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Glutamatergic Regulation of Remyelination After Spinal Cord Injury

Abstract

Chronic demyelination, resulting in slowed conduction velocity and impaired recovery, is a hallmark of spinal cord injury (SCI), and is partially due to the loss of oligodendrocytes (OLs). OLs are terminally differentiated and cannot self-renew; thus, the endogenous repair that occurs after SCI is largely attributed to the robust proliferation of oligodendrocyte progenitor cells (OPCs) that differentiate into mature OLs. Since OPCs are the primary source of new myelin, it is critical to understand how OPCs are regulated at various times pre and post-injury. Evidence from chemical demyelination studies show glutamate is packaged in vesicular glutamate transporter (Vglut)-positive vesicles and stored along demyelinated axon shafts. Activity-dependent release of glutamate from these axons stimulates OPC migration, differentiation, and ultimately remyelination. Since spinal tracts are mostly glutamatergic, we expect a similar phenomenon to occur after SCI. Here, we tested the hypothesis that Vglut2 accumulates in axons after SCI and predicts OPC-axon contact points. For this, we collected spinal cords from naïve and SCI mice from 7 days to 6 months post-injury (mpi) to examine Vglut2 distribution in axons. This revealed the number of Vglut2+ puncta in spared white matter increased continuously after SCI to a peak 9-fold greater than naïve at 6mpi. The number of Vglut2+ puncta within axons contacted by OPC processes was also quantified. In naïve tissue, ~10% of Vglut2+ puncta were contacted by an OPC process. OPC/Vglut2 contacts increased significantly by 28dpi and rose to a peak at 6mpi when 40% of Vglut2 puncta had an OPC contact. These results suggest axonal glutamate release promotes OPC contacts with axons after SCI and may play a role in remyelination. Overall this work shows for the first time that OPCs contact Vglut2-enriched

regions of axons after CNS trauma, and suggests that loss of such contacts may contribute to chronic post-SCI demyelination.

Introduction

Spinal cord injury (SCI) is a devastating trauma that affects over 17,000 individuals in the United States per year[1]. The initial impact from a SCI severs axons, causing immediate and irreversible damage that impairs fine and gross motor function below the level of injury[2]. After a spinal contusion, total axon numbers continue to decrease in the days following injury, with axon degeneration increasing 2-fold between 2-7 days post injury (dpi)[3]. Most axons that survive the initial trauma and secondary degradation are myelinated, creating a rim of spared white matter along the lesion border. These spared axons remain functional but become demyelinated, which is detrimental to axon health after SCI. Importantly, oligodendrocytes (OLs) are the resident myelinating cells of the central nervous system. However, 50% of OLs in spared tissue die within 24 hours of injury[4], and since each OL myelinates up to 50 axons[5], this extensive OL death leads to widespread demyelination after injury[6]. Demyelination occurs within 24 hours after injury and persists for at least 10 years in humans[7].

Remyelination is the process of generating new myelin sheaths to wrap axons in the adult CNS. Remyelinated axons have thinner myelin and shorter internodes, yet ion channel organization is re-established and saltatory conduction is restored after injury[6,8,9]. Surviving OLs are post-mitotic and cannot remyelinate axons, thus new mature OLs are produced by proliferation and differentiation of NG2 cells[10]. NG2 cell proliferation peaks within the first week post-SCI, but NG2 cells continue to proliferate for at least 4wpi and differentiate into OLs chronically[11,12].

One potential mechanism for OL-mediated remyelination is neural activity[13–16]. NG2 cells are not excitable and instead express growth factor and neurotransmitter receptors to monitor and respond to changes in extrinsic factors[17]. Neurotransmitter release was traditionally thought to be isolated to the synapse, but NG2 cells are located along axons far away from the synapse, suggesting that neurotransmitters are also released along axons. Indeed, NG2 cells contact myelinated axons at nodes of Ranvier[18], a spot of glutamate release[19]. Interestingly, glutamate release has also been observed from unmyelinated axons (Figure 1)[20,21]. In zebrafish, vesicular glutamate release influences the selection of axons that undergo myelination[15]. Glutamate is packaged into vesicular glutamate transporter (Vglut)-positive vesicles and, upon neural stimulation, calcium influx stimulates glutamate release via vesicle fusion with the axonal membrane[20,21]. Glutamate then binds to AMPA receptors on neighboring NG2 cells to stimulate NG2 cell migration and ultimately differentiation[20–24].

