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Chronic nerve compression alters Schwann cell myelin architecture in a murine model

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Abstract

Introduction—Myelinating Schwann cells compartmentalize their outermost layer to form actin-rich channels known as Cajal bands. Here, we investigate changes in Schwann cell architecture and cytoplasmic morphology in a novel mouse model of carpal tunnel syndrome.

Methods—Chronic nerve compression (CNC) injury was created in wild-type and slow-Wallerian degeneration (*Wld^S*) mice. Over 12 weeks, nerves were electrodiagnostically assessed, and Schwann cell morphology was thoroughly evaluated.

Results—A decline in nerve conduction velocity and increase in g-ratio is observed without early axonal damage. Schwann cells display shortened internodal lengths and severely disrupted Cajal bands. Quite surprisingly, the latter is reconstituted without improvements to nerve conduction velocity.

Discussion—Chronic entrapment injuries like carpal tunnel syndrome are primarily mediated by the Schwann cell response, wherein decreases in internodal length and myelin thickness disrupt the efficiency of impulse propagation. Restitution of Cajal bands is not sufficient for remyelination post-CNC injury.

Keywords

Schwann cells; demyelination; Cajal bands; Chronic Nerve Compression Injury; DRP2

1. Introduction

To facilitate efficient transmission of nerve impulses, Schwann cells in the peripheral nervous system (PNS) produce insulating layers of cytoplasm, called myelin, which ensheath large-caliber axons. The outermost, or abaxonal, layer of myelin is polarized to create a network of abundant cytoplasm flanked by patches of minimal cytoplasm. Ramón y Cajal first identified this unique microstructure underlying the plasma membrane of myelinating Schwann cells and ascribed to them a role as trophic supporters of the myelin sheath.¹ These longitudinal and transverse cytoplasmic trabeculae are known as Cajal bands and have long been the subject of much interest; however, little has been discovered about their function, their role in facilitating Schwann cell maturation, and their response to nerve injury.

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For proper action potential propagation to occur, adequate myelin thickness and Schwann cell internodal length (IL) must be maintained. Recent studies using periaxin-null mice suggest that the Cajal bands facilitate the microtubule-based transport of proteins and organelles necessary for Schwann cell elongation and maturation.² Aberrations from this architecture coincide with irregularities in transmembrane signaling, specifically within the dystroglycan-dystrophin axis, which is necessary for myelin maintenance.

Studies have focused on hereditary models of demyelination as a means of investigating the relationship between impulse propagation, myelin thickness, IL and Cajal band integrity. However, little has been done to investigate the role of these factors in acquired injury. Entrapment neuropathies, such as carpal and cubital tunnel syndromes, have been effectively reproduced in rat models through chronic nerve compression (CNC) injury.³ Characterized by limited cytokine activation and delayed macrophage recruitment⁴, CNC injury differs dramatically from the rapidly activated network of cytokines and macrophages associated with Wallerian degeneration (WD).⁵ Deficiencies in motor function following CNC injury are thought to result from long-term demyelination and decreases in IL.^{3,6}

The current rat model is limited by inapplicability to transgenic studies. We generated a novel mouse model of CNC injury and evaluated differences in Schwann cell function and architecture between wild-type and slow-WD (*Wld^S*) strains. Our goal was to elucidate the role that demyelination plays in the development of CNC injury and to characterize changes in Schwann cell architecture that inhibit the efficient propagation of nerve impulses.

2. Materials and Methods

Mouse model of Carpal Tunnel Syndrome

Two strains of mice, 6 weeks old, were used: (1) the WT C57BL/6 (Harlan Laboratories, UK), which display normal WD, and (2) the mutant C57BL/6-WLD/OLA/NHSD (Harlan Laboratories, UK), which display a neuroprotective phenotype and abnormally slow-WD.

