Nodes of Ranvier are Incompletely Repaired and Continually Disrupted after Spinal Cord Injury

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Introduction

In the United States, approximately 17,500 cases of spinal cord injury (SCI) are reported each year; however, fewer than 1% of these patients experience significant motor, sensory, and autonomic recovery [1]. This is, in part, due to the limited capacity for the injured spinal cord to repair itself. There are several therapeutic currently endeavors focused in stimulating regeneration of spared axons; however, these axons typically remain unmyelinated thereby limiting their ability to efficiently propagate signals. Thus, strategies that aim to promote the spontaneous remyelination of spared axons are integral to enhancing functional recovery for patients of spinal cord injury.

In the healthy brain and spinal cord, myelin compactly ensheathes axons thereby providing the insulation necessary for timely conduction of nerve signals ([2]). Following spinal cord injury, myelin sheaths retract and degenerate, rendering the axons demyelinated within 15 minutes post injury and persists for at least 3 weeks post injury [3–5]. While the CNS is largely limited in its capacity for self-repair, trauma induces a robust regeneration of oligodendrocytes, the primary myelinating cells of the CNS for at least 3 months post injury [6–11]. Whether demyelinated axons persist chronically after spinal cord injury or whether endogenous oligodendrocyte regeneration has the capacity to remyelinate all spared axons remains contentious. Recent work suggests that there is no evidence for demyelination in the chronically injured mouse cord [12]; however, endogenous regeneration of oligodendrocytes, a metabolically demanding process, persists for at least 3 months post injury (mpi) [8] suggesting that

oligodendrocytes are continually attempting to remyelinate spared, demyelinated axons. While acute endogenous myelin biogenesis following trauma and therapeutics targeting towards improving myelin health acutely have been widely studied, our knowledge regarding myelin status and spared axons chronically is poor and incomplete. Herein, we propose nodal structure as a novel and sensitive method to assess myelin status after CNS trauma and if endogenous oligodendrogenesis has the capacity to fully remyelinate spared axons.

Rapid, saltatory conduction of nerve signals is enabled via complex myelin-axon interactions that establish distinct domains of structural proteins and include the paranodal protein, Caspr, flanked by juxtaparanodal voltage gated K+ channels (Kv1.2) [13,14]. Nav 1.6 voltage-gated Na+ channels are also clustered in the Node of Ranvier, a break in the myelin sheath where the axolemma is exposed, to support regeneration of signals [15]. However, OL death is several demyelinating conditions has been shown to disrote the discreet organization of these nodal proteins, resulting in compromised signal propagation, aberrant excitability patterns, and neuronal dysfunction [16]. Functional OL remyelination of spared axons restores the proper nodal organization.

Currently, structural abnormalities in the node induced by SCI have not been evaluated. The present study aimed to characterize structural abnormalities in/around the Node or Ranvier as an index of functional or suboptimal endogenous remyelination. We hypothesized that endogenous oligodendrogenesis does not have the capacity to fully remyelinate spared axons. To test this, we evaluated the structural organization of nodal proteins as an index of function remyelination or persistent axon demyelination. If axons are spontaneously remyelinated, the discreet organization of nodal proteins should be restored; however, if axons remain chronically

demyelinated, the specific arrangement of proteins within and around the Node will be lost and, instead, diffusely expressed outside of the node on spared axons.

Data confirm that moderate contusion SCI in a preclinical mouse model induces aberrant spreading of nodal proteins outside of the node. Disruption of all nodal domains persists chronically for at least 24wpi. Moreover, we show here that despite robust oligodendrogenesis that persist for at least 3mpi, nodes of Ranvier are not only chronically disrupted but are also actively and persistently broken down distal from the injury epicenter.

Materials and Methods

Contusion spinal cord injuries. All surgical procedures, post-operative care, and experimental paradigms were in accordance with The Ohio State University Institutional Animal Care and Use Committee (IACUC). C57Bl/6 mice ~12 weeks of age (insert n and gender) were deeply anesthetized a ketamine/xylazine cocktail (insert mg/kg).

Figure 1: Experimental group sizes and time points.

