In Vivo Longitudinal MRI and Behavioral Studies in Experimental Spinal Cord Injury

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Abstract

Comprehensive *in vivo* longitudinal studies that include multi-modal magnetic resonance imaging (MRI) and a battery of behavioral assays to assess functional outcome were performed at multiple time points up to 56 days post-traumatic spinal cord injury (SCI) in rodents. The MRI studies included high-resolution structural imaging for lesion volumetry, and diffusion tensor imaging (DTI) for probing the white matter integrity. The behavioral assays included open-field locomotion, grid walking, inclined plane, computerized activity box performance, and von Frey filament tests. Additionally, end-point histology was assessed for correlation with both the MRI and behavioral data. The temporal patterns of the lesions were documented on structural MRI. DTI studies showed significant changes in white matter that is proximal to the injury epicenter and persisted to day 56. White matter in regions up to 1 cm away from the injury epicenter that appeared normal on conventional MRI also exhibited changes that were indicative of tissue damage, suggesting that DTI is a more sensitive measure of the evolving injury. Correlations between DTI and histology after SCI could not be firmly established, suggesting that injury causes complex pathological changes in multiple tissue components that affect the DTI measures. Histological evidence confirmed a significant decrease in myelin and oligodendrocyte presence 56 days post-SCI. Multiple assays to evaluate aspects of functional recovery correlated with histology and DTI measures, suggesting that damage to specific white matter tracts can be assessed and tracked longitudinally after SCI.

Key words: behavioral assays; diffusion tensor imaging; rat spinal cord injury; magnetic resonance imaging

Introduction

MUCH OF OUR CURRENT KNOWLEDGE about the progression of spinal cord injury (SCI) is based on histological studies. While histology has greatly contributed to our understanding of the pathologic processes in SCI, it is difficult to fully evaluate the spatial and temporal progression of the injury because histology provides only a static view of the injured spinal cord. More recently, magnetic resonance imaging (MRI) has been increasingly employed to non-invasively follow both spatial and temporal changes in SCI (Bilgen et al., 2007; Deo et al., 2006; Lammertse et al., 2007; Mihai et al., 2008; Narayana, 2001; Narayana et al., 2004a, 2004b).

High resolution MRI studies have been employed to quantify the lesion volumes in SCI (Bilgen et al., 2007; Ditor et al., 2008; Gonzalez-Lara et al., 2009; Mihai et al., 2008; Nout et al., 2009; Runge et al., 1997). Mihai and associates (2008) employed these MR techniques to compare different grades of injury and observed that as the hyperintense lesion representing edema resolved, the animals showed improved

functional recovery on the Basso-Beattie-Bresnahan (BBB) locomotor rating scale (Basso et al., 1995). Serial studies were performed *in vivo* in rats with SCI to quantify pathology and compare with end-point histology and behavioral performance (Falconer et al., 1994; Narayana et al., 1999). Bilgen and colleagues (2007) performed MRI scans up to 28 days post-injury in mice, and provided a longitudinal description of the changes in both the lesion length and volume following a contusive injury.

More recently, diffusion tensor imaging (DTI) has been used to probe the spatial and temporal changes in white matter pathology following SCI (Deo et al., 2006; Ellingson et al., 2008; Kim et al., 2007; Kozlowski et al., 2008; Wang et al., 2009; Zhang et al., 2009). However, to fully appreciate the changes that occur in SCI and their effects on functional outcome, correlations with neurobehavioral assays need be explored. Deo and associates reported *in vivo* longitudinal DTI studies up to 2 months post-SCI, and correlated DTI measures in selected regions of white matter to BBB scores (Deo et al., 2006). Mihai and colleagues (2008) evaluated animals up to 3 weeks post-SCI and correlated a histological stain for myelin,

as well as BBB scores, paw preference, and grooming tasks with the lesion volume calculated from high-resolution anatomical images. While these studies have provided valuable information, the relationship between longitudinal changes in white matter integrity and lesion volume, comprehensive neurobehavioral assays, and histopathology remains largely unexplored. Integrated studies that relate these various measures have the potential to provide important information about the spatial and temporal pathological changes, and allow the interpretation of the MRI measures on a rational basis.

