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The impact of myelination on axon sparing and locomotor function recovery in spinal cord injury assessed using diffusion tensor imaging

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Abstract

The dysmyelinated axons of shiverer mice exhibit impaired conduction characteristics similar to early postnatal axons before myelination, while the patterns of neuronal activity and connectivity are relatively comparable to those of wild-type myelinated axons. This unique dysmyelination pattern is exploited in the present study to determine the role of compact myelin on the loss and recovery of function following traumatic spinal cord injury (SCI). We applied in vivo diffusion tensor imaging (DTI) and post-mortem immunohistochemistry analysis to examine changes in myelin and axonal integrity and evaluated these changes in concert with analysis of locomotor function from 1 to 4 weeks following a mid-thoracic contusion injury in homozygous shiverer and heterozygous littermate mice. The DTI biomarkers, axial and radial diffusivities, are noninvasive indicators of axon and myelin integrity in response to SCI of both myelinated and dysmyelinated spinal cord. We show that myelin is critical for normal hind limb function in open field locomotion. However, when the functional outcome is limited during chronic SCI, the extent of recovery is associated with residual axonal integrity and independent of the extent of intact myelin at the lesion epicenter.

Keywords

diffusion tensor imaging; myelin; dysmyelination; white matter; spinal cord injury

INTRODUCTION

The interaction between myelin sheath and axon has long been a major focus targeting the therapeutic intervention for the central nervous system (CNS) white matter disease (1-8). With the advent of diffusion tensor imaging (DTI) (9,10), the study of the axon-myelin interaction could be conducted noninvasively for investigating its correlation to the neurological function (11-16). Early DTI studies have examined the myelination process in developing brain (17-21) and in demyelinating diseases (22-25). The changes of DTI parameters, such as fractional anisotropy (FA) and mean diffusivity (MD), are found to correlate with the evolution of the white matter packing and myelination. Several DTI studies also compared the diffusion measurements between the myelin-deficient mutant and

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the normal myelinated wild-type animals (26-29). They found that the degree of myelination could contribute to the increased radial diffusivity (, describing water diffusion across the fiber tract) without affecting axial diffusivity (, describing water diffusion along fiber tracts) (30). FA and are also shown sensitive to detect the donor-derived corpus callosum myelination in the shiverer mice after neural precursor cell transplant (31). Since the hypothesis that and could respectively reflect the extent of axon and myelin damage (30,32-35), numerous studies have applied these DTI parameters in the past decade to study the axon-myelin interrelation in brain injury (36,37), multiple sclerosis (38-41), retinal ischemia (32), Alzheimer's disease (42) and spinal cord injury (SCI) (43-47).

The objective of this study is to evaluate the capability of the DTI indices in assessing the impact of myelin sheath on the progression of white matter pathology and recovery of function after SCI. We employed the myelin-deficient shiverer mutants and their heterozygous littermates to test the hypothesis that long tract myelination is essential for locomotor function during the course of SCI. Comparing to the heterozygous littermates with the normal myelin sheath, shiverer mice represent a unique model without pre-existing axonal degeneration nor inflammation in the CNS (48) for investigating the effect of myelin sheath in the traumatic CNS injury. The extent of spared axon and myelin sheath after mid-thoracic contusion SCI was assessed longitudinally using in vivo DTI followed by end-point histology. The DTI findings were correlated with the extent of hindlimb locomotor function assessed using Basso Mouse Scale (BMS) (49). Results suggest that the presence of the myelin sheath is critical to maintain the higher hind limb function performance before SCI. In contrast, at the chronic phase of SCI where significant myelin loss was severe, hind limb function was primarily correlated with the extent of spared axons.

MATERIAL AND METHODS

All surgical preparations and pre- and postsurgical care were provided in accordance with Public Health Service Policy on Humane Care and Use of Laboratory Animals and Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, 1996), and with the approval of the Washington University Animal Studies Committee.

Animals and spinal cord injury

Adult (8 to 10-week-old) female shiverer mice (shi $-/-$), and their heterozygous littermates (shi $+/-$) were used in this study. SCI and sham operation were performed on both shiverer and heterozygous mice (N = 6, each group) as described previously (50,51). The mice were anesthetized with an isoflurane/oxygen mixture and received severe contusion SCI (displacement: 1.1mm, velocity: 0.2m/s) utilizing a modified Ohio State University impactor after dorsal laminectomy at the T9 vertebral level. All mice showed complete paralysis with no ankle movement at 24 hours after injury. The sham-operated animals (uninjured) underwent a laminectomy without contusion and served as the control. The surgical site was closed in layers with 4-0 vicryl and nylon sutures. Injections of enrofloxacin (2.5 mg/kg) and lactated ringers (1.5 ml) were given subcutaneously. Manual bladder expression of the injured mice was performed twice daily throughout the duration of the study (52,53). Softened rodent chow was provided. Body weights were measured and compared daily with the preoperative weight. High-calorie nutrient paste (Nutrical; Evsco Inc., Buena, NJ) was given to the mice to maintain body weights at 90% of their preoperative values. Daily BMS scoring was performed on all mice from 1 to 21 days post injury (DPI).