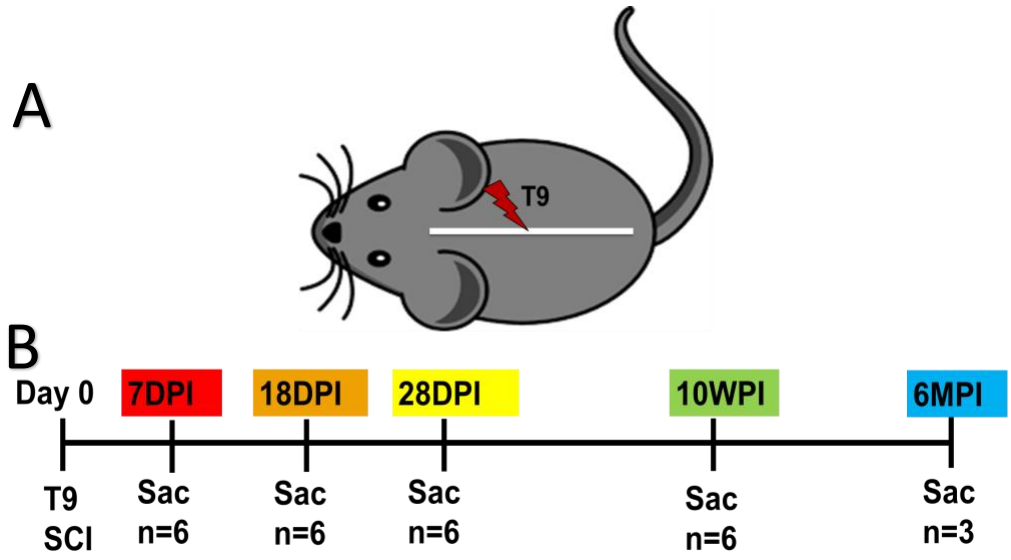

Figure 1: A) Schematic of clinically relevant mouse model of contusion spinal cord injury sustained at mid-thoracic vertebrae (T9). ***B)*** Time course time line of various acute and chronic time points at which mouse spinal cords were evaluated for nodal pathology.

A mid-thoracic (T9) laminectomy was performed to expose the spinal cord and all mice received a moderate contusion injury (75KDyne force) using the Infinite Horizons device (Precision Systems and Instrumentation, Lexington, Kentucky). Muscles around the spinal cord were sutured and sterile wound clips were used to close the skin, after which all mice received a subcutaneous saline injection (2ml) to help maintain proper hydration and were kept on a heating plate (37C) overnight to ensure proper body temperature. Saline and Gentomicin were administered subcutaneously for the first 5 days post injury and bladders were voided twice a day until voluntary expression returned. See below for experimental group sizes and time points.

Perfusion and tissue processing. Naïve animals and animals from acute and chronic time points were anesthetized with a ketamine/xyalazine mixture (1.5x body weight) and transcardially perfused with 0.1M PBS and ~100 ml of 4% paraformaldehyde in PBS. Spinal cords were dissected, post-fixed in 4% PFA at 4C for 2 hours, and incubated in 0.2PB overnight. The next day, spinal cords were cryoprotected in 30% sucrose dissolved in 0.1M PBS (4C, 48 hours). Spinal cords were embedded in OCT compound (Electron Microscopy Science), frozen on dry ice, and cut at 14um longitudinal sections on a cryostat. Tissue was serially mounted on super frost slides and stored at -20C until use.

Immunoflourescence. Sections were rinsed in 0.1M PBS and subsequently incubated in a 4%BSA/PBS/0.3% Tx-100 (BP3+) solution for 40 minutes to prevent non-specific antigen binding. Next, sections were incubated in rabbit anti-Caspr primary antibody (1:1000) for 3 hours in room temperature after which they were incubated with an AlexaFluor secondary antibody (1:500; Abcam) for 1 hour. Heat induced antigen retrieval was used to unmask any antigens that are otherwise prevented from binding to the primary antibody due to tissue fixation. Here, a high pH citrate buffer (insert pH and vendor) was heated to 90C and sections were placed

in the buffer for 10 minutes. After cooling for 10 minutes, sections were rinsed in 0.1M PBS and High salt PBS to prevent high background and subsequently incubated in additional primary antibodies overnight (rabbit anti- Kv1.2 and chicken anti-NFH or rabbit anti-Nav1.6 and chicken anti-NFH). The next day, sections were incubated in AlexaFluor secondary antibodies for 90 minutes, rinsed, and coversliped with Immunmount. See below for a list of primary and secondary antibodies used.