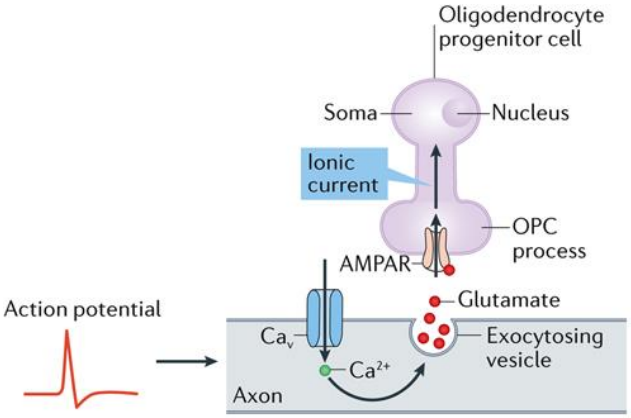

Figure 1. Schematic of glutamatergic signaling to NG2 cells. Modified from Micu et al., Nature Reviews Neuroscience, 2018.

It is well accepted that OLs remyelinate axons after SCI; however, the extent of demyelination and remyelination is debated within the field. We hypothesize that remyelination is robust and long-lasting, but a subset of spared axons remain chronically demyelinated. To test this, changes in nodal pathology were tracked over time after a thoracic spinal cord contusion injury. This revealed aberrant spreading and elongated nodal profile lengths at acute and chronic times post-SCI compared to uninjured controls. Then, we used a reporter mouse to track new

OLs and new myelin over time, and observed GFP+ labeling indicative of OL differentiation and remyelination between 5-10wpi. Therefore, some, but not all axons are chronically remyelinated after injury. The mechanism promoting remyelination, however, is unknown.

One potential mechanism is glutamate, the main excitatory neurotransmitter. We predict that similar to demyelination models where glutamate drives remyelination[25], glutamate release also contributes to repair after SCI. Therefore, we tested the hypothesis that Vglut2 accumulates in axons after SCI and predicts NG2 cell-axon contact points. Here, our data show a progressive increase in Vglut2+ puncta and Vglut2+ puncta co-localization with NG2 cell processes after SCI. Collectively, our data highlight that demyelination persists for at least 6 months post-SCI with a subset of these spared axons becoming remyelinated after injury, which may be due to active NG2 cell/glutamate signaling.

Methods

Animals: Two SCI mice experiments were performed in this study. First, a time course analysis on female C57Bl/6 mice 10-12 weeks of age was conducted. Second, *PDGFRα-CreER*^{T2} mice (generous gift from Dr. William Richardson) were crossed with *Tau-loxP-STOP-loxP-mGFP-IRES-NLS-LacZ-pA* mice (Jackson Laboratories, strain Taum^{GFP}) in which new OLs and new myelin express membrane-bound GFP. Recombination was induced by oral administration of tamoxifen (300mg/kg) for 4 consecutive days starting at 5wpi, and animals were sacrificed at 10wpi.

Spinal cord injury: All surgical and postoperative care procedures were performed in accordance with the Ohio State University Institutional Animal Care and Use Committee. Adult mice were anesthetized with a ketamine/xylazine mixture (80mg/kg) and received a single-level laminectomy at vertebral level T9. The mice then received a moderate (75kD force) spinal cord

injury using the Infinite Horizons device (Precision Instruments). The muscles overlaying the spinal cord were then sutured and the skin was closed using surgical staples. Postsurgical care included 5-day administration of antibiotics (gentomicin, 5mg/kg) and 2ml of saline. Bladders were manually expressed twice a day for the duration of the study.