In vivo DTI

In vivo DTI were conducted on all mice at five time-points: naïve, hyperacute (~3hrs), sub-acute (7 DPI), sub-chronic (14 DPI), and chronic (21 DPI) phases. A magnetic resonance imaging (MRI)-compatible device was utilized to stabilize the vertebral column as reported previously (39). Mice were anesthetized with an isoflurane/oxygen mixture (4.5 – 5% for induction and 0.7 – 1.5% for maintenance) to be placed in the MRI scanner. Core body temperatures were maintained at 37°C with a circulating warm-water pad. The inhalant anesthetic was delivered to the mice through a custom-made nose cone. The respiratory exhaust line was connected to a pressure transducer to synchronize DTI data collection with the respiratory rate. An actively-decoupled surface coil covering vertebral segments T6 – T12 (15 mm × 8 mm) was used as the receiver. A 9-cm (inner diameter) Helmholtz coil was employed as the radio frequency transmitter. The entire preparation was placed in a 4.7 T magnet (Oxford Instruments, Abingdon, UK) equipped with a 15-cm (inner diameter) actively shielded Magnex gradient coil (60 G/cm, 270 μs rise time). The magnet, gradient coil, and IEC gradient power supply were interfaced with an Agilent DirectDrive console (Agilent Technologie, Santa Clara, CA).

A spin-echo sequence, modified by adding Stejskal-Tanner diffusion-weighting gradient (54), was used with the following parameters: echo time (TE) = 38 ms, diffusion gradient interval (Δ) = 18 ms, diffusion gradient time (δ) = 6 ms, diffusion gradient amplitude = 8.5 G/cm, b value = 1.02 ms/μm², six diffusion-sensitizing gradients: (G_x, G_y, G_z) = (1, 1, 0), (1, 0, 1), (0, 1, 1), (-1, 1, 0), (0, -1, 1), and (1, 0, -1), number of averages = 4, field of view (FOV) = 1×1 cm², and data matrix = 128×128 (zero-filled to 256×256). The repetition time (TR≈1.2s) was varied according to the period of the respiratory cycle (≈270ms). Nine transverse images (slice thickness = 0.75 mm) were collected covering vertebral segments T8 – T10. The acquisition time was approximately 1.3 hrs. Three eigenvalues were calculated from the diffusion tensor matrix by least square regression. The eigenvalue-derived parameters (λ₁, λ₂, λ₃) were calculated from the diffusion tensor matrix (30,32). MD was calculated by MD = (λ₁ + λ₂ + λ₃)/3. Axial diffusivity was defined as λ₁. Radial diffusivity was defined as λ₂ + λ₃/2. The relative anisotropy (RA) was calculated as:

$$RA = \sqrt{\frac{(\lambda_1 - MD)^2 + (\lambda_2 - MD)^2 + (\lambda_3 - MD)^2}{3MD}} \quad (1)$$

All parameters were derived from diffusion-weighted images using software written in Matlab (MathWorks, Natick, MA).

Data analysis

Contrast between gray matter (GM) and WM was clearly seen in the RA maps of both heterozygous and shiverer spinal cords allowing the delineation of total cord area (Fig. 1a, b). The FA map, reflecting the integrity of axons, was used to determine the area of spared axons in the ventral-lateral WM region (44,45,51) (Fig. 1c, d). The histograms of FA maps were first collected from the GM and ventral-lateral WM regions in six sham-operated heterozygous and shiverer mice to determine the means and standard deviations (SD) of normal appearing FA (Fig. 1e, f). The spared axon area was defined by a threshold of mean ± 2×SD of the normal appearing FA within the ventral-lateral WM region. On the other hand, contributing from both axonal and myelin integrity, the RA map provided a WM contrast suggesting the extent of ventral-lateral WM free from axon or myelin injury in the later part of the time course by the threshold of RA. In the naïve and acute injury mice, when RA appears normal, the RA threshold allows the estimate of the total white matter. The spared axon areas were normalized to the total cord or total ventral-lateral WM areas of their naïve cords (i.e. percent spared axon areas) for comparing between groups and with the gold-

standard histology. The group averaged values of DTI parameters were obtained from the region of interest delineating the total ventral-lateral WM.

Behavioral assessment of hind limb locomotor function

The mouse hindlimb locomotor function was assessed daily using BMS for 21 days after injury by three raters scoring from 0 (worst) to 9 (best) with a sub-score tally from 0 (worst) to 11 (best) to evaluate angle movement, plantar placement, stepping, coordination, paw position, trunk instability, and tail position.