The goal of this study was to evaluate spatial and temporal pathological changes in SCI using a multi-modal approach that includes *in vivo* longitudinal MRI studies, a battery of neurobehavioral tests, and end-point histology, in the same cohort of animals. Rats were imaged at multiple time points up to 2 months post-injury so that the evolution of injury as seen by lesion spread and damage to the white matter tracts could be followed within the same animals. Neurobehavioral tests for locomotion, motor performance, sensory-motor integration, and changes to the sensory system were evaluated at the same time points so that these measures could be compared to MRI data. Finally, the end-point histology was performed to correlate with MRI measures and behavioral assays.

Methods

The protocol used in this study was reviewed and approved by our Institutional Animal Welfare Committee, and the guidelines provided in National Institutes of Health Guide for the Care and Use of Laboratory Animals were followed.

Animals and spinal cord injury procedure

A total of 24 adult male Sprague-Dawley rats (300–320 g) were used in these studies. Fifteen rats received a contusion injury and nine animals were assigned to a sham group which received a laminectomy only. All surgical procedures were performed as described previously (Herrera et al., 2009). Briefly, animals were anesthetized with 4% isoflurane, intubated, and maintained under anesthesia with a mixture of 2% isoflurane, air, and oxygen, administered through a rodent ventilator (Model 683; Harvard Apparatus, Holliston, MA). The vertebral processes of the sixth and eighth thoracic vertebrae (T6 and T8) were clamped to stabilize the vertebral column, and a laminectomy was performed at T7. A 150-kDyne force was delivered to the exposed cord using the Infinite Horizon Impactor (Precision Systems and Instrumentation, LLC, Lexington, KY) to cause a moderate level SCI. The sham animals underwent the same surgical procedure, but received only a laminectomy.

For improved signal-to-noise ratio (SNR) and MRI image quality, an 11×35-mm rectangular radio frequency (RF) coil was implanted over the spinal column and centered on the site of injury (Deo et al., 2006; Fenykes and Narayana, 1998). The implanted coil was held in place with two sutures and tuned to 300 MHz. The overlying paraspinal muscles were then sutured at the midline and the skin was closed.

The animals were allowed to recover in a warmed cage and received subcutaneous injections of Baytril 100 (2.5 mg/kg; Bayer Healthcare LLC Animal Division, Shawnee Mission, KS) twice a day for 3–5 days, and Buprenex (0.01 mg/kg; Hospira, Inc., Lake Forest, IL) twice a day for 5 days. Animals were also administered subcutaneous injections of saline

twice daily for 5 days. The animals' bladders were manually expressed twice daily until the return of spontaneous urination. The animals were housed in pairs in plastic cages, maintained on a 12-h light/dark cycle, and had free access to food and water.

Animal MRI preparation

The animals underwent spinal cord MRI at post-surgery days 7, 14, 28, 42, and 56. The animals were intubated and the anesthesia level was maintained using the Harvard Apparatus rodent ventilator. For MRI scans, the animals were placed on a custom-made acrylic glass bed, and respiration and rectal temperature were continuously monitored with a physiologic monitoring unit (Model 1025; SA Instruments, Inc., Stonybrook, NY), and heart rate and oxygen levels were monitored with the Nonin pulse oximeter (Model 8600V; Nonin Medical Inc., Plymouth, MN). The animal's body temperature was maintained at 37°C using the SA Heating System (Model 11007B; SA Instruments).

MRI protocol

All MR studies were carried out on an actively shielded Bruker Biospec 70/30 URS scanner operating at a field strength of 7 T (Bruker, Karlsruhe, Germany). Prior to each animal MRI study, a quality control scan was performed on a gel phantom to ensure scan-to-scan consistency and to assess the SNR. The SNR analysis consisted of a spin echo sequence of a single slice with a square field of view (FOV) size of 5×5 cm. Imaging parameters included: echo time (TE) = 13.4 msec, repetition time (TR) = 1000 msec, matrix size of 256×256, one average, and one repetition. For the animal scans, a 35×40-mm external surface coil was placed under the animal's back and inductively coupled to the implanted RF coil. Respiratory gating was employed for minimizing breathing artifacts in the images.

