspinal plasticity that occurs following SCI, results in detrimental dysfunction of multiple organ systems. Therefore, individuals suffering with SCI are at greater risk for a plethora of diseases associated with these multi-organ dysfunctions, greatly affecting their quality-of-life. Thus, it is important for future studies to examine the effectiveness of potential therapeutics in improving not only motor and sensory function but also autonomic function, as mitigating SCI-induced organ dysfunction would greatly and broadly benefit those living with SCI.

Unfortunately, there are no current treatments to repair the damage to spinal cord after SCI. This is in part due to the lack of complete understanding of what factors limit repair after the CNS. However, this is a very active area of research. Cell-based treatments have significant promise in treating individuals living with SCI. Various cell types, such as mesenchymal stem cells (MSCs), neural progenitor cells (NPCs), Schwann cells, and induced pluripotent stem cells (iPSCs), have all been explored as potential therapies (Assinck et al., 2017). Cell-based transplantation methods are advantageous due to the fact that specific populations of cells can be selected for grafting. For instance, we recently showed that transplanting NPCs that contained serotonergic neurons – a population that normally modulates activity of the spinal sympathetic circuit – into a high-level SCI site increased the number of serotonergic fibers innervating the intermediolateral cell column below the SCI, improved cardiovascular regulation after SCI in a serotonin-dependent manner (Hou et al., 2020). Additionally, transplanted cells can be modified to secrete specific growth factors for neuroprotection, as wells a promote things like axon regeneration and synapse formation (Assinck et al., 2017). Similarly, peripheral nerve grafts can provide growth-supportive substrates that allow damaged axons to regenerate (Côté et al., 2011). These natural scaffolds may possibly serve as a means to restore innervation from the brain to neurons and circuits below the level of injury.

Recovery of autonomic function may not need a therapy focused on cell replacement or promoting axon regeneration that would reform circuits. As mentioned above, plasticity within the spinal cord after SCI contributes to sympathetic dysregulation. While the mechanisms underlying this plasticity are still being elucidated, several have been identified. This knowledge allows these mechanisms to be targeted for therapeutic effects.

For instance, as previously mentioned, treatment with gabapentin immediately following SCI reduces autonomic dysreflexia-induced immunosuppression, splenic atrophy, and maintained B and T cell levels post SCI (Brennan et al., 2021). Gabapentin binds to α2δ calcium subunits and is associated with decreased excitability of neuronal circuits, which is why it is often used to treat neuropathic pain following SCI. It has also been shown to decrease excitatory synaptogenesis (Eroglu et al., 2009; Risher and Eroglu, 2012; Risher et al., 2018). Acute treatment with low- and midrange-dose gabapentin after SCI have been shown to reduce the magnitude of experimentally induced autonomic dysreflexia (Rabchevsky et al., 2011, 2012; Eldahan et al., 2020; Brennan et al., 2021). Additionally, chronic treatment with a very high dose of gabapentin also reduced autonomic dysreflexia (Brennan et al., 2021). However, chronic treatment of a midrange dose of gabapentin was conversely showed to increase naturally occurring autonomic dysreflexia frequency (Eldahan et al., 2020). While the mechanism in which gabapentin is working to reduce SCI-induced autonomic dysreflexia is likely layered, one possible thought is that acute gabapentin treatment is working to prevent the formation of excitatory synapses by blocking TSP (Eroglu et al., 2009) as well as block secretion of intraspinal glutamatergic secretion. While these data provide interesting insights on gabapentin as a potential therapeutic for sympathetic dysregulation, additional studies on dosing and timing is needed to understand the full therapeutic potential of using gabapentin.

An additional potential treatment is XPro1595, a biologic that inhibits the pro-inflammatory cytokine TNFα. TNFα has been implicated with plasticity of the spinal sympathetic circuit, such as primary afferent sprouting and recruitment of interneurons into the circuit. Intrathecal administration of XPro1595 following complete high-level SCI attenuates the frequency and magnitude of autonomic dysreflexia events (Mironets et al., 2018, 2020). This was associated with decreased immunosuppression and detrimental remodeling and increased sensitivity of the peripheral vasculature to vasopressors normally observed after high-level SCI (Mironets et al., 2018, 2020). While this is promising, it there appears to be a finite therapeutic window for XPro1595. Starting administration 2 weeks after SCI does not affect autonomic dysreflexia (O'Reilly et al., 2021), indicating that starting treatment in the subacute timeframe would be needed.

High-level SCI increases levels of norepinephrine that act on β2-adrenergic receptors (β2AR) (Lucin et al., 2007; Zhang et al., 2013). In uninjured individuals, activation of SPNs results in release of norepinephrine and glucocorticoids from postganglionic neurons and the adrenal gland which, in turn, activate β2ARs and play a role in normal immune function. However, prolonged SPN activation following SCI ultimately leads to prolonged activation of β2ARs, resulting in an immunosuppressive state in the SCI population (Lucin et al., 2007; Nance and Sanders, 2007). Therefore, a possible therapeutic for the immunosuppression associated with high-level SCI could be utilizing β2AR blockers to prevent prolonged activation of β2ARs associated with an immunosuppressive state (Lucin et al., 2007; Zhang et al., 2013).