Immunohistochemistry analysis

Immediately after imaging at 21 DPI, mice were perfused under deep anesthesia with 50 mL of 0.1 M phosphate-buffered saline (pH 7.4) followed by 200 mL of 0.1 M PBS containing 4% paraformaldehyde (pH 7.4). Following fixation, the spine was excised, left in the fixative overnight, decalcified for 48h, embedded in paraffin, and sectioned on a sliding microtome (5 μ m) with the decalcified vertebral column intact. Tissue sections were stained for eriochrome cyanine and cresyl violet (EC/CV) to delineate myelin/neuron survival (55), or immunostained with antibodies directed against MBP (rabbit polyclonal anti-MBP, 1:2000; Sigma-Aldrich, St. Louis, MO) and phosphorylated neurofilament protein (SMI-31) (mouse clone SMI-31, 1:1000; Covance Inc., Princeton, NJ) to identify the presence of MBP and large diameter spared axons, respectively. Antibodies were detected using biotinylated secondary antibodies and avidin-biotin *Elite®* complex (Vector Labs, Burlingame, CA) with diaminobenzidine or vector red as chromagens. Stained sections at the injury epicenter were digitally imaged using a Zeiss Axioskop microscope equipped with a 2.5 $\times$ objective, and the images were captured with a Sony CCD 970 analog camera using MCID image acquisition software (Interfocus Imaging Ltd, Cambridge, UK) (56). The cross sectional areas and areas of WM or positive MBP and neurofilament staining were determined for the entire tissue section and expressed as μ m². Then, the spared myelin or axon areas were normalized as a percentage (%) of total cord area and total ventral-lateral WM area of the control cords to correct for atrophic effect of injury and shrinkage during fixation. All slides were assessed blindly with respect to the genotype of the subject.

Statistical Analysis

In order to compare the difference of injury response between shiverer and heterozygous mice, a two-way analysis of variance (ANOVA) with repeated measures was used on the locomotor function, evolution of total cord areas, spared myelin and axon areas, and the DTI parameters (OriginPro v8.5.1, OriginLab Corp., Northampton, MA and Prism 5.0, Graphpad Inc. Carlsbad, CA). Tukey's honestly significant difference (HSD) post-hoc test was used to examine the difference between groups with significant level predetermined at $\alpha=0.05$. Bonferroni's correction for multiple comparisons was used when main effects were significant. In addition, a Pearson correlation analysis was also performed to delineate a possible relationship between the chronic hindlimb locomotor function and ventral-lateral WM integrity. For this purpose, we extracted the values of α and the percent spared axon area (normalized by the total cord area), representing the myelin and axon integrity, to correlate with the BMS scores.

RESULTS

In the presence of profound tremors, the sham shiverer mice exhibited impaired hindlimb function with consistent plantar stepping, no or some coordination, and rotated paws at initial contact and lift off (BMS=5). The sham heterozygous controls showed normal hindlimb function (BMS=9). Severe contusion injury resulted in complete paralysis (BMS=0) right after injury on both heterozygous and shiverer mice (Fig. 2). Interestingly,

neither heterozygous or shiverer mice exhibited tremors after injury. Both groups of severe injured animals showed similar hindlimb function deficits throughout the 21-day period after SCI, though a slightly faster recovery was seen in shiverer mice from day 3 to day 8 (BMS scores tested between day 3 and day 8; shiverer: $t(100)=4.28$, $p=0.04$; heterozygous: $t(100)=3.59$, $p=0.44$). The repeated measures ANOVA showed a main effect of time ($F(20, 100)=51.05$, $p<0.0001$) on the BMS scores, as was the main effect of shiverer phenotype ($F(3, 15)=131.13$, $p<0.0001$). The interaction of these two factors was also significant ($F(60,300)=10.46$, $p<0.0001$).

Longitudinal DTI maps of the contusion injured spinal cords of heterozygous and shiverer mice are shown in Figure 3. Hemorrhage areas were detected in T2W images and confirmed the location of injury epicenter between the heterozygous (Fig. 3a) and shiverer mice (Fig. 3b). The threshold allowed the estimate of spared axon areas longitudinally after SCI. The maps clearly revealed the difference in myelination status. The GM to WM contrast in RA maps resulted from the combined effect of axon and myelin sparing after injury.

While the spinal cord of both groups showed similar patterns of initial swelling and then shrinkage, the shiverer spinal cords exhibited a faster and greater contracture of the lesion epicenter over time than their heterozygous littermates (Fig. 4a). The repeated measures ANOVA showed a main effect of time ($F(4, 20)=91.44$, $p<0.0001$), as well as the main effect of shiverer phenotype ($F(1, 5)=7.55$, $p<0.05$). The interaction of these two factors was also significant ($F(4, 20)=8.70$, $p<0.001$). The post-hoc Tukey's HSD tests showed differences in the total cord area between heterozygous and shiverer mice at 7 DPI ($t(20)=6.81$, $p<0.001$) and 21 DPI ($t(20)=3.56$, $p<0.05$). Initially, the shiverer spinal cords had a smaller area of ventral-lateral WM ($t(20)=5.68$, $p<0.001$), consistent with the absence of myelin sheaths. After injury, however, no significant difference was seen in the spared axon areas between heterozygous and shiverer mice (Fig. 4b). Because the initial cord sizes were different, the cross sectional ventral-lateral WM areas were also expressed as a proportion of the uninjured cord areas (Fig. 4c and 4d). The spared axon areas normalized to the total cord area exhibited no significant differences between heterozygous and shiverer mice throughout the time course of the study (Fig. 4c). When normalized to the naïve total ventral-lateral WM area, pronounced atrophic change was seen in both strains of mice (Fig. 4d). At the hyperacute phase, the heterozygous mice showed a more rapid decrease of spared axon area normalized to ventral-lateral WM ($t(20)=16.63$, $p<0.00001$) than that of shiverer mice ($t(20)=11.26$, $p<0.0001$). However, there were no significant differences in normalized areas of spared axons between the two strains of mice from 7 DPI through the end of the time course.