A tripilot scan was performed to ensure proper positioning of the rat within the MRI scanner. A quality assurance scan for magnetic field homogeneity assessment was performed. The magnet was shimmed to adjust the magnet homogeneity initially using the autoshim routine provided by the manufacturer, followed by manual adjustment of linear and Z² shims (Cohen et al., 2009).

High-resolution images were acquired using the rapid acquisition by relaxation enhancement (RARE) sequence. For this sequence, 35 1-mm-thick contiguous and interleaved axial slices were obtained. The images were acquired using the following parameters: rare factor of 4, TE₁/TE₂/TR = 21.2/63.6/3150 msec, image matrix of 256×256, square FOV of 2.62×2.62 cm.

DTI was used in these studies to evaluate the integrity of the white matter fiber tracts. The geometry that was utilized for DTI studies was identical to that used in the RARE sequence. Diffusion-weighted images were acquired using a spin echo echo planar imaging (EPI) readout sequence with 4 shots, TR/TE = 3000/39.73 msec, receiver bandwidth of 200 kHz, an encoding scheme with 21 positive and negative encoding directions that was balanced and unbiased (42 gradient encoding directions total) (Madi et al., 2005), acquisition matrix of 128×128, square FOV of 2.62×2.62 cm, slice thickness of 1 mm, and 25 slices; DTI images were reconstructed to 256×256.

To assure consistent positioning of the animal in these longitudinal studies, the epicenter slice in the RARE images was determined by critically observing the anatomy surrounding the spinal cord. The laminectomy site was visible, allowing the determination of the approximate epicenter slice. The signal voids from the conductors of the implanted coil also provided additional reference points.

Evaluation of MRI lesion volume

Lesion visualization and quantification were based on the RARE images. In the acute phase of injury, hyperintense lesions correspond to edema, and hypointense lesions correspond to hemorrhage, while in the chronic stages of injury they are associated with demyelination and necrosis, respectively (Weirich et al., 1990). RARE images were analyzed using Image-Pro Plus 5.1 software (Media Cybernetics, Inc., Silver Spring, MD). Intensity thresholds for hypo- and hyperintense lesions were established by inspecting images from uninjured animals. The images of normal, uninjured spinal cords were evaluated to determine normal white and gray matter intensity values, and to set thresholds for hypo- and hyperintensities. The thresholds were then applied to areas of interest encompassing the spinal cord, and the total area of the hyper- or hypointense lesion was automatically recorded for each image slice. Once the areas were determined for a given image slice, the lesion volume for that slice could be calculated. Summing the volumes of all MRI image slices provided the total volume for the hyper- and hypointense lesions. Volumes were calculated for each time point in order to evaluate the temporal evolution of the injury during the course of the study.

DTI data processing

EPI images generally have low SNR and are prone to ghosting and noise artifacts, therefore the DTI data required some post-processing to minimize artifacts. A pipeline for processing data in batches with minimal operator intervention was used (Bockhorst et al., 2010a). This custom program, written in IDL (ITT Visual Information Solutions, Boulder, CO), identified and eliminated images with significant ghosting (Bockhorst et al., 2008, 2010b). First, a digital tuning program corrected ghosts in the time domain (Ye and Xiang, 2007). The corrected data were reconstructed using the "FRECO" macro in ParaVision (Bruker), and magnitude images were created. The ghosted magnitude images were then replaced by unghosted images. The images were cropped to a 71×71 matrix to reduce the amount of data processing. The data were filtered using a Rician filter (Hahn et al., 2004, 2005). Extrameningeal tissues were manually cropped using MRICro (<http://www.sph.sc.edu/comd/rorden/mricro.html>) for a set of images acquired with one gradient encoding direction. This mask was applied to images acquired with all other encoding directions, and to the b0 images (images without any diffusion gradients) for spinal cord extraction. The diffusion-weighted images were warped to the corresponding b0 images using AIR (align_warp, 3D, model 2; Woods et al., 1998) to minimize the image distortions caused by the eddy currents induced by strong diffusion gradients. Finally, the data were written in DTI Studio (Jiang et al., 2006) (<http://cmrm.med.jhmi.edu/>) readable format. All of the above-mentioned processing steps, aside from the application of the mask, were fully automatic and were processed as batch jobs.