Table 1: Primary antibodies used for immunofluorescence

Primary Antibody	**Vendor**
Caspr (rabbit)	Abcam
Kv1.2 (rabbit)	Alomone
Nav1.6 (rabbit)	Alomone
NF-H (chicken)	Aves

Results

SCI disrupts Nav+ clustering in the Node of Ranvier

In healthy, myelinated CNS axons, several studies have confirmed that Nav1.6 is the primary voltage gated sodium channel clustered in the Node of Ranvier. Previous studies in EAE mouse models have also shown diffuse Nav1.6 immunolabeling along demyelinated axons in MS plaques ([18]). Indeed, we our data confirms Nav1.6 is clustered in the Node of Ranvier and is diffusely expressed along demyelinated axons after traumatic spinal cord injury. In healthy, uninjured mice (naïves), approximately 100% of voltage-gated sodium channels (Nav1.6) are ≤5μm but within 1wpi, 5μm length Nav1.6 clusters in the Node are decreased by 10% and within 2.5wpi, decreased by 15%, with some Nodes extending to lengths over 2-fold greater than

Nav1.6 cluster lengths in Naïves (Figure 1a). This pathology persists for at least 24wpi suggesting that axons are chronically demyelinated and sodium channels that regenerate nerve signals are chronically dysregulated after traumatic mouse contusion spinal cord injury. The percent area of Nav1.6 immunolabeling along spared, demyelinated axons was used to confirm that sodium channels are persistently disprupted and clustering in the Node of Ranvier is lost (Figure 2b).

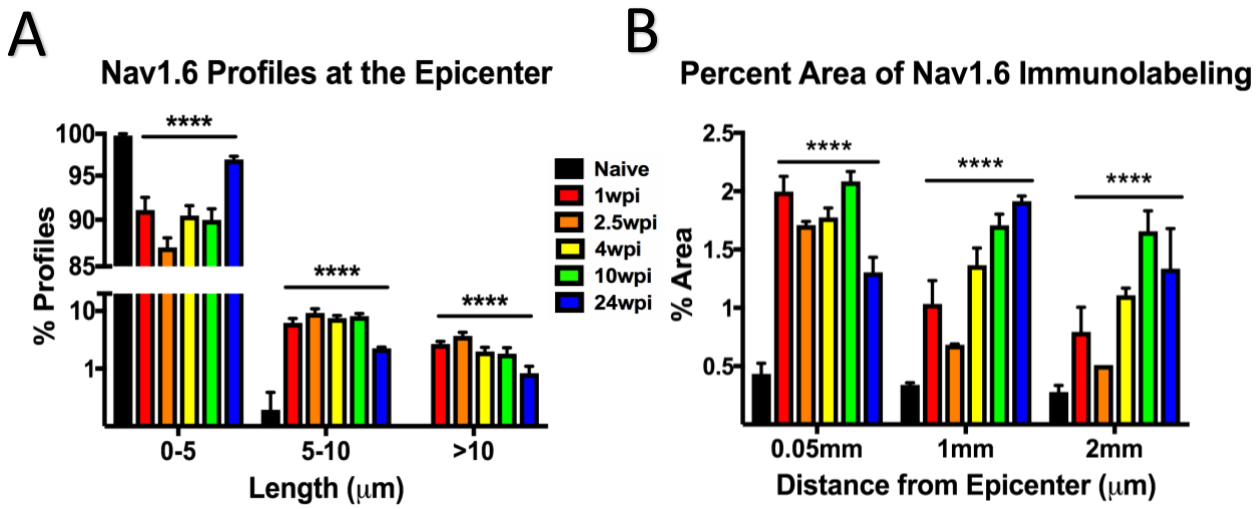

Figure 2: A) *Approximately 100% of Nodes of Ranvier in uninjured mice are ≤5μm. By 1wpi, 5μm length Nodes are decreased by 10%, and by 2.5wpi, decreased by 15%, with some Nodes extending >10μm. This pathology persists for at least 24wpi.* **B)** *Disruption of Nav channel is observed distal from the injury epicenter and persists chronically.*

Sustained dysregulation of paranodal proteins that anchor the myelin sheath to the axon membrane

Discreet Caspr localization establishes the axo-glial junction by forming septate-like functions between the axon membrane and the compact myelin sheath [19,20]. Paranodal junctions have also been shown to prevent the lateral diffusion of ion channels clustered in the Node of Ranvier and the adjacent juxtapranode [21]. As previously reported in mouse models of EAE and SCI, discreet

localization of the paranodal protein, Caspr, is disrupted and diffusely expressed along demyelinated axons [18]. Approximately 95% of Naïve Caspr profiles are ≤5μm (Figure 3). By 1wpi, 40% of Caspr profiles are >5μm, some of which are >20μm, a length that is 4-fold greater than Naïve Caspr profiles. 20% of Caspr profiles revert to ≤5μm between 2.5wpi and 4wpi, only to aberrantly increase in length by 10wpi suggesting that Caspr pathology is dynamic. Excessively long Caspr profiles that extend outside of their paranodal domain persists for at least 24wpi. Data suggests that despite previous evidence for a protracted and robust oligodendrogenesis [13], the dysregulation of paranodal proteins that establish the axo-glial junctions and prevent the lateral diffusion of nodal and juxtaparanodal ion channels is sustained.