The intensity change in the maps corresponds to the evolving integrity of longitudinally oriented axons after SCI (Fig. 5a). Prior to SCI, the was lower in shiverer ($1.71\pm0.14 \mu\text{m}^2/\text{ms}$) than in heterozygous ($1.95\pm0.10 \mu\text{m}^2/\text{ms}$) mice ($t(20)=3.73$, $p=0.01$). After SCI, there was no difference between heterozygous and shiverer mice (Fig. 5a). Both groups exhibited the greatest decrease of at 3 hrs after injury, and became indistinguishable from the lowest value at 7 days after injury. In contrast, the , reflecting the diffusion of water molecules in the medial-lateral and dorsal-ventral planes, was markedly different between groups (Fig. 5b). In the uninjured shiverer mice, was $0.42\pm0.02 \mu\text{m}^2/\text{ms}$, significantly higher than that of the heterozygous mice with $0.27\pm0.03 \mu\text{m}^2/\text{ms}$ ($t(20)=8.90$, $p<0.0001$). After SCI, the in shiverer mice remained unchanged up to 14 DPI. In contrast, the of heterozygous mice increased but not exceeding the baseline value of shiverer mice from 3 hrs after injury up to 14 DPI. Both shiverer and heterozygous increased above the baseline value of the shiverer mice without significant difference at 21 DPI. The diffusion anisotropy from heterozygous mice was higher than that of shiverer mice at the baseline (Fig. 5c). After injury, the differential reduced becoming equal from 7 DPI until 21 DPI.

Figure 6a shows the correlation between the BMS scores and the β from all animals. At 21 DPI, the β of the uninjured shiverer and all of the injured mice (filled circle and all open symbols) was higher than that of the uninjured heterozygous mice (filled square). Figure 6b shows the correlation between the BMS scores and the percent spared axon areas estimated based on β . In the injured group, the locomotor function was better when mice had more axons spared, regardless of shiverer or heterozygous mice ($r(12)=0.57$, $p<0.05$). The locomotor function of the injured mice was all limited below BMS=4. Based on β , the uninjured heterozygous had normal axon area (~50%) and completely normal BMS scores. The uninjured shiverer mice also had nearly normal axon areas, comparing to the uninjured heterozygous mice, but instead showed limited locomotor function with BMS=5.

To further clarify the underlying pathological differences between shiverer and heterozygous mice, histological analyses were performed on fixed tissue sections obtained at the end of the study. Myelin/neuron integrity of uninjured and injured cords from both groups of mice was examined using EC/CV staining respectively (Fig. 7a-h). Uninjured heterozygous mice showed normal demarcation of GM and WM regions and the myelin in the ventral-lateral WM stained densely with EC (blue color) (Fig. 7a, e). In contrast, uninjured shiverer mice showed good CV staining (violet color), but lacked evidence of compact myelin as stained with EC (Fig. 7c, g). After 21 DPI, there was pronounced shrinkage of the spinal cord in both heterozygous and shiverer mice. The heterozygous mice showed reduced intensity of EC staining in the spared rim of ventral-lateral WM (Fig. 7b, f), consistent with loss of myelin over time, while shiverer mice again had no EC staining, but showed intense cellular staining in the ventral-lateral WM with the CV stain (Fig. 7d, h). Similar to the DTI results (Fig. 4a), measures of the histological cross sectional total cord area were comparable in uninjured shiverer and heterozygous mice. After injury, however shiverer mice endured more severe atrophy than that of the heterozygous mice (Fig. 7i). Two way ANOVA revealed a strong interaction effect of genotype and injury on total cross sectional area ($F(1,13) = 19.01$, $p=0.008$), and by post-hoc analysis, the injured shiverer epicenter sections were significantly smaller than controls ($t(13)= 7.61$, $p<0.0001$). Similarly, the ventral-lateral WM of the control shiverer mice was significantly smaller than that of the heterozygous mice both before and after injury (Fig. 7j; cf. Fig. 4b) (genotype effect; $F(1,13)=40.99$, $p<0.0001$). After injury, significant atrophy of ventral-lateral WM was seen in both groups of mice (injury effect; $F(1,13)=66.8$, $p<0.0001$). The ventral-lateral WM area normalized to the total cord area was thus smaller in shiverer mice both before and after injury ($F(1,13)=22.88$, $p=0.0004$; Fig. 7k), but with this correction, the proportion was not significantly changed following injury (injury effect; $F(1,13)=2.51$, $p=0.137$). Finally, measures of the lesions in shiverer mice revealed a larger proportion of total cord area than that in heterozygous ($t(6)=2.8$, $p=0.03$; Fig. 7l), again reflecting the reduced overall size of the WM rim and the general increase in contraction of the epicenter in the shiverer mice.

Immunostaining for MBP was performed on sections from shiverer and control mice. Normal MBP immunostaining was seen in heterozygous mice both before and after injury (Fig. 8a, e, i) while shiverer mice exhibited negative MBP staining (Fig. 8c, g, i). At 21 DPI, there was an interaction effect on the area of MBP staining ($F(1,13)=5.34$, $p=0.038$), as heterozygous mice showed a loss of total MBP staining intensity consistent with myelin loss (Fig. 8b, f, i). The spinal cords of shiverer remained MBP negative at 21 DPI (genotype effect; $F(1,13)=51.06$, $p<0.0001$; Fig. 8d, h, j).