Evaluation of white matter fiber tracts

In these studies, DTI-derived maps of fractional anisotropy (FA), transverse diffusivity (λ_T), and longitudinal diffusivity (λ_L) were generated using DTI Studio (Jiang et al., 2006) (<http://cmrm.med.jhmi.edu/>). Region of interest (ROI) analysis was performed for each image slice using the "ROI Manager" application of ImageJ software (<http://rsb.info.nih.gov/ij/>). Methods similar to those described previously (Deo et al., 2006) were used to determine values for each of the DTI measures in specific regions within the spinal cord white matter. Briefly, an elliptical ROI was placed on three regions: ventral, dorsal, and lateral white matter (V-, D-, and L- respectively). Care was taken in the placement of the ROI to minimize the overlap with gray matter.

For DTI analysis, data were grouped into regions that were defined by the distance to the injury epicenter. Sections located up to 10 mm rostral and 10 mm caudal to the epicenter were evaluated; thus a total of 21 mm of spinal cord tissue was assessed. For the analysis, the spinal cord was divided into seven regions, each containing three 1 mm slices. The seven total regions included: caudal regions 1 through 3 (C1–C3), epicenter (EC), and rostral regions 1 through 3 (R1–R3). The EC region contained the epicenter slice plus one slice rostral and one caudal; C1 and R1 consisted of slices that were 2–4 mm from the epicenter slice, C2 and R2 contained MRI slices that were 5–7 mm from the epicenter, and C3 and R3 encompassed slices that were 8–10 mm from the epicenter. As each spinal segment is approximately 3 mm long in rats, each region corresponds to one segment of the spinal cord. Therefore, R3 corresponds to T4, R2 to T5, R1 to T6, EC to T7, C1 to T8, C2 to T9, and C3 to T10. Dividing the cord into these regions also allowed us to reduce the number of multiple comparisons that would have diluted the statistical significance.

Neurobehavioral assays

The animals underwent neurobehavioral assays that included tests of open-field locomotion, inclined plane performance, grid walk analysis, and measures of activity using computerized activity boxes, as well as evaluations of sensory function using the Von Frey filament test. All behavioral assays were performed on the same days as the MRI scans, prior to the administration of anesthesia.

The most widely used outcome measure in SCI rats is the BBB test (Basso et al., 1995; Sedy et al., 2008). It is an assay of open-field locomotion that characterizes the animal's recovery with a score of 0–21, for which an increasing score indicates better functional outcome. For our studies, the animals' performance on the BBB assay was evaluated on days 3, 7, 14, 28, 42, and 56.

Once the animals reached a BBB score of 9, which is indicative of the animal's ability to maintain plantar placement of the paws in a weight-supported stance (Basso et al., 1995), the inclined plane assay was incorporated into the study. The inclined plane test evaluates the animal's strength and postural control (Rivlin and Tator, 1977). For this task, the animals were placed horizontally on a 61×45-cm board covered with a grooved rubber mat which was raised to an incline of 30°. The angle of the plane was then increased or decreased in 5° increments to determine the maximum angle at which a rat could maintain its balance for at least 5 sec. This task evaluates

FIG. 1. Example of injury pathology in an MRI RARE image of a spinal cord 28 days post-SCI. Hypointense lesions correspond to hemorrhage and necrosis, while hyperintense lesions correspond to edema and demyelination (MRI, magnetic resonance imaging; RARE, rapid acquisition by relaxation enhancement; SCI, spinal cord injury).

the integrity of the rubrospinal, vestibulospinal, raphespinal, and reticulospinal tracts, but not the corticospinal tracts (DeLiagina et al., 2006; Fehlings and Tator, 1995; Lyalka et al., 2005; Matsuyama and Drew, 2000; Sedy et al., 2008).