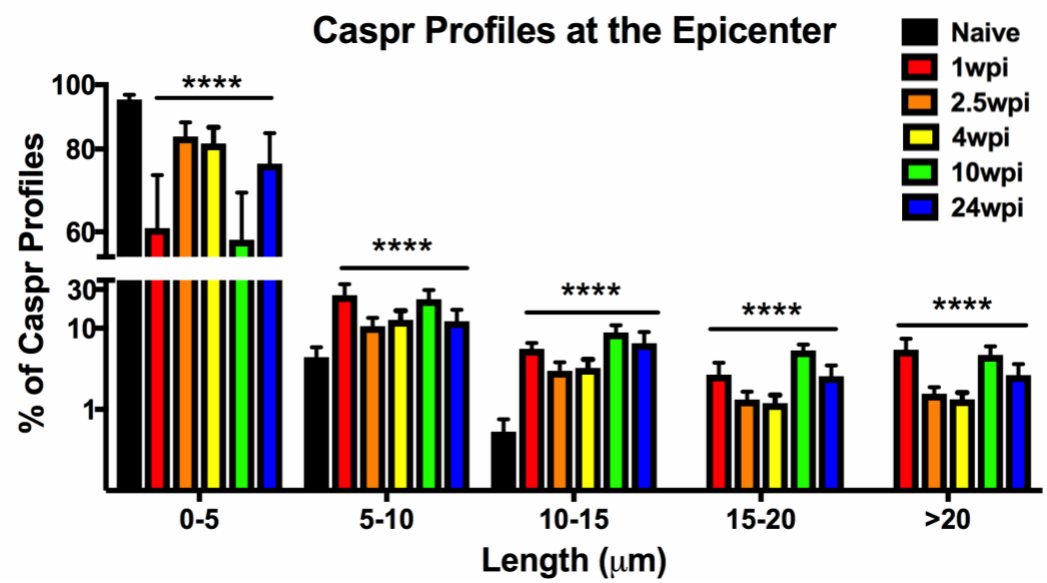

Figure 3*: 95% of Naive Caspr profiles are ≤5μm. By 1wpi, 40% of profiles are >5μm, some of which are >20μm. 20% of Caspr profiles revert to ≤5μm between 2.5-4wpi, only to aberrantly increase in length by 10wpi suggesting that Caspr pathology is dynamic. Excessively long Caspr profiles persist for at least 24wpi.*

Disruption of juxtaparanodal proteins that regulate membrane excitability

Delayed rectifier voltage gated K+ channels (Kv1.2) are discreetly clustered under the myelin sheath in the juxtaparanodal where they restore resting membrane potential after signal regeneration in the Node of Ranvier [22,23]. Approximately 90% of Kv1.2 profiles in Naïve mice

are ≤5μm (Figure 4). By 1wpi, Kv1.2 profiles aberrantly increase in length such that less than 70% of profiles are ≤5μm. Disrupted profile length persists for at least 24wpi, with some profiles extending 4-fold greater than Naïve Kv1.2 profiles suggesting that juxtaparanodal proteins that regulate membrane excitability are chronically disrupted on spared axons.