Although measures of WM and myelin staining with both EC and MBP were clearly different between heterozygous and shiverer mice, the density of axons was quite similar as revealed with antibodies to SMI-31 (Fig. 9). Control cords of both shiverer and heterozygous mice showed strong SMI-31 staining with a homogeneous pattern of immunopositive axon profiles in the ventral-lateral WM (Fig. 9a, c). The stain density

appeared higher in heterozygous spinal cord sections (Fig 9e, g). After injury, SMI-31 staining was dramatically reduced in both groups (injury effect; $F(1,13)=98.52$, $p<0.0001$; Fig. 9b, d), suggesting severe axonal damage independent of the presence of myelin (Fig. 9f, h). After correcting for tissue shrinkage, ~40% loss of SMI-31 positive area was seen in both groups ($F(1,13)=33.25$, $p<0.001$; Fig. 9j:). Notably, there was no effect of the dysmyelinated phenotype on the density or total area of axon staining in the spared WM regions.

DISCUSSION

Dysmyelinated shiverer mutants lack the essential myelin basic protein (MBP) required to form the major dense line of the myelin sheath (57). Compact myelin is absent in shiverer mice when the MBP level is less than 25% of the normal (58). Although neurons and fiber tracts develop normal patterns of neuronal activity and functional connectivity (59), the resulting dysmyelination causes conduction deficits and neurological consequences (60). The hallmark symptomatic tremor of shiverer mice derives from a loss of spinal motor and reflex control due to the lack of compact myelin (57,61). This shivering behavior starts about 12 days after birth and progresses with time leading to increased frequency and duration of tonic seizures with age (61). Shiverer mice therefore have a shorter life span ranging between 50 and 100 days. Interestingly, despite the deficits in myelination, shiverer mice exhibit neither axonal degeneration nor inflammation in the brain or spinal cord (48). Comparing to their heterozygous littermates (+/-) with the normal myelin sheath, shiverer mice represent a unique model to investigate the effect of myelin sheath on the progressive pathology and recovery of function after SCI.

Comparable to previous reports of SCI time course studies, we observed a rapid decrease of ΔFA in the hyper-acute phase and a gradual increase of ΔFA from the sub-acute to chronic phase (43,46,62,63). Several investigations also looked into the correlations between the DTI and histological markers of white matter injury in SCI suggesting that ΔFA is reflective of the acute axonal injury and ΔMD is sensitive to the delayed myelin damage following SCI. For instance, Zhang et al. applied DTI in studying Wallerian degeneration after dorsal root axotomy and found a strong correlation between ΔFA and the optical density of SMI31, and between ΔMD and the optical density of LFB staining in the lesion (62). After the initial trauma, collapsed axon debris and myelin ovoid may hinder water molecule diffusion along injured axons and therefore causes a rapid decrease in ΔFA . Subsequently, the slow clearance of axon and myelin debris may account for the sustained level of reduced ΔFA from the sub-acute to the chronic phase following SCI. On the other hand, ΔMD in the lesion increased significantly after the sub-acute phase coinciding disintegration of the myelin sheath and clearance of myelin and axon debris. Studies using cat model of contusive SCI also reported that the DTI derived parameters were capable of detecting the evolving pattern of axonal dying back and Wallerian degeneration in the time course of SCI (64,65). Subtle changes in the white matter microstructure, such as axon diameter, the break-down of the axolemma or change in its permeability, loss of neurofilaments and microtubules, axon swelling and increase in the extracellular space were also found contributing to the change of DTI directional diffusivities in the white matter following SCI (62,66-68).

In the present study, both heterozygous and shiverer mice underwent the identical degree of contusion injury resulting in comparable acute axonal damage indicated by the decreased ΔFA . Interestingly, the peak severity of axonal injury was reached immediately after contusion suggesting that the primary damage to the axon resulted from the direct mechanical insult. The secondary axonal degeneration, neuronal dieback and Wallerian degeneration, progressed slowly over time leading to significant axonal loss at 21 DPI as seen in postmortem histology (62,64). The slow secondary degeneration was also evident by the gradual elevation of ΔMD reflecting the slow myelin loss after axonal damage. The pattern of

the injury to myelinated and dysmyelinated ventral-lateral WM in response to contusion SCI suggested that dysmyelinated axons in shiverer mice suffered lesser, although not statistically significant, primary injury than the myelinated axons after the mechanical insult (Fig. 4a, 5a). However, faster secondary axonal degeneration is seen in the dysmyelinated axon in the later time points indicating that compact myelin may be required to support the axonal survival from the detrimental secondary degeneration.