Neuromodulation *via* electrical stimulation is a non-pharmacological therapy that has been shown to improve functional recovery after SCI. Epidural electrical stimulation of the lower thoracic spinal cord after a high-level SCI attenuates extreme blood pressure fluctuations, bladder, and sexual dysfunctions normally observed after such an injury (Phillips et al., 2018; Walter et al., 2018; Darrow et al., 2019). Although epidural stimulation provides a variety of promising evidence for functional recovery following injury, a main downfall to epidural stimulation is that it requires a fairly invasive surgery in order to implant the electrodes on the dura of the spinal cord itself. Due to the invasive nature of epidural stimulation, researchers have begun to explore other, less invasive stimulation techniques such as transcutaneous stimulation. Similar to epidural stimulation, transcutaneous stimulation works to provide electrical currents to activate neuronal circuits. However, in the case of transcutaneous stimulation, the electrodes are placed on the skin of the injured individual rather

than on the spinal cord itself. Transcutaneous stimulation has shown to also ameliorate cardiovascular dysfunction as seen by attenuating orthostatic hypotension normally seen in SCI individuals (Phillips et al., 2018), as well as improving bladder capacity and decreasing detrusor contractions following injury (Doherty et al., 2019).

Another type of stimulation being explored as a therapy for SCI individuals is vagus nerve stimulation. Stimulation of the vagus nerve after SCI in preclinical models decreases heart rate, however this decrease is only seen while the stimulation is happening (Sachdeva et al., 2020). Importantly, vagus nerve stimulation does not appear to exacerbate autonomic dysreflexia events, alluding to its safety as a possible therapeutic to improve autonomic function after SCI (Sachdeva et al., 2020). Interestingly, vagus nerve stimulation in a rodent SCI model reduces neuroinflammation, i.e., expression of pro-inflammatory cytokines while also increasing the expression of anti-inflammatory cytokines, after SCI (Chen et al., 2022), suggesting that vagus nerve stimulation has broad effects and any recovery may be through multiple mechanisms. Although the overall concept of neuromodulation as a therapy for a myriad of dysfunctions seen after SCI is very exciting and full of potential, the devil is in the details on how the electrical stimulation is delivered, as there are ultimately pros and cons to each type of electrical stimulation technique. Additionally, it is likely that neuromodulation will be needed to be used in conjunction with other therapeutic treatments to provide optimal treatment to individuals living with SCI.

Although the spinal cord research field is constantly developing new therapeutic strategies, it is not likely that one therapy will produce complete recovery in spinal cord injured individuals. Because of the complex, multifaceted nature of SCI, it is likely a combination of therapies targeting various aspects will be needed to provide the best possible chance of recovery for SCI individuals. It is imperative that the research community continue to increase our mechanistic understanding of the consequences of SCI in order to shed light on what therapies need to target in order to improve the overall health and quality of life for those living with SCI.

Author contributions

MW prepared the first draft of the manuscript. MW and VT edited and revised the manuscript. Both authors contributed to the article and approved the submitted version.

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Conflict of interest

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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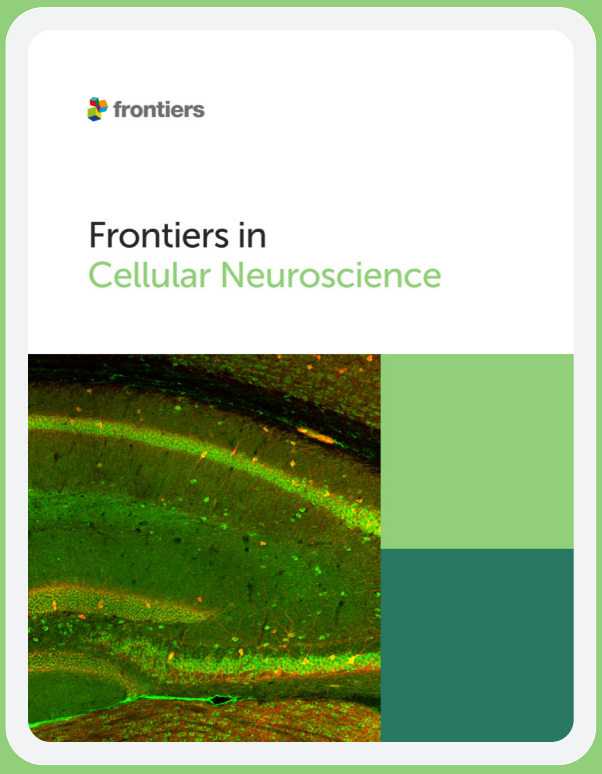

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