Chronic nerve entrapment was introduced through a novel surgical approach. Mice were anesthetized by intraperitoneal injection of ketamine/xylazine, and a dorsal gluteal-splitting approach was used to isolate and mobilize the sciatic nerve. To minimize the inflammatory response, all tubing was placed in a Petri dish the night prior to surgery, soaked in 70% ethanol, and allowed to dry in a vacuum hood. A 3 mm biologically-inert silastic tube (Cole-Palmer, Vernon Hills, IL) with an inner diameter of 0.51 mm was placed atraumatically around the ipsilateral nerve below the level of the sciatic notch (Figure 1A). Preliminary measurements of sciatic nerves in both WT and slow-WD mice (n=6) demonstrated an average diameter of 0.378 ± 0.029 mm. Consequently, after the experimental nerve was returned to the host bed, the tube readily glided along the nerve. Based on the characteristics of the tube polymer and pre-application processing, the tube was easy to remove at all times of specimen harvest, and no gross changes to nerve structure were observed (Figure 1b). The contralateral sciatic nerve was isolated using the same technique and mobilized without placement of tubing in order to serve as a control. Wounds were closed in all layers and tension-free skin closure was performed on all mice.

As positive controls for demyelination, a separate cohort of mice received crush injury. As previously described⁷, the right sciatic nerve was carefully exposed, mobilized, and crushed immediately distal to its emergence from the gluteus maximus using hemostatic forceps for 30 seconds. The left sciatic nerve was mobilized and returned to its host bed without inducing crush. Approval for animal use and all experimental procedures were obtained from the Institutional Animal Care and Use Committee at the University of California, Irvine, CA.

Electrodiagnostic evaluation

Electrodiagnostic studies of nerve conduction velocity were performed on (n=10) pre-operatively and serially at weekly post-operative time points. Recordings of both the ipsilateral experimental and contralateral limbs were gathered *in vivo* under ketamine/xylazine anesthesia using a Cadwell Sierra LT machine (Cadwell Laboratories, Kennewick, WA). Motor conduction in the sciatic-tibial nerves was assessed by stimulating at the sciatic notch and knee using a monopolar needle electrode. The reference for the stimulating electrode was placed in the ipsilateral lumbar paraspinal muscle. The compound muscle action potential (CMAP) from the tibial-innervated ankle plantar extensor muscle (tibialis anterior) was recorded by placing subdermal EEG electrodes in the muscle approximately 2 mm above the heel. The reference-recording electrode was inserted into the dorsal aspect of the foot, and the CMAP amplitude and motor nerve conduction velocity were measured.

Light microscopy and morphometric analysis

Pre-operatively and at 2 and 6 weeks after injury, the sciatic nerve in the area of compression was harvested from wild-type and *Wld^S* mice (n=4). Nerve segments were coded for blind analysis and fixed in 4% glutaraldehyde in a 0.1M phosphate buffered saline solution (PBS, pH 7.4) at 10 °C. Following fixation, specimens were postfixed in 1% osmium tetroxide in 0.1 M PBS, dehydrated in serial ethanol washes, and treated with propylene oxide. Samples were incubated in a 1:1 propylene oxide and Epon resin, and then transferred to Epon resin. Specimens were transferred to Beem Flat Embedding Molds and baked at 60°C for 24 hours. Blocks were cut with an ultramicrotome to obtain 1 µm sections and stained with Toluidine Blue. Whole nerve maps of cross sections were captured at 100X magnification using an Olympus 1×71 inverted microscope (Olympus Imaging America Inc, PA). G-ratios were calculated as the ratio of axon diameter to the total fiber diameter for 1000 axons per group per time point. Total axon counts, and number of myelinated axons were evaluated in uninjured and injured WT samples for over 1000 axons per time point. Distributions of axon diameter were also evaluated in uninjured and compressed specimens, and fibers were categorized as either small ($d \leq 2\mu\text{m}$), medium ($2\mu\text{m} < d < 4\mu\text{m}$), or large ($d \geq 4\mu\text{m}$) sized. All measurements were taken using SlideBook software (Intelligent Imaging Innovations).

IL measurements

Contralateral and ipsilateral sciatic nerves were harvested at post-operative time points (n=4). Following fixation in glutaraldehyde, samples were postfixed in 1% osmium tetroxide at 37°C for 2.5 hours. Each sample was then serially treated for 24 hours with 44%, 66%, and 100% glycerin at 37°C. Under a surgical microscope, single myelinated fibers were teased apart using ultrafine forceps. Over 25 fibers were teased per nerve sample for measurements of IL. For compressed nerve samples, IL was measured in the zone of injury. IL was measured with Visiopharm Integratory System Software (Visiopharm, Denmark).