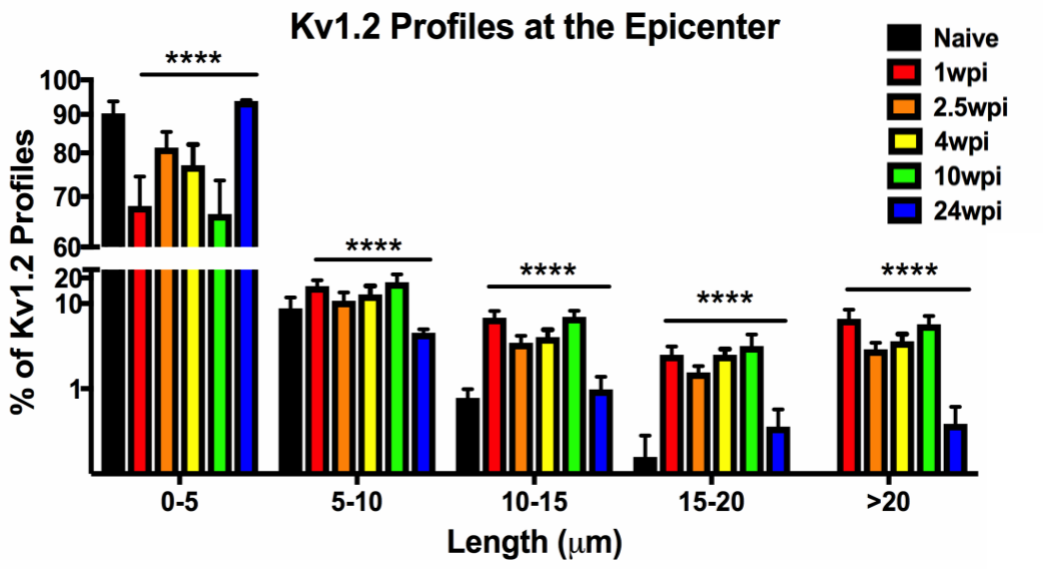

Figure 4: *~90% of Kv1.2 profiles in Naive mice are ≤5μm. By 1wpi, Kv1.2 profiles aberrantly increase in length such that less than 70% of profiles are ≤5μm. Disrupted profile length persists for at least 24wpi, with some profiles extending >20μm*

Node of Ranvier disruption is ongoing and not completely repaired after SCI

Despite evidence of sustained disruption of nodal proteins, there was a trend of decreasing length of Nav1.6, Caspr, and Kv1.2 profiles by 24wpi. To evaluate if this decrease in the percent of profiles at aberrant lengths suggested or preceded an increase in intact Nodes of Ranvier, the percent of axons with intact nodes and percent of axons with disrupted nodes was quantified. Intact nodes were defined as those that have the proper and discreet organization of nodal proteins while disrupted nodes were defined as those that were expressed beyond their proper

domains (Figure 5b, c).