Axonal loss is the primary reason for atrophy in the WM diseases (69,70). In the current study, heterozygous and shiverer mice showed different atrophic patterns in response to the same contusion SCI. Initially after the primary mechanical injury, the spinal cords of heterozygous and shiverer mice swelled to the similar size as a consequence of injury-induced edema (Fig. 4a). The compact myelin sheaths in the heterozygous mice, however, might cause more mechanical strain or channel dysfunction at nodes of Ranvier and thus produce greater axonal injury in their myelinated WM (71). As indicated by in the acute phase, a rapid decrease was seen in the injured heterozygous ventral-lateral WM and lesser axon area was spared (Fig. 4d). Secondly in the sub-acute phase, compared to the mild atrophy of the heterozygous mice, shiverer cord exhibited more severe atrophy in this period with mild shrinkage afterward suggesting that initial tissue shrinkage is greater in the absence of space-filling myelin. Finally in the chronic phase, the heterozygous mice cord size remained larger with some myelin remaining, although there was comparable amount of axons spared as that in shiverer mice. Notably, although the time course of the degenerative responses to the injury were different, both heterozygous and shiverer cords eventually resulted in similar axonal sparing and similar level of functional outcome.

Through the serial in vivo DTI, the noninvasive detection of WM injury allows direct correlation of WM damage with the animal's locomotor function. In the hyper-acute to sub-acute phase, BMS scoring did not show a significant difference between the two groups due to the effects of spinal shock and profound inflammation. In the chronic phase when the injury site stabilized, the DTI and histology results suggested that the spared axons of heterozygous and shiverer mice were comparable as well as the resultant locomotor function. In a series of graded contusion SCI, Kim et al. (44) demonstrated that the locomotor function is nearly linearly correlated to the area when spared WM area was below 50% (proportional to total ventral-lateral WM) of the baseline. The locomotor function was not affected if the spared WM area was above 50% of the baseline. In the current study, we further demonstrated that the area of spared axons correlated with the hindlimb locomotor function at 21 DPI in both shiverer and heterozygous mice with BMS less than 5 (Fig. 6a, b). However, full myelination is required for higher locomotor functions underlying coordination, paw placement and trunk stability, i.e., BMS > 5, in mice.

Without compact myelin, the dysmyelinated axons of shiverer mutant exhibit decreased axonal caliber, increased cytoskeletal densities, reduced slow axonal transport rates, reduced neurofilament phosphorylation, increased mitochondria stability, irregular axoglial junctions and scattered distribution of axonal K⁺ channels (59,72-74). As a result, deteriorated axonal conduction is seen in shiverer spinal cord as evidenced by decreased amplitude, increased latency and decreased conduction velocity (60). The deficient myelin sheath was also evidenced by the increased in uninjured shiverer mice compared to the myelinated controls (26,30). The structural changes resulting from the absence of myelin basic protein lead to an erratic and less organized white matter structure in shiverer mice. The dysmyelinated axons exhibited lower potentially, according to literature evidence, resulting from more axon counts, smaller axon calibers, and less extracellular space and intra-axonal congestion of organelles due to shiverer's smaller axon diameter (e.g. mitochondria, microtubule, and neurofilament), (59,60,66,72-74). These potential factors are consistent with smaller RA observed in the ventral-lateral WM of the shiverer mice.

In a traumatic event, axons and glia suffer direct mechanical injury. The ensuing secondary injury leads to the necrotic and apoptotic death of neurons and glial cells. In addition to its role in efficient axonal conduction, the myelin sheath is thought to be an important contributor to secondary pathology and recovery of function after SCI. The myelinating oligodendrocytes can provide protection of neurons and axons from the excessive glutamate activity (75,76) and may contribute to production of essential neurotrophins for neuron survival (48,77,78). On the other hand, the loss of oligodendrocytes and the myelin sheath partly contributes to the glutamatergic excitotoxicity, free radical damage, and inflammation (76,79-83), and may also contain inhibitors of axonal growth and regeneration (84-86). In shiverer mice, the dysmyelinated axon has been shown to act like an immature axon in which the tubulin protein is elevated to increase the density of axonal microtubules (59). In addition, the elevated mitochondrial activity in the dysmyelinated axon may provide more energy for maintaining conduction (72). These alterations in response to the absence of myelin, together with reduced myelin inhibitors presented in shiverer mice may mediate greater relative recovery due to axonal sprouting and plasticity.

During the course of SCI, injury epicenter contains the least amount of spared white matter and the most severe axonal injury and myelin damage (43,87). Spinal cord white matter tracts are found relatively preserved as close as 2-mm away from the epicenter acutely after injury in the mouse contusion SCI (43,45). Later in the injury course, the extent of injury spared above or below the epicenter depends on neuronal dying back and Wallerian degeneration (46,64). The segmental injury to the white matter tract would interrupt the neuronal conduction resulting in the neurological dysfunction, thus the integrity of the epicenter correlates better ($r=0.57$, $p<0.05$) than that of the rostral ($r=0.23$, $p>0.05$) and caudal sites ($r=0.18$, $p>0.05$) with the locomotor function at 21 days-post injury (data not shown). The epicenter with least amount of spared white matter tracts would be representative in reflecting the severity of SCI and crucially important in assessing to the treatment effect (88,89).