Tissue preparation for immunohistochemistry

At 2, 4, 6 and 12 week post-operative time points, mice (n=4) received intracardiac perfusion using 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS, pH 7.4). Ipsilateral and contralateral sciatic nerves were harvested, post-fixed in 4% PFA for 30 minutes and stored at -80° C. Under a surgical microscope, the endoneurium and perineurium were stripped, and myelinated fibers were manually teased using ultrafine forceps. Previous studies suggest that myelin abnormalities following chronic injury occur initially on outermost fibers.⁸ Thus, we selected these fibers for evaluation through immunohistochemistry.

Teased fibers were blocked and permeabilized with 0.1% Triton X-100, 5% fish skin gelatin (Sigma) in PBS for 1 hr at room temperature. Primary antibodies were applied in the same blocking/permeabilizing solution overnight at 4°C. Subsequently, fibers were washed in PBS with 0.1% Triton X-100. Secondary antibodies were applied in blocking/permeabilizing solution for 3 hr at room temperature. After several washes, excess PBS was removed, and fibers were mounted in Vectashield (Vector Laboratories). Images were acquired using an Olympus 1×71 inverted microscope.

Primary/Secondary Antibodies and Dyes

The following antibodies and dyes, sources and dilution were used: Rabbit anti-DRP2 (gift from P. J. Brophy, University of Edinburgh, Edinburgh, UK; 1:200), FITC and rhodamine-conjugated phalloidin (Sigma, 1:400), mouse anti-S100 (Chemicon, 1:600), goat anti-rabbit FITC (Jackson Immunoresearch, 1:400), goat anti-rabbit tetramethylrhodamine isothiocyanate (Jackson Immunoresearch, 1:400), and 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma, 4 µg/ml). Teased samples were immunostained to determine the structural integrity of Cajal bands using mouse anti-S100, phalloidin-TRITC, and DRP2. As previous studies have utilized f-actin to outline the location of Cajal bands, double-immunostaining using phalloidin-FITC and DRP2 was completed to visualize Cajal bands and the appositions they border.

Morphological analysis and f-ratio

Using ImageJ (NIH), DRP2 and phalloidin staining were adjusted (after RGB color split) using the threshold function. The threshold (in black and white) was set arbitrarily for each image to match most closely the size and shape of trabeculae and patches. The Pearson R Coefficient was calculated (n=20, from 4 animals) at each time point using the "Intensity Correlation Analysis" plugin. The combination of channel color was established as TRITC vs. FITC, and pixels were analyzed in both channels for overlap. Perfect correlation gives an R value of 1, and values approaching 1 indicate reliable colocalization.

Schwann cell compartmentalization at the light microscope level was determined as previously described.⁹ Calibrated images of the complete Schwann cell volume immunostained with antibodies against DRP2 and phalloidin-FITC were obtained. At least 20 fibers from 4 animals were analyzed. The f-ratio, defined as the ratio of area occupied by cytoplasmic rich Cajal bands (f-actin signal) to DRP2-filled plaques, was calculated in chronically compressed nerve segments. DRP2 staining was adjusted using the threshold function. DRP2 patches have defined edges, and the use of a different threshold for each image does not add significant errors, but was necessary because of differences in overall DRP2 staining intensities between samples processed at different times. The area occupied by the DRP2 signal was measured using the "Analyze particles" option. The Cajal bands/trabeculae area was defined as region of the Schwann cell compartment lacking DRP2 staining. These open cytoplasmic regions were estimated by measuring the whole Schwann cell area and subtracting the corresponding DRP2 area.

Statistical Analysis

An equal number of samples and data points were obtained from experimental and control groups for each time point. Electrophysiological measurements and g-ratio data are expressed as mean ± SEM and were evaluated using the Student t-test and one-way ANOVA followed by Tukey-Kramer post-hoc testing. Differences were considered significant at p<0.01.

3. Results

CNC Injury causes sustained decreases in nerve conduction velocity

For an animal model of compression neuropathy to recreate the human condition, there must be a progressive decline in nerve conduction velocity in the area of compression. To determine the degree of neuropathy resulting from CNC injury, we conducted serial electrodiagnostic evaluations through a 12-week time course (Figure 2). In wild-type mice, conduction velocity decreased progressively after CNC injury from a baseline of 51.5 ± 1.6 (m/s) to 37.5 ± 2.5 (m/s) 6 weeks after injury. After the 6-week time point, the conduction velocity plateaued and remained consistently low through the 8, 10, and 12-week time points. To confirm that this decline resulted primarily from demyelination rather than axonal damage, we analyzed CMAP amplitudes at each time point. CMAP amplitudes represent all the axon bundles comprising the nerve. A decrease in the total number of axons resulting from nerve damage would cause a reduction in the evoked amplitude. At all time points, there was no statistically significant discrepancy in amplitude between experimental and control groups.