The grid walk analysis, sometimes known as the foot-fault test (Bresnahan et al., 1987), evaluates both the sensory and

motor systems and appraises their connection to each other (Sedy et al., 2008). This test was performed once the animals were able to take weight-supported steps. In this task, the animals are required to traverse an elevated wire grid with 4-cm square gaps, and scores were recorded as the number of hindlimb footfalls in a total of 30 steps. To perform well on this examination, the animals must demonstrate normal limb coordination, which is mediated by ventrolateral tracts, have an intact reticulospinal system for initiation of stepping rhythm, and possess voluntary motor control, which is mediated by corticospinal and rubrospinal systems (Metz et al., 2000; Sedy et al., 2008). Because this task evaluates more complex behavior, this assay can expose deficits that are not evident in tests of normal locomotion, and helps to identify the underlying mechanisms involved.

Computerized activity boxes were also employed once the animals could take weight-supported steps, at post-injury days 14, 28, 42, and 56. These units evaluate behaviors such as fine motor movements, locomotion, and rearing (Mills et al., 2001). These assessments of exploratory behavior provide insight into the general motor behavior and overall health of the animal (Metz et al., 2000). For this assay, the animals were placed in the activity chamber for 30-min testing periods, and data were collected using the software and hardware provided by the Photobeam Activity System (PAS) with Flexfield (San Diego Instruments, Inc., San Diego, CA). The PAS acquires data reflecting the animal's movements within the activity chamber by recording the number of times photobeams are obstructed in an x, y, and z axis-oriented grid

FIG. 2. Temporal profile of hyperintense and hypointense lesions in injured animals. **(A)** There was slight hyperintense injury in sham animals, but there were no significant changes in volume seen over the course of the study. **(B)** There was slight hypointense injury seen in sham animals, but there were no significant changes in volume throughout the 8 weeks. **(C)** In the injured animals on days 7 and 14 they had significantly more hyperintense lesioning than the more chronic time points at days 28, 42, and 56. **(D)** There were no significant differences in hypointense lesion volume between any of the time points.

system, and activity can be categorized as fine motor movements, gross movements, and rearing. Rearing behavior is thought to reflect vestibulomotor function (Himes et al., 2006) and trunk function (Cain et al., 2007). Previous studies have also suggested that changes in exploratory behavior and rearing may reflect chronic central pain following SCI (Cruz-Orengo et al., 2006; Houghton et al., 1997; Mills et al., 2001; Mills and Hulsebosch, 2002). Because the PAS system is entirely automated it provides simple, direct, and continuous measures which are free from any user bias (Cain et al., 2007).

The von Frey filament test was utilized to assess the development of mechanical allodynia. This test was incorporated at the same time points as other neurobehavioral studies, once the animals demonstrated weight support with the body weight distributed equally between the front and hind paws. The rats were placed in clear acrylic glass cubicles on an elevated metal mesh floor and allowed to acclimate to the new environment for 15 min. Following the acclimation period, the paw-withdrawal response to mechanical stimuli was recorded using an Electronic Von Frey Anesthesiometer (IITC Life Science, Inc., Woodland Hills, CA). In uninjured animals the stimulus is innocuous and does not elicit a paw-withdrawal response at low levels of pressure; however, a decreased threshold for the stimuli in injured animals demonstrates the increased sensitivity of the paw's mechanoreceptors. The von Frey filament was applied to the plantar surface of each paw, and the maximum pressure required to elicit a response was automatically recorded (Peng et al., 2006). Five scores for each paw were recorded; the lowest and highest values were discarded and the middle three were used for analysis. To determine whether the animals were experiencing allodynia at a given time point, the average of the three pressure values recorded for each paw were compared to the baseline scores that were recorded prior to receiving injury. Animals that showed a reduction in the pressure required to elicit a paw-withdrawal response compared to baseline values were defined as having increased sensitivity to the mechanical stimulus at that particular time point. For these studies, animals consistently demonstrating heightened sensitivity in both forelimbs from day 28 onward were qualified as expressing persistent allodynia.

The order of assessment, once the animals had achieved the required BBB scores (above 10), was as follows: activity box, von Frey filament test, inclined plane, and grid walk. To allow for adequate recovery time between tests, roughly 15–20 min was provided between testing sessions for each test.