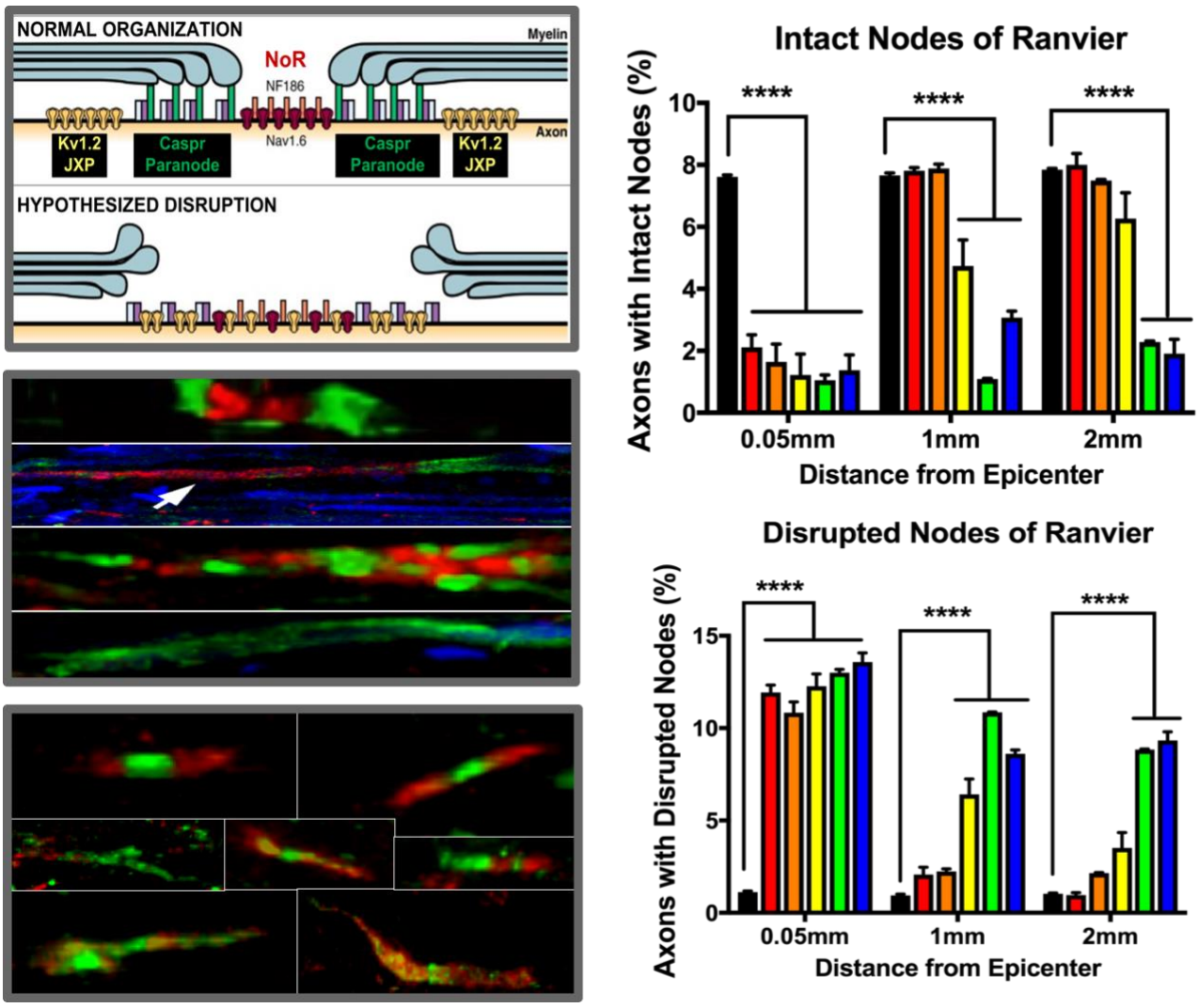

Figure 5: A) *Schematic of normal Node of Ranvier domains and the hypothesized SCI-induced disruption. Modified from: Arancibia-Carcamo and Attwell. Acta Neuropathol. 2014 Aug;128(2):161-75.* ***B, C)*** *Examples of normal and aberrant Kv1.2, Caspr and Nav1.6 localization after SCI.* ***D, E)*** *Intact and disrupted Nodes per axon profile were quantified. 8% of axons in Naïve tissue had intact Nodes, while only ~2% of axons at the epicenter had intact Nodes from 1w-24wpi and 10-14% of axons had disrupted nodes. Nodal pathology spread distally over time suggesting active Nodal breakdown chronically.*

If Nodes of Ranvier were restored, the percent of axons with intact nodes would increase. Instead, while 8% of axons in Naïve tissue had intact nodes, only 2% of axons at the epicenter had intact Nodes regardless of the time post injury (Figure 5d). Morevoer, injured animals consistently had between 10-14% of axons with disrupted nodes suggesting that spared axons at

the injury epicenter are never fully remyelinated (Figure 5e). Interestingly, pathology was not contained to the injury epicenter and spread distally over. At chronic time points, there were progressively fewer intact nodes and a concurrent increase in disrupted nodes suggesting that the SCI environment is dynamic, persistently pathologic, and actively and continually breaks down intact Nodes of Ranvier chronically.

Discussion

Widespread dysregulation of structural proteins that comprise the nodal area can contribute to aberrant signal propagation and widespread neural dysfunction (Arancibo-Carcamo and Attwell 2014). Herein, data shows that despite previous evidence of robust oligodendrogenesis that persists for at least 3mpi, structural nodal proteins are chronically disrupted and expressed diffusely outside of their domains suggesting that there are additional mechanisms that prevent functional remyelination of spared axons and the restorations of nodal proteins in their domains. Moreover, the persistent disruption of axo-glial nodal domains despite a slight trend towards shorter profile lengths of Caspr, Kv1.2, and Nav1.6 suggests that profile length of individual domains is not necessarily reflective of the integrity of the domain. Rather, to assess nodal domain integrity, the profiles must be assessed with regards to their proper localization relative to the abutting domain.

Data also shows that demyelination is not a static event. In fact, demyelination and chronic injury microenvironment is dynamic as evidenced by the active and sustained breakdown of Nodes of Ranvier for at least 6mpi. Overall, data not only reveal a novel and sensitive approach to evaluate the extent of demyelination but also demonstrate that endogenous oligodendrogenesis after CNS trauma is not sufficient to remyelinate all spared axons. The results suggest that nodal proteins whose dysregulation persists after injury may, potentially,

serve as novel therapeutic targets that can restore meaningful clinical recovery for patients of spinal cord injury. We anticipate these findings can be applied to other conditions whose pathology is largely driven by aberrant nodal structure such as MS and epilepsy – all of which affect large patient populations.

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