To further assess the role of axonal damage in the progression of CNC injury, we evaluated nerve conduction velocities in mice with delayed onset of WD. The *Wld^S* mouse is a spontaneously occurring mutant with a triplication of the fusion gene *Ube4b/Nmnat* and a phenotype of axon protection in both the central and peripheral nervous systems.^{10, 11} If CNC injury induces early axonal pathology, such a finding would not be evident in the mutant strain until later time points. Following CNC injury, *Wld^S* mice exhibited an immediate and progressive decline in conduction velocity, similar to their WT counterparts. Non-compressed (contralateral) nerves maintained a baseline conduction velocity of 56.1 ± 3.61 (m/s). As early as one week post-CNC injury, average velocity declined and reached a plateau of 34.6 ± 6.38 (m/s) by the four week time point (Figure 2C). There were no significant discrepancies of CMAP amplitudes between compressed and non-compressed groups.

CNC injury induces changes in fiber size and myelination

To morphometrically evaluate axonal and axoglial integrity following CNC injury, we compared total axon counts with the number of myelinated axons in uninjured and compressed nerve specimens from WT mice. No significant change in overall axon numbers was observed between normal samples and those harvested at 2 and 6 week time points after CNC injury (Figure 3A). Comparison of total axon counts versus the number of myelinated fibers in each group demonstrated a statistically significant decline in myelinated axons 2 and 6 weeks after CNC injury, with more pronounced demyelination observed at the later time point ($p < 0.01$).

We next sought to evaluate changes in axon fiber diameter at various time points following CNC injury. The diameters of 1000 axons per time point were measured and categorized as small ($d \leq 2\mu\text{m}$), medium ($2\mu\text{m} < d < 4\mu\text{m}$), or large ($d \geq 4\mu\text{m}$) (Figure 3B). A significant increase was observed in the number of small-sized fibers by 6 weeks after CNC injury, which coincided with decreases in the proportion of large-sized fibers at the same time point ($p < 0.001$). Although the fraction of medium-sized axons fluctuated between normal, 2 and 6 week post-CNC injury samples, these changes were not statistically significant.

CNC injury induces sustained decreases in myelin thickness

To determine the effect of CNC injury on myelin thickness, we calculated the g-ratio in large-caliber fibers from WT and *Wld^S* nerve samples (Figure 4G). Average g-ratio values for WT uninjured nerves approximated 0.62 ± 0.0012 . We found a statistically significant

elevation in this value 2 weeks following compression ($p < 0.001$). 6 weeks after CNC injury, g-ratio values peaked (0.792 ± 0.0076) (Figure 4A-C,H). Such elevation in the g-ratio corresponds to progressive myelin thinning. In *Wld^S* mice, the average g-ratio on the control side resembled the WT counterpart, with a value of 0.62 ± 0.0008 . Average values increased progressively after CNC injury, peaking at 0.76 ± 0.0008 by the 6 week time point (Figure 4D-F, H).

As positive control, we measured changes in myelin thickness after acute crush injury. In the WT mouse, sciatic nerve crush caused a sharp increase in the average g-ratio that peaked 2 weeks after injury and approached baseline values 6 weeks after injury. Due to the neuroprotective phenotype of *Wld^S* mice, the average g-ratio remained normal 2 weeks after nerve crush, and it elevated in a delayed fashion 6 weeks after injury (Figure 4H).

Decrease in IL over time following chronic compression injury

In conjunction with myelin thickness, IL also affects the speed of impulse propagation along the axon. Previous studies have demonstrated a correlation between decreased nerve conduction velocity and IL^{9, 12}, corroborated by increases in nodal frequency in various models of peripheral neuropathy.¹³ We sought to determine whether CNC injury affects the length to which Schwann cells can elongate. Analysis of single teased nerve fibers from sciatic nerves of WT mice showed a significant decrease ($p < 0.0001$) in IL over a 12 week time course (Figure 5). Baseline ILs for teased fibers approximated $633.5 \pm 15.4 \mu\text{m}$. 2 weeks following compression, ILs decreased to 74.8% of normal, declining further to 56.6% of normal 6 weeks following CNC injury. IL remained shortened 12 weeks after injury. Following CNC injury, Schwann cells were unable to properly elongate and form internodes of normal length.