Tissue processing: Mice were euthanized with a ketamine/xylazine mixture (1.5X surgical dose) and perfused transcardially with 0.1M PBS, followed by 4% paraformaldehyde (PFA) in PBS (10ml/min flow rate). Spinal cords were removed, post-fixed in 4% PFA for 2 h, then transferred to 0.2 M PB overnight. The next day, tissue was cryoprotected for 3 days in 30% sucrose dissolved in dH_2O. Fixed spinal cords were embedded in optimum current temperature compound (Electron Microscopy Sciences) and frozen on dry ice. Blocked spinal cords were cut in serial longitudinal sections at 10 μm, mounted on SuperFrost Plus slides (Thermo Fisher Scientific), and stored at -20℃.

Immunofluorescence: Sections were rinsed in 0.1M PBS and blocked for nonspecific antigen binding using 4% BSA/0.3% TritoX-100/PBS (BP+) for 1 h. Primary antibody was diluted in BP+ and sections were incubated overnight at room temperature. Next, sections were rinsed and treated with an Alexa Fluor secondary antibody (1:1000; Invitrogen) for 1 h. Sections were rinsed and cover slipped with Immu-Mount (Thermo Scientific).

For double-labeled immunofluorescence when two primary antibodies were raised in the same species (rabbit anti-Caspr; rabbit anti-Kv1.2), heat-mediated antigen retrieval was performed. Sections were placed in antigen retrieval solution containing 0.1% high pH solution in dH2O heated to 90℃ for 10 min, then cooled at room temperature for 10 min. Sections were rinsed in 0.1M PBS, blocked in BP+ for 1 h, then incubated in Caspr for 3 h. Next, sections were

rinsed and treated with Alexa Fluor secondary antibody for 1 h. The remainder of the stain was completed as outlined above.

Primary antibody: specificity	Concentration	Host species	Vendor
Caspr	1:1000	Rabbit	Abcam
Kv1.2	1:1000	Rabbit	Alomone
NF-H: large diameter axons	1:1000	Chicken	Aves Labs
GFP: green fluorescent protein	1:1000	Chicken	Aves Labs
NG2: progenitor cells	1:1000	Rabbit	Millipore
Vglut2	1:4000	Guinea pig	Millipore

Quantification of nodal pathology: Longitudinal spinal cord sections were stained for Caspr, Kv1.2, and NF as outline above. Confocal z-stack images (1 μm thick) were collected at 40X at 0.5 mm, 1 mm, and 2 mm rostral and caudal to the lesion epicenter on a Leica SP8 confocal microscopy system. Maximum projection images were used for analysis with MIPAR, a novel image analysis software[26]. All sections were imaged with the same confocal laser intensity, and analyzed with the same MIPAR threshold (Kv1.2, 103 Bright; Caspr, 99 Bright). Nodal protein lengths were measured using the Color by Measure feature in MIPAR's Image Processor.

Quantification of Vglut2/NG2 co-localization: Longitudinal spinal cord sections were stained for Caspr, Kv1.2, and NF as outline above. Confocal z-stack images (1 μm thick) were collected at 40X at 1 and 2 mm rostral and caudal to the lesion epicenter on a Leica SP8 confocal microscopy system. All sections were imaged with the same confocal laser intensity, and analyzed with the same MIPAR threshold (Vglut2, 84 Bright; NG2, 99 Bright). Total Vglut2+ puncta in the spared white matter where quantified using a 3D-reconstruction of the z-stack. Vglut2+ vesicles are at least 4 pixels in size[27], therefore labeling smaller than 4 pixels was excluded from Vglut2 counts. Data are averages of 1 mm and 2 mm analysis.

Data analysis: All data were collected in a blinded manner. Data were analyzed using GraphPad Prism 7.0. Immunofluorescence data were analyzed by a 2-way repeated-measures ANOVA and Bonferroni post-hoc tests where significance was reported when $p < 0.05$.