Tissue processing and immunohistological staining

To correlate with the DTI measures, tissue was stained with antibodies to myelin and oligodendrocytes, phosphorylated neurofilament, and glial fibrillary acidic protein. At the conclusion of the MR studies, the animals were transcardially perfused with 0.1 M phosphate-buffered saline (PBS), followed by 4% paraformaldehyde (PFA). The spinal cords were removed, post-fixed overnight in 4% PFA, and then immersed in 30% sucrose/phosphate-buffered saline (0.1 M PBS) for 2–3 days at 4°C. The spinal cords were coronally sectioned at 35 μ m using a cryostat (CM1800; Leica, Bannockburn, IL), and stored at –20°C in Tissue-Tek® OCT Compound (Sakura Finetek, U.S.A., Inc., Torrance, CA).

We compared histological sections that were taken from the same regions of the spinal cord as the corresponding MRI images (Deo et al., 2006; Herrera et al., 2008), to correlate with DTI metrics. We examined sections ($n = 8/\text{animal}$) from rostral and caudal areas spanning a 1-cm section of cord that was centered on the injury region.

Tissue sections were processed as free-floating and incubated in the following primary antibodies: myelin and oligodendrocytes (MAB 328, 1:1200; Millipore, Billerica, MA), neurofilament 200-kD protein (SMI-31, SMI-31R, 1:1000; Covance, Princeton, NJ), and glial fibrillary acidic protein (GFAP, Z0334, 1:1000; Dako, Glostrup, Denmark). Primary antibodies were diluted with blocking solution (0.1 M PBS containing 5% goat serum and 0.3% Triton X-100). Appropriate Alexa Fluor dye-conjugated secondary antibodies (Invitrogen, Carlsbad, CA) were used at a dilution of 1:600 in 0.1 M PBS. Tissue sections were viewed and captured using a Spot Flex digital camera (Diagnostic Instruments, Inc. Sterling Heights, MI) attached to a Leica RX1500 upright microscope, and the images were collected using Spot software (Diagnostic Instruments, Inc.). The operator acquiring the images was blinded to the group assignment.

Immunohistological image analysis

Histology slides were analyzed using ImagePro Plus software (Media Cybernetics, Inc., Silver Spring, MD). The percent areas of myelin, neurofilament, and GFAP were measured in a defined ROI using intensity thresholding. The thresholds were established by inspecting randomly selected

TABLE 1. EXAMPLES OF DTI MEASURES AT POST-SURGERY DAY 56

Region	Measure	Injured	Sham	p Value
C3	FA	0.60 $\pm$ 0.070	0.80 $\pm$ 0.091	<0.00001
	λ_L	1.14 $\pm$ 0.296	1.38 $\pm$ 0.152	0.0131
	λ_T	0.41 $\pm$ 0.093	0.24 $\pm$ 0.084	<0.00001
C2	FA	0.59 $\pm$ 0.085	0.86 $\pm$ 0.045	<0.00001
	λ_L	1.18 $\pm$ 0.309	1.47 $\pm$ 0.122	0.0015
	λ_T	0.44 $\pm$ 0.140	0.19 $\pm$ 0.056	<0.00001
C1	FA	0.52 $\pm$ 0.100	0.85 $\pm$ 0.048	<0.00001
	λ_L	1.13 $\pm$ 0.346	1.48 $\pm$ 0.129	0.0007
	λ_T	0.50 $\pm$ 0.229	0.20 $\pm$ 0.061	<0.00001
EC	FA	0.39 $\pm$ 0.128	0.86 $\pm$ 0.040	<0.00001
	λ_L	1.56 $\pm$ 0.426	1.53 $\pm$ 0.091	0.1905
	λ_T	0.90 $\pm$ 0.382	0.20 $\pm$ 0.048	<0.00001
R1	FA	0.50 $\pm$ 0.120	0.86 $\pm$ 0.053	<0.00001
	λ_L	1.25 $\pm$ 0.343	1.55 $\pm$ 0.149	0.0060
	λ_T	0.58 $\pm$ 0.285	0.20 $\pm$ 0.073	<0.00001
R2	FA	0.59 $\pm$ 0.094	0.82 $\pm$ 0.060	<0.00001
	λ_L	1.27 $\pm$ 0.237	1.49 $\pm$ 0.127	0.0035
	λ_T	0.47 $\pm$ 0.173	0.24 $\pm$ 0.073	<0.00001
R3	FA	0.64 $\pm$ 0.077	0.81 $\pm$ 0.082	<0.00001
	λ_L	1.34 $\pm$ 0.188	1.45 $\pm$ 0.124	0.1521
	λ_T	0.44 $\pm$ 0.153	0.26 $\pm$ 0.096	<0.00001

An example of injured and sham FA, λ_L , and λ_T values taken from the lateral white matter on day 56.