Actin cytoskeleton in the outermost cytoplasmic layer is interrupted following CNC injury

Fluorescently labeled phalloidin toxin binds to and labels filamentous-actin in the cell cytoskeleton.¹⁴ As Cajal bands are largely comprised of a network of filamentous actin, we assessed morphological changes in microstructure along the length of teased nerve fibers by staining with phalloidin-FITC (Figure 6, left). Immunohistochemistry revealed a dramatic disturbance to Cajal bands immediately following CNC injury. Specifically, the regular pattern of actin channels was severely disrupted 2 weeks after injury. Quite surprisingly, partial reconstitution of this actin scaffold became evident at the 6 week time point; though irregular in pattern, a discrete network of Cajal bands was identifiable. 12 weeks after injury, the integrity of the actin scaffold resembled uninjured specimens: Cajal bands outlined appositions of similar shape and size, and were symmetric in pattern. Immunostaining of teased fibers for the Schwann cell cytoplasmic protein S100 (Figure 6, right) confirmed the pattern of Cajal band disruption and subsequent reconstitution after CNC injury.

Cajal band disorganization compromises apposition integrity

Currently, only one intracellular marker, DRP2, has been identified as being uniquely localized to the cytoplasmic appositions that are outlined by Cajal bands.² Using this marker, we sought to evaluate the spatio-temporal interplay between Cajal bands and the localization of DRP2 to cytoplasmic appositions. Immunostaining for DRP2 in uninjured samples revealed deposits of uniform shape and size and of a regularly repeating pattern throughout the Schwann cell internode (Figure 7). 2 weeks after CNC injury, DRP2 clusters were disrupted, and diffused staining was observed throughout the length of the internode. Similar to the pattern of disruption and reconstitution observed in Cajal bands, a gradual re-convergence of DRP2 into discrete plaques occurs at later time points. 6 weeks after injury, DRP2 localized to form appositions, although the shape and size of plaques were irregular

and incomplete. By 12 weeks post-CNC injury, DRP2 staining approximated uninjured samples, with plaques of regular pattern and shape.

Double-immunofluorescence confirmed that the pattern of DRP2 delocalization and convergence to cytoplasmic appositions coincided temporally with the disruption and subsequent reconstitution of Cajal bands (Figure 8). To assess the degree of overlap between DRP2 and phalloidin-FITC, we determined colocalization levels through the Pearson R Coefficient. As expected, uninjured samples demonstrated minimal overlap between Cajal bands and appositions. Post-injury, this overlap spiked most dramatically at the 2 week time point and decreased progressively thereafter, and the degree of colocalization approximated near normal values 12 weeks after injury ($p<0.01$) (Figure 8B). This finding is unique from investigations into genetic models of demyelinating neuropathies and may be attributable to the dual processes of demyelination and remyelination occurring concurrently.

To quantitate the changes in cytoplasmic morphology that were observed following CNC injury, we calculated the f-ratio, defined as the ratio of the internodal area occupied by cytoplasmic-rich Cajal bands to the internodal area occupied by DRP2-positive appositions, in normal and chronically compressed nerve segments. Normal nerves exhibited an average f-ratio value of 1.39 ± 0.25 , indicating an approximately equal distribution between the areas occupied by Cajal bands and appositions. F-ratio spiked to a maximum of 4.46 ± 0.55 2 weeks after injury ($p<0.01$). Subsequent time points revealed a return to near-baseline values, with average f-ratios for 6 and 12 week time points equaling 2.36 ± 0.65 and 1.86 ± 0.21 , respectively ($p<0.01$) (Figure 8C).

4. Discussion

The goals of this study were three-fold. As the previously described rat model of CNC injury represents a reliable yet scientifically limited injury model for the study of entrapment neuropathies, we first sought to develop a mouse model of CNC injury. Secondly, we sought to evaluate the role of Wallerian degeneration in this injury model. Our third aim was to assess morphological changes resulting from CNC injury, specifically with respect to myelin thickness, IL, and the integrity of the Cajal band network.