Results

Loss of axonal nodes of Ranvier occurs chronically in the injured mouse spinal cord.

Myelin comprises numerous layers of compact plasma membranes that form an insulating sheath around axons[28]. Periodic gaps in myelin allow for highly specialized structures called nodes of Ranvier that are essential for proper signal transduction[29]. These nodes are organized with the sodium channel Nav1.6 concentrated at the node of Ranvier, contactin-associated protein (Caspr) in the paranode, and the potassium channel Kv1.2 restricted to the juxtaparanode[30]. However, after SCI, OL death and demyelination disrupts ion channel localization causing nodal proteins to spread along demyelinated axons (Fig. 1A, B), which contributes to axon dysfunction after injury[31]. The extent and chronicity of axon demyelination remains highly contentious.

To examine demyelination over time, we performed a time course study where mice were injured and sacrificed at acute and chronic time points. Spinal cords were analyzed at 7d, 18d, 28d, 10w, and 6mpi and at 0.5 mm, 1 mm, and 2 mm from the lesion epicenter for changes in nodal pathology to identify demyelination. Because myelin does not label well with immunohistochemistry, a common technique to assess myelin is to label nodes of Ranvier. We first used nodal pathology to examine whether nodal disruption could be detected in chronically injured mice. Intact nodes of Raniver were defined as Kv1.2 flanking Caspr protein. Aberrant spreading of Caspr and Kv1.2 was present along axons for at least 6mpi (Fig. 1A). In naïve tissue ~88% of nodes were intact, whereas intact nodes close to the lesion decreased significantly to

~15% at acute and chronic times post-SCI (Fig. 1B). Distal to the lesion, the number of intact nodes decreased 3-fold compare to naïve mice at acute time points and continued to decrease to a peak 9-fold lower than naïve at 6mpi (Fig. 1B). To further confirm demyelination, Caspr and Kv1.2 profiles were quantified in the white matter bordering the SCI lesion site. Average nodal length on spared axons at the lesion epicenter were significantly longer in acute and chronically injured tissue compared to uninjured tissue. Naïve tissue had no Caspr or Kv1.2 lengths greater than 20 μm; however, 20 μm profiles were present 7dpi and persisted for at least 6mpi (Fig. 1D, E). This demonstrates that the loss of nodes of Ranvier is maintained long-term after spinal cord injury.