During the current experiments, we were surprised to observe that the shiverer mice did not show severe tremors after SCI. This may be due to the complex reaction in WM after SCI blocking both dysfunctional and normal axonal conduction in shiverer mice. Notably, the injured shiverer mice then recovered after SCI to a level very close to their initial locomotor function. Thus, considerable locomotor function after SCI can be attributed to axonal function that is independent of successful myelination. However, another consideration of these data is that there is a greater relative improvement in recovery in shiverer mice. The possible underlying mechanisms for the contribution of mature myelin to recovery remain unknown. One speculation may be that developmental compensatory mechanisms or a reduced inhibitory environment in shiverer mice facilitate recovery. An abundance of studies have used shiverer mice to study the interaction of myelination and axon functions in the uninjured central nervous system, including axon maturation (59,73), myelination interactions with axonal structure and conduction (60,72), the distribution and expression of the K^+ channel subunits Kv1.1 and Kv1.2 (90,91) and glutamate excitotoxicity in WM injury (76,83). Shiverer mice have also been used as a model organism to confirm the capacity of remyelination therapy (74). However, the present findings demonstrate that these mice are also very well suited for important further studies to elucidate mechanisms of myelin function in secondary injury processes and axonal plasticity and regeneration after CNS trauma.

CONCLUSIONS

In vivo DTI was used in this study to investigate the role of axonal sparing and myelin integrity in locomotor function on the dysmyelinated shiverer mice and their heterozygous

littermates. Since uninjured shiverer mice have neither axonal injury nor evidence of neuroinflammation, the lower hindlimb function may be primarily due to the deficient myelin sheath as evidenced by the higher λ and the absence of MBP and EC stainings, with relatively normal λ and the absence of the loss of SMI-31 staining. At 21 days after severe SCI, however, the hindlimb locomotor function was not different between heterozygous and shiverer mice. Although at this time point, there was still some myelin in the heterozygous WM, the contribution of the residual myelin sheath to the recovery of locomotor function was limited. Thus, the present study confirms that the myelin sheath is critical for a higher hindlimb function in uninjured mice. However, when the locomotor function is limited by the damage to long fiber tracts, such as the chronic phase after mid-thoracic SCI, recovery is independent of the presence of residual myelin or the degree of radial diffusivity, instead, the degree of spared axons is critical, and is reflected by measures of axial diffusivity in DTI.

ABBREVIATIONS

SCI	Spinal cord injury
DTI	Diffusion tensor imaging
FA	Fractional anisotropy
RA	Relative anisotropy
MD	Mean diffusivity
	Axial diffusivity
	Radial diffusivity
CNS	Central nervous system
BMS	Basso Mouse Scale
DPI	Days post injury
MRI	Magnetic resonance imaging
TR	Repetition time
TE	Echo time
FOV	Field of view
T2W	T2-weighted
GM	Gray matter
WM	White matter
SD	Standard deviations
EC	Eriochrome cyanine
CV	Cresyl violet
MBP	Myelin basic protein
SMI-31	phosphorylated neurofilament protein
ANOVA	Analysis of variance
HSD	Honestly significant difference
CSF	Cerebrospinal fluid

VLWM   Ventral-lateral WM

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Figure 1. Determination of total cord and spared axon areas

Clear gray and white matter tissue contrast was seen in $\lambda_{\parallel}$ maps in both the sham-operated heterozygous (a) and shiverer spinal cord (b). The total spinal cord area is readily determined in the $\lambda_{\parallel}$ maps taking advantage of the surrounding bright cerebrospinal fluid (CSF). After the determination of total cord area from the $\lambda_{\parallel}$ map, the segmentation of GM (green) and ventral-lateral WM (VLWM, red) allows quantification of the $\lambda_{\parallel}$ in these regions (c, heterozygous; d, shiverer). Normal distributions of $\lambda_{\parallel}$ from the manually defined GM and VLWM regions were seen in the spinal cord from the sham-operated heterozygous (e) and shiverer (f) mice (N=6 in each group). Mean $\pm$ 2 $\times$ SD of the normal appearing VLWM ($\lambda_{\parallel}$ in red) was used as the threshold to define the region of spared axons in the control and injured spinal cords of shiverer and heterozygous mice. The green and red ROI designate GM and VLWM determined by these $\lambda_{\parallel}$ thresholds.

Figure 2. Locomotor recovery of the shiverer and heterozygous mice after SCI

Impaired locomotion was evident from lower BMS scores uninjured (Control) shiverer mice. Values represent mean $\pm$ SD (N=6 in each group). After contusion SCI, both shiverer and heterozygous mice showed initial paralysis (shiverer: $t(15)=31.41$, $p<0.0001$; heterozygous: $t(15)=79.43$, $p<0.0001$) followed by a moderate recovery in the sub-acute phase (7~9 DPI). Neither group recovered consistent plantar stepping after injury, and BMS scores, were not significantly different between the injured shiverer and heterozygous mice throughout time course ($F(1, 5)=16.77$, not significant).

Figure 3. Longitudinal DTI maps of the contusion injured spinal cords of heterozygous (a) and shiverer (b) mice

The hemorrhage associated hypo-intense regions were clearly seen in T2W images throughout the time course. The area of axonal preservation shown as hyper-intense regions in $\lambda_{||}$ maps were marked in yellow regions of interest in heterozygous and shiverer mice. Prior to the contusion injury, the gray-to-white matter contrast was lower in dysmyelinated shiverer mice comparing to the heterozygous spinal cord in the $\lambda_{||}$ map. After injury, the gray-to-white matter contrast reduced in heterozygous mice while no change in contrast of shiverer mice was observed in $\lambda_{||}$ maps. The preserved total area of ventral-lateral WM was also seen in RA maps throughout the time course (outlined in green). The time course of DTI maps revealed acute tissue swelling followed by significant shrinkage 7 days after injury, reflecting the evolution of spinal cord damage.