Prior investigations into chronic compression injuries have commonly utilized rat animal models.¹⁵⁻¹⁹ However, such models are limited from the use of transgenic and knock-out techniques. We thus sought to establish an easily reproducible mouse model wherein CNC injury can be more aggressively investigated. The shared hallmark of all entrapment neuropathies is a progressive and sustained decline in nerve conduction velocity post-injury. Our electrodiagnostic data demonstrates this trend, as decreases in nerve conduction velocity were sustained throughout the 12 week time course. Analysis of CMAP amplitudes demonstrate that demyelination, rather than axonal damage, plays the primary role in diminishing nerve conduction velocity. Our mouse model thus exhibits the classical hallmarks of entrapment neuropathy. As our electrophysiological findings suggested demyelination in the absence of axonopathy, we sought to characterize this phenomenon morphometrically through counts of total axons and myelinated axons. As expected, there were no significant changes in total axon numbers, however, demyelination was observed at both the 2 and 6 week time points. This finding supports our hypothesis that the Schwann cell response following CNC injury plays the primary role in the development of the ensuing neuropathy.

While overall axon numbers did not change between uninjured and experimental samples, we observed a decrease in the proportion of large-diameter fibers at 6 weeks post-CNC injury that temporally correlated with an increase in the proportion of small-diameter fibers.

Previous studies in rat models of entrapment neuropathy have illustrated that, following CNC injury, a phenotypic switch occurs in neurons within the dorsal root ganglia that is characterized by increased sprouting, elevated expression of the small-fiber markers CGRP and IB4, and coinciding decreases in the large-fiber marker NF-200.²⁰ Consequently, the increases in small diameter axons and decreases in large-sized fibers we observed may be a function of the increased sprouting which occurs after CNC injury.

We next assessed whether, in conjunction with demyelination, the process of Wallerian degeneration plays a significant role in the development of CNC injury. Naturally occurring mutant *Wld^S* mice express a fusion protein known to delay WD after neuronal injury and demonstrate a multi-faceted neuroprotective phenotype.²¹ We hypothesized that if WD did play a role in mediating the neuropathology, the decline in nerve conduction velocity would be delayed in *Wld^S* mice. Electrophysiological evaluation *Wld^S* mice mirrored the WT counterpart and demonstrated an immediate but progressive decline in NCV that was sustained throughout all time points. No significant discrepancies in CMAP amplitudes were observed between injured and non-injured groups. These findings strongly suggest that axonal damage and WD are not key players in the pathogenesis of CNC injury, and rather substantiate Schwann cells as the primary agents of the ensuing neuropathy.

We next sought to examine the morphological changes that occur after CNC injury in myelinating Schwann cells. g-ratio calculations confirmed a significant progressive thinning of the myelin sheath after injury in both WT and *Wld^S* mice. In the absence of WD, the same pathological state ensues. Increases in g-ratio occur on a similar time course and exhibit a similar progressive trend as the observed decline in nerve conduction velocity. Sciatic nerve crush was used as a positive control to which the trends in g-ratio following CNC injury were compared. After crush, the average g-ratio value increased sharply and re-approximated baseline values by the 6 week timepoint, indicating effective axonal regeneration and remyelination after the initial insult. This differed dramatically from the progressive rise in g-ratio observed after CNC injury, which remained elevated at the 6 week timepoint. Such findings confirm the existence of intrinsic differences between the pathogenesis of CNC injury and acute nerve injury. Specifically, the secondary role of axonal trauma in the CNC injury model makes it a primarily Schwann cell mediated injury state.

In conjunction with myelin thickness, Schwann cell IL is a major determinant of the efficiency with which action potentials are propagated along the axon. We found dramatic decreases in IL 2 weeks following CNC injury in both WT and *Wld^S* mice. Similar to observations on myelin thickness, the decline in IL occurred progressively and plateaued at later time points. Shortening of the internode coincided temporally with changes in g-ratio and nerve conduction velocity. Consequently, we propose that decreases in myelin thickness and IL mediate the ensuing aberrations in impulse propagation.