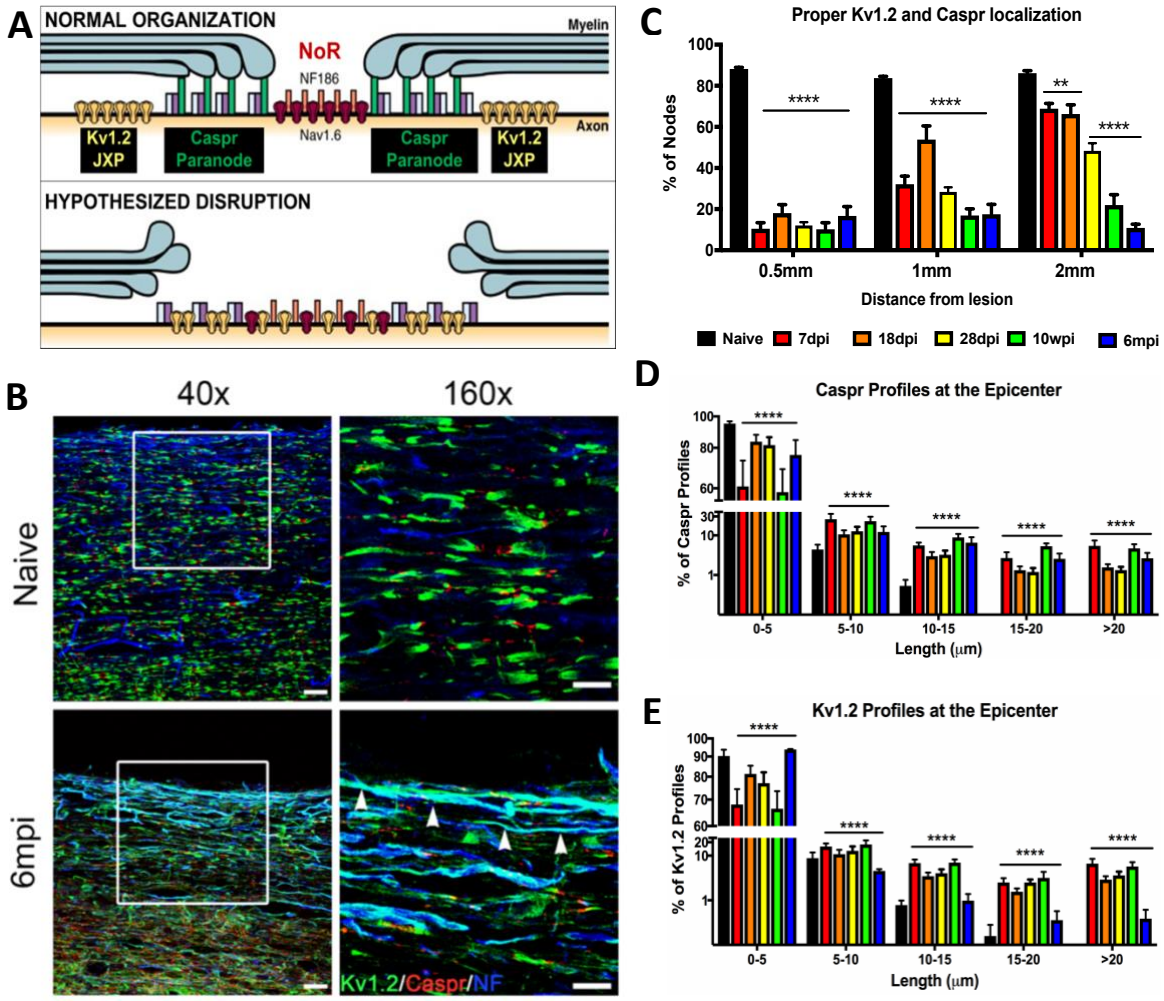

Figure 1. Quantification of nodal protein localization after SCI at acute and chronic time points. **(A)** Schematic of normal node of Ranvier domains and the hypothesized SCI-induced redistribution of these domains. Modified from: Arancibia-Carcamo, I. L., & Attwell, D. (2014). Acta Neuropathologica, 128(2), 161–75. **(B)** Intact nodes were quantified based on proper Kv1.2 and Caspr localization (Kv1.2, Caspr, Caspr, Kv1.2). The number of intact nodes were significantly decreased at 0.5, 1, and 2mm from the lesion at all times post-SCI compared to naïve mice. At 7d and 18dpi, there were more intact nodes 2mm distal to the lesion epicenter. **(C)** Confocal z-stacks of longitudinal sections from naïve and 6mpi spinal cords showing normal and aberrant nodes of Ranvier. Nodes are identified by **Kv1.2+** segments (green) flanking **Caspr+** segments (red). **Axon neurofilaments** are labeled with blue. Ion channel and Caspr spreading indicated by arrowheads. Scale bars = 25 μm (40x), 10 μm (160x). **(D-E)** Quantification of nodal profile lengths. White matter bordering SCI lesion sites after injury reveal marked spreading of **(D)** Caspr and **(E)** Kv1.2, an indication of demyelination.

Newly generated OLs and new myelin can be tracked over time with membrane-targeted GFP expression.