Figure 4. Evolution of total cord area (a), spared axon area (b), percent spared axon proportional to total cord area (c), and percent spared axon proportional to total area of ventral-lateral WM

The cord sizes were similar between the shiverer and non-shiverer mice before and immediately after SCI. Both groups suffered severe whole cord atrophy, worse in shiverer than heterozygous mice at 7 DPI (a). The size of total ventral-lateral WM in shiverer mice was smaller initially (b). After SCI, both groups exhibited similar spared axon area. When normalized to the whole cord atrophy, the percent spared axon was not different between heterozygous and shiverer mice throughout the time course of the study (c). The total ventral-lateral WM normalized spared axon areas exhibited different patterns between the two groups of mice (d). The percent of spared axon area in total ventral-lateral WM immediately after SCI decreased more rapidly in heterozygous than that in the shiverer mice. There was no significant difference in total ventral-lateral WM normalized percent of axon preservation between shiverer and the heterozygous mice from 7-21 DPI. Values represent mean + SD (N=6 in each group). (* p<0.05, ** p<0.001)

Figure 5. Time course of the group averaged (a), (b) and RA (c) from the total ventral-lateral WM

is lower in naïve shiverer mice. After SCI, was not significantly different between heterozygous and shiverer mice. The of the shiverer mice did not change after injury suggesting no further demyelination in these dysmyelinated animals. In contrast, the of the heterozygous mice continued to increase suggesting progressive demyelination. There was not statistically significant difference in between shiverer and heterozygous mice 14 days after injury. RA was significantly different between the two groups before injury (c). Similar extent of decrease in RA was seen between naïve and 3 hours after injury. RA was comparable between shiverer and heterozygous mice beginning at 7 days after injury. (* $p < 0.01$, ** $p < 0.0001$)

Figure 6. Correlation plots between BMS vs. (a), and BMS vs. total cord area normalized spared axon area in ventral-lateral WM (b) at 21 days post injury

The BMS of the injured heterozygous and shiverer, and the non-injured shiverer, mice was significantly higher than that of the uninjured heterozygous mice ($t(28)=8.90, p<0.0001$), reflecting the difference in myelin integrity (a). A positive correlation ($r(12)=0.57, p<0.05$) between BMS vs. the percent spared axon area was seen among the injured mice with hindlimb function below BMS=4 (b). The significant difference in BMS score between the uninjured shiverer and heterozygous mice was seen with comparable extent of the spared axon area.

Figure 7. EC/CV staining for myelin/neuron integrity of control (a, e) and injured heterozygous (b, f), and control (c, g) and injured shiverer (d, h) mice

Images of the entire cord (a-d) are presented with high power expansion on the marked regions (e-h). Although the total cord areas of the control mice are comparable, the injured cord of shiverer mice endured more significant atrophy than that of heterozygous mice (i). The ventral-lateral WM area of the control shiverer mice was significantly smaller than that of the heterozygous. The shiverer also suffered more severe shrinkage than heterozygous mice after injury (j). The injured cords of both groups showed comparable percentage of ventral-lateral WM area after normalizing to the total cord area (k). Lesions in shiverer mice occupied a larger proportion of the cord areas than that in heterozygous after correcting for shrinkage (l). Values represent mean + SD (N = 6) for heterozygous (filled bar) and shiverer (open bar) mice. Scale bar in d represents 200 μm , common for a – d. Scale bar in h = 100 μm , common for e – h. (* $p < 0.01$, ** $p < 0.0001$, *** $p < 0.00001$)

Figure 8. Staining for myelin basic protein (MBP) in control (a, e) and injured heterozygous (b, f), and control (c, g), and injured shiverer mice (d, h)

Positive MBP staining was seen in heterozygous mice (a, e, i). Shiverer mice congenitally lacking MBP were largely MBP negative (c, g, i). At 21 days after SCI, heterozygous mice exhibited slightly reduced staining area suggesting demyelination (b, f, i). When corrected for cord shrinkage at the epicenter, the proportion of MBP positive area normalized to total cord area was not different between control and injured heterozygous mice (d, h, j). Values represent mean + SD (N = 6) for heterozygous (filled bar) and shiverer (open bar) mice. Scale bar in d represents 200 μm , common for a – d. Scale bar in h = 100 μm , common for e – h. (***) $p < 0.00001$

Figure 9. Staining for phosphorylated neurofilament protein (SMI-31) in control (a, e) and injured heterozygous (b, f), and control (c, g) and injured shiverer mice (d, h)
 Both control shiverer and heterozygous mice were positive in SMI-31 staining appearing comparable intensity (a, c). The stain density of the heterozygous cord was higher than that of the shiverer mice (cf. e, g). Total SMI-31 staining is dramatically reduced after injury in both groups, suggesting severe axonal damage in the chronic phase (i). After correcting for cord shrinkage, the percent SMI-31 areas show a ~40% loss in both groups (j). Values represent mean + SD (N = 6) for heterozygous (filled bar) and shiverer (open bar) mice. Scale bar in d represents 200 μ m, common for a – d. Scale bar in h = 100 μ m, common for e – h. (***) $p < 0.00001$