To further investigate changes in myelin architecture, we evaluated the integrity of Cajal bands after CNC injury. Cajal bands are believed to provide trophic support to the myelinating Schwann cell by facilitating the transport of critical proteins and nutrients within the myelin sheath.²² They are thought to play an essential role in Schwann cell elongation and growth.¹² A rigorous 12 week immunostaining workup revealed a dramatic disruption of Cajal bands as early as 2 weeks after injury which coincided with dispersal of DRP2 throughout the length of the internode. The f-ratio, defined as the ratio between the area occupied by Cajal bands and DRP2-filled appositions, increased dramatically, corresponding to disruption of internodal architecture. These early findings support the theory that Cajal bands provide trophic support and that in their absence, Schwann cells cannot elongate to appropriate lengths.

Since Schwann cell internodes remain shortened throughout the 12 week time course, we had initially expected Cajal bands to remain disrupted. Quite surprisingly, our results for the 6 week and 12 week time points revealed a progressive reconstitution of Cajal bands. f-ratio values reflected these findings and indicated a gradual but incomplete regression to baseline levels of localization. A plausible explanation for this phenomenon is that in a chronic injury model such as CNC, mechanical stimuli are consistently applied. Consequently, the opposing processes of demyelination and remyelination occur simultaneously. Ultimately, the continued presence of the mechanical stimuli may result in equilibrium between the opposing processes of demyelination and remyelination. This also may explain the observed plateau of nerve conduction velocity, g-ratio and ILs. Alternatively, the restitution of Cajal bands, despite the prevalence of diminished IL, may indicate that other factors play a role in perpetuating the neuropathological state. Chronic ischemia may play a factor as well, as hypoxia and limited nutrient delivery are believed to play a role in entrapment injuries.²³

CNC injury mimics the pathogenesis and clinical manifestations of entrapment neuropathies, such as carpal and cubital tunnel syndromes. Studies have suggested that the neuropathology that follows CNC injury is induced by changes in the interaction between myelinating Schwann cells and their extracellular environment.^{4, 20, 23, 24} Mechanical stimulation via shear stress is known to alter the basal lamina and extracellular matrix, affecting major signaling proteins such as fibronectin and the family of laminins.²⁵⁻²⁷ Cell surface receptors for these extracellular components, such as integrins and the dystroglycan complex, consequently provide Schwann cells with mechanosensitive properties.^{28, 29} Given these findings, it is probable that changes incurred in the extracellular microenvironment as a result of CNC injury are internalized by Schwann cells. Studies have demonstrated a striking number of shared signaling molecules, such as the $\alpha 6\beta 4$ and $\alpha 6\beta 1$ integrins and DG^{30, 31}, and overall pathways, such as ERK1 and ERK2³²⁻³⁴, between CNC injury and other demyelinating neuropathies, including Charcot-Marie-Tooth disease, multiple sclerosis and leprosy.³⁴⁻³⁶ Our current ongoing investigations are aimed at elucidating the changes to the extracellular microenvironment after CNC injury, with a greater goal of uncovering the molecular determinants that cause the altered myelin architecture observed in this study.

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Abbreviations

CNC	chronic nerve compression
DRP2	dystrophin-related protein 2
IL	internodal length
WD	Wallerian degeneration
Wld^S	slow Wallerian degeneration mouse
WT	wild-type

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Figure 1.

Mouse model of CNC injury. (A) A bioinert silastic tubing is placed around the sciatic nerve, providing chronic compression and mechanical stimulation in a manner consistent with entrapment neuropathies. (B) A representative image of whole nerve cross-section from a compressed specimen is shown. At all points of specimen harvest, no gross changes in nerve structure were observed.

Figure 2.

Electrodiagnostic assessment of nerve conduction velocity and CMAP amplitude after CNC injury. (A) WT mice demonstrate a progressive decline of nerve conduction velocity following chronic nerve compression injury of the affected nerve. (B) No significant discrepancies in CMAP amplitudes were observed between CNC and control groups. (C) Nerve conduction velocities in *Wld^S* mice following CNC injury mirrors the trend observed in the WT counterpart, demonstrating a progressive, sustained decline in the speed of impulse propagation from one to 6 weeks post-compression. (D) CMAP amplitudes remain unaffected in *Wld^S* mice.

Figure 3.