Characterized as non-neuronal glial cells in the CNS, NG2 cells are identified by their dual expression of the proteoglycan nerve/glial antigen 2 (NG2) and platelet-derived growth factor receptor alpha (PDGFRα)[32,33]. NG2 cells are highly proliferative and comprise ~5% of CNS cells[34], a portion of which differentiate into OLs. Extensive work from our lab and others have characterized NG2 responses post-SCI, leading to the overall conclusion that NG2 cell proliferation and differentiation is essential for repair and functional recovery in the acute and chronic injury environment[35–37]. The extent of this repair after SCI, however, it not yet known.

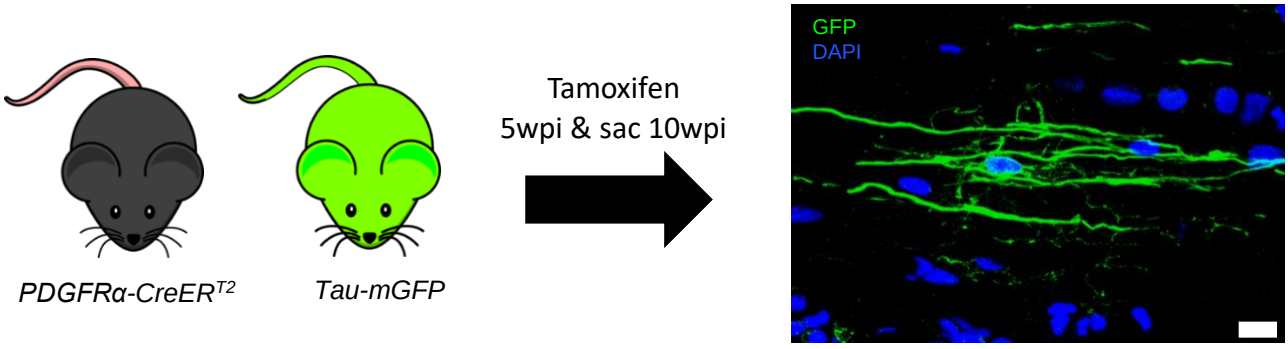

Figure 2. Oligodendrogenesis occurs between 5-10wpi. *PDGFRα-CreER^{T2}* mice crossed with *Tau-mGFP* mice received tamoxifen 5wpi and were sacrificed 10wpi.

To track new OLs and new myelin over time, *PDGFRα-CreER*^{T2} mice (in house) were crossed with *Tau-loxP-STOP-loxP-mGFP-IRES-NLS-LacZ-pA* mice (*Tau-mGFP* mice; in house). Mice were given a moderate spinal cord injury, gavaged with tamoxifen at 5wpi, then spinal cords were collected at 10wpi (Fig. 2). In this mouse line (referred to as mTau), GFP is bound to the membrane and expressed following tamoxifen induced Cre-mediated recombination. Because NG2 cells do not express the active Tau promoter, only NG2 cells that differentiate into OLs after tamoxifen administration will be tagged with GFP[38]. Therefore, only OLs and myelin generated between 5-10wpi expressed membrane-targeted GFP. GFP+ cells were present in the spared white matter, indicating the presence of a myelinating OL (Fig. 2). This provides a snapshot of NG2 cell differentiation and chronic remyelination after spinal cord injury.

Vglut2+ puncta increase in spared white matter after SCI and associate with NG2 cells.

Neural activity is known to stimulate the release of Vglut2+ positive vesicles from unmyelinated axons and promote NG2 cell migration, differentiation, and myelination[13,15,20,21,24,25]. Thus, we examined Vglut2 distribution in naïve and SCI axons. Mice received a moderate (75kD) T9 spinal cord injury and were sacrificed at 7d, 18d, 28d, 10w, and 6mpi. This revealed that SCI significantly increased the number of VGlut2+ puncta in spared white matter relative to naïve axons, increasing continuously to a peak 9-fold greater than 3 month naïve and 2-fold greater than 6 month naïve at 6mpi (Fig. 3A, B). SCI also increased the number of contacts between NG2 cells and VGlut2+ puncta ratio with a 3-fold increase in 28dpi tissue and 1.5-fold increase in 6mpi tissue compared to age-matched naïve tissue (Fig. 3D). Overall, SCI increased the total number of VGlut2 puncta for at least 6 months after injury, which may stimulate increased OPC-axon contact.

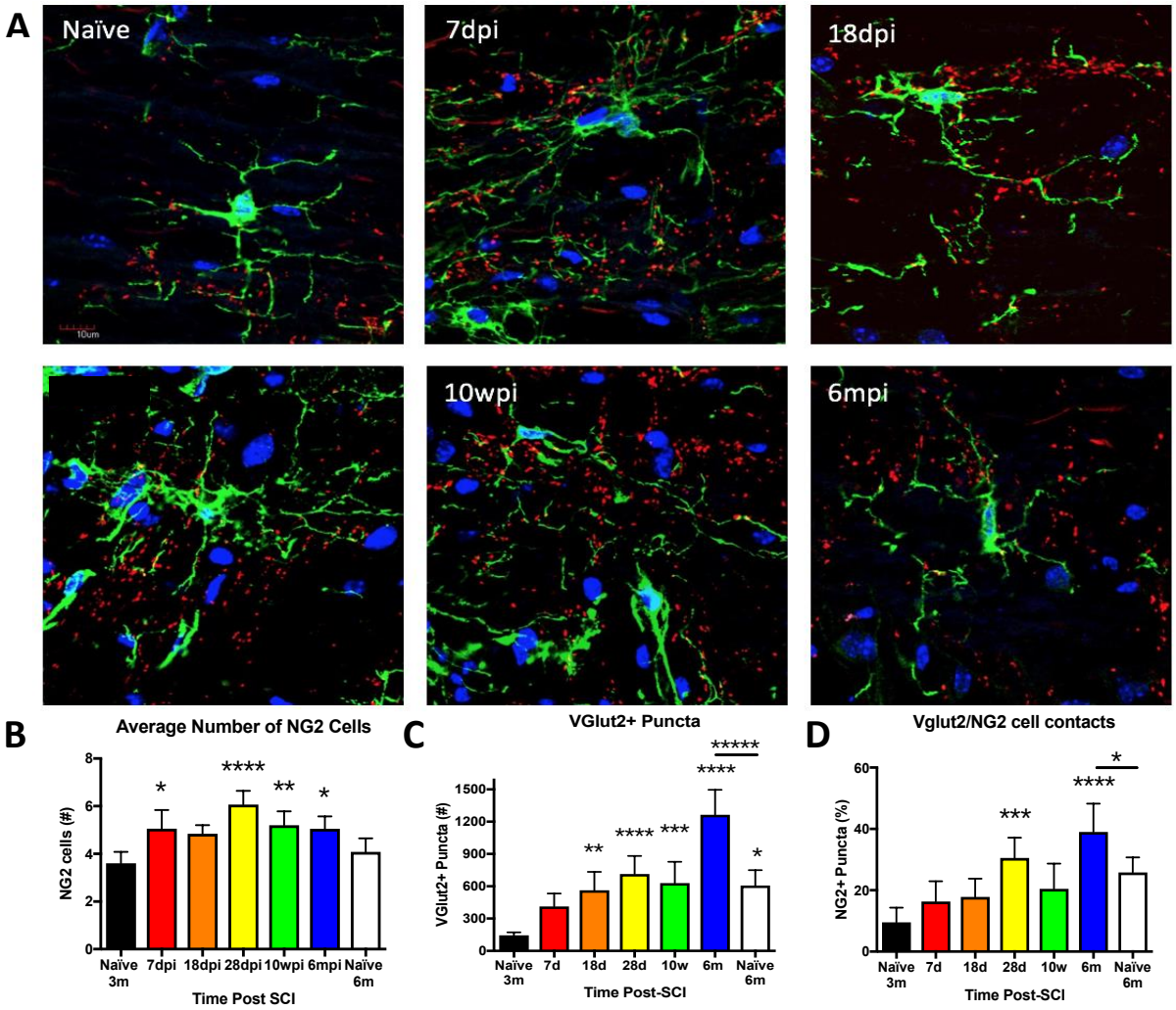

Figure 3. Quantification of Vglut2+ puncta and Vglut2+/NG2+ contacts at acute and chronic times post-SCI. (**A)** Immunohistochemical labeling of **NG2** (green), **Vglut2** (red), and **nuclei** (DAPI, blue) in naïve and injured spinal cord white matter, from 7d post-injury through 6m post-injury. Scale bar = 10μm. (**B)** Quantification of total Vglut2+ puncta in spared white matter. Vglut2 increases continuously after SCI to a peak 9-fold greater than 3 month naïve and 2-fold greater than 6 month naïve at 6mpi. Counts represent averages of 1mm and 2mm images. **(C)** Despite elevated NG2 cells after injury, **(D)** the ratio of Vglut2+/NG2+ puncta per OPCs rises 3-fold in 28dpi tissue and 1.5-fold in 6mpi tissue compared to age-matched naïve tissue. Counts represent averages of 1mm and 2mm images.

Conclusions and Future Directions

SCI results in functional deficits despite a large number of axons surviving the injury. One reason could be the loss of myelin on these axons, preventing saltatory conduction at the site of injury. Thus, a common therapeutic strategy is to promote remyelination to restore axon

transduction. Here we describe the demyelination and remyelination patterns of axons at acute and chronic time points post-SCI, and propose a potential mechanism for remyelination.

Nodes of Ranvier are gaps in the myelin sheath fundamental to transmitting electrical signals down axons. SCI results in retraction of the myelin sheath from the paranode, initiating dispersion of nodal proteins and loss of their highly organized distribution along the axon[30] (Figure 1A, 1B). This nodal disruption prevents saltatory conduction and contributes to functional impairments. Here we quantify anatomical changes in nodal pathology to characterize the extent of demyelination over time. The present data show that proper Kv1.2 and Caspr localization significantly decreases after SCI. Interestingly, at 7d and 18dpi a larger percent of Kv1.2 and Caspr profiles are localized to the node of Ranvier distal to the lesion compared to the lesion border (Figure 1C). This suggests that there is prominent demyelination along the lesion border and, to a lesser extent, in the distal white matter acutely after spinal cord injury, which persists for at least 6 months post injury. Electron microscopy experiments currently underway will confirm the presence of chronically demyelinated axons.

Other labs have shown that remyelination occurs beyond 2-weeks post injury[6,39,40]. Our experiment used GFP-expressing reporter mice to confirm new myelin is generated chronically in the spared white matter (Figure 2). Future studies will focus on characterizing the extent of GFP+ myelin at the lesion border and distal to the lesion. In addition, we will use reporter mice to determine when peak remyelination occurs after injury. Finally, we will track OLs over time to examine OL survival at acute and chronic injury time points.

The present data show that the number of Vglut2+ puncta are elevated at 18dpi and remain upregulated until at least 6 months post injury (Figure 3A, 3C). Acutely after SCI there was no change in Vglut2+/NG2 cell contacts, despite the increase in Vglut2+ puncta. However,

at 28dpi and 6mpi, the percent of Vglut2+/NG2 cell contacts significantly increased compared to naïve mice (Figure 3D). Since NG2 cells are exquisitely sensitive to their environment, we predict that glutamate release from demyelinated axons promotes OL differentiation and remyelination. Future studies will examine the remyelination of glutamatergic axons using transgenic mice.

In summary, this work demonstrates that the periods of demyelination and remyelination after SCI is more robust than previously reported. The reported data suggest that glutamate may promote NG2 cell migration to demyelinated axons, but future studies manipulating glutamatergic signaling are necessary to clarify its specific role in remyelination. This is clinically relevant as understanding cellular responses in the injury environment may uncover better therapeutic targets to maximize endogenous and functional repair after SCI.

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