Morphometric evaluation of overall axon number, myelination, and fiber-size distribution. (A) Overall axon counts remain consistent between normal, 2 week and 6 week CNC injury groups. Significant decreases in number of myelinated axons are observed at both the 2 and 6 week time points ($p < 0.01$). (B) Evaluation of fiber-size distribution reveals a significant increase in the proportion of small-diameter fibers and a corresponding decrease in large-diameter fibers after 6 week of CNC injury ($p < 0.001$).

Figure 4.

Myelin thinning post-CNC injury in wild-type (top row) and *Wld^S* (bottom row) mice. (A, D) Uninjured samples demonstrate normal myelin thickness. (B, E) 2 weeks after CNC injury, both wild-type and *Wld^S* mice exhibit decreases in myelin thickness (arrows). (C, F) Demyelination is sustained 6 weeks after injury in both mouse models (scale bar = 10 μ m). (G) Myelin thickness was quantitatively assessed by calculating g-ratios. (H) Uninjured samples exhibit baseline g-ratios of 0.623 ± 0.0012 (WT) and 0.62 ± 0.0008 (*Wld^S*). CNC injury induces significant increases in g-ratio ($p < 0.001$). In wild-type mice, crush injury (control) induces an immediate spike in g-ratio, coinciding with the rapid onset of Wallerian degeneration, which returns to baseline values by 6 weeks. *Wld^S* mice demonstrate the neuroprotective phenotype through a delayed spike in g-ratio after crush injury.

Figure 5.

Schwann cell elongation is hindered after CNC injury. ILs were measured for mice (n=4) at 2, 6, and 12 weeks post-CNC injury. (A) Representative images of uninjured teased nerve fiber (top) compared to 6 week compressed fiber (bottom) (scale bar = 100 μm). (B) Quantification of IL demonstrates a progressive decrease in IL that is sustained to the 12 week time point ($p < 0.0001$).

Figure 6.

Cajal bands dissociate following CNC injury, but are reconstituted at later time points. In the left column, uninjured nerves display an intact network of Cajal bands as identified by immunostaining with phalloidin-FITC (green). Cajal bands outline appositions of similar shape and size (arrowheads). 2 weeks after CNC injury, Cajal bands are severely disrupted, and appositions cannot be determined. 6 weeks after injury, an irregular pattern of distinct bands is visible that outlines appositions that are irregular in shape and size. By 12 weeks, a more regular pattern of Cajal bands is observed, more closely approximating uninjured samples. Immunostaining with phalloidin also revealed Schmidt-Lanterman Incisures (asterisks). In the right column, immunostaining of teased fibers for the Schwann cell cytoplasmic protein S100 (red) confirms the pattern of Cajal band disruption and subsequent reconstitution after CNC injury (scale bar = 100 μ m).

Figure 7.

DRP2 complex is disrupted after CNC injury but is reconstituted at later time points. Uninjured nerves immunostained with DRP2 (red) reveal distinct, symmetric plaques along the internode. DRP2 deposits are disrupted 2 weeks after CNC injury. By 6 weeks, DRP2 appears to converge to form plaques, although the shape, size, and organization of these plaques are irregular and often incomplete. 12 weeks after injury, samples display more uniformly filled DRP2 plaques, although the shapes remain non-uniform and oblong (scale bar = 100 μ m).

Figure 8.

Immunohistochemistry reveals a correlation between the integrity of Cajal bands and DRP2 localization to cytoplasmic appositions. (A) Uninjured nerve fibers immunostained with phalloidin-FITC (green) and DRP2 (red) display an organized pattern of DRP2 localization and regular Cajal band pattern, which becomes disrupted 2 weeks after injury and is gradually reconstituted at the 6 and 12 week time points. (B) To assess the degree of colocalization, the Pearson R coefficient was calculated at each time point (n=20, from 4 animals). Overlap between DRP2 and phalloidin-FITC is least among uninjured nerve samples. Dissociation of Cajal band structure and the DRP2 complex at 2 weeks post-CNC injury corresponds to the greatest degree of overlap. This overlap decreases 6 and 12 weeks after CNC injury, coinciding with the re-convergence of DRP2 to discrete appositions and the constitution of Cajal bands. (C) f-ratio measurements confirm gross disruptions to myelin morphology following CNC injury followed by gradual but incomplete restitution to baseline values (scale bar = 100 μm). f-ratios are abnormally high 2 weeks after injury and gradually return to near-baseline values 6 and 12 weeks after injury (p<0.01).