C3, caudal region 3; C2, caudal region 2; C1, caudal region 1; EC, epicenter; R1, rostral region 1; R2, rostral region 2; R3, rostral region 3; FA, fractional anisotropy; λ_L , longitudinal diffusivity; λ_T , transverse diffusivity; DTI diffusion tensor imaging.

tissue sections from regions encompassing thoracic levels 5–9 from multiple uninjured controls to determine baseline levels of the fluorescent stain. ROIs were drawn in the lateral, dorsal, and ventral white matter in a similar manner to the DTI analysis, and all regional ROIs were the same size for each animal.

Statistical analysis

Statistical analyses were performed using STATA (Intercooled Stata 9.2; StataCorp LP, College Station, TX). The

Wilcoxon rank-sum test was chosen for its robustness and lack of distribution restraints, and used for comparisons between groups at fixed time points, as well as time-point comparisons within one group; multiple comparisons were corrected for using the Bonferroni correction for alpha. Generalized estimating equation (GEE) analysis (Twisk and de Vente, 2002) was used to evaluate the temporal correlations within each group (Cohen et al., 2009), and Pearson's correlation analysis was used to evaluate links between end-point histology and MRI and behavior measures. All values are reported as mean $\pm$ standard deviation unless otherwise

FIG. 3. Diffusion tensor imaging measures within lateral white matter at day 56. **(A)** Fractional anisotropy (FA) values in injured spinal cord tissue decrease with proximity to the injury epicenter. FA values at all points, up to 1 cm rostral and 1 cm caudal to the epicenter, are significantly less in injured animals. **(B)** Longitudinal diffusivity (λ_L) in the lateral region was lower in injured animals, but only significantly lower in region C1. **(C)** Transverse diffusivity (λ_T) values in the lateral region increased with proximity to the epicenter, indicating damage to myelin. λ_T Values in all regions were significantly higher than the values in control animals.

FIG. 4. Diffusion tensor imaging values from region C3, 1 cm from the epicenter. **(A)** Injured animals had significantly lower fractional anisotropy (FA) values at days 7 ($p = 0.0002$), 14 ($p = 0.0009$), 28 ($p < 0.00001$), 42 ($p < 0.00001$), and 56 ($p < 0.00001$). **(B)** Longitudinal diffusivity (λ_L) in the lateral region was significantly lower in injured animals at days 7 ($p = 0.0014$) and 28 ($p < 0.00001$). **(C)** Injured animals had significantly higher transverse diffusivity (λ_T) values at days 28 ($p = 0.0005$), 42 ($p = 0.0012$), and 56 ($p < 0.00001$).

stated. Statistical significance was set at $p < 0.05$, with corrections for multiple comparisons (Bonferroni adjustment).

Results

MRI lesion volume

The RARE images were analyzed for both the hypo- and hyperintense lesions, which correspond to hemorrhage/necrosis and edema/demyelination, respectively (Fig. 1). As expected, the injured animals contained significantly greater hypo- and hyperintense lesions at all time points compared to controls. Interestingly, the control animals displayed some small hypo- and hyperintense lesions (Fig. 2A and B), indicating that the laminectomy itself in some cases resulted in slight injury to the cord. However, the lesions that resulted in those animals remained minimal throughout the course of the study, and were significantly smaller than the lesions seen in injured animals at all time points ($p < 0.002$).

By 7 days post-SCI, the injured animals demonstrated substantial hyper- and hypointense lesions that extended both rostrally and caudally from the epicenter. The hyperintense lesion diminished in size over the course of the study, and there were significant differences in lesion volume between the subacute (days 7 and 14) and chronic (days 28, 42, and 56) time points ($p < 0.0059$; Fig. 2C). From day 28 onward there were no significant changes in the hyperintense volume. The hypointense lesion volume in injured animals remained relatively constant over the entire 8-week time period, with no significant differences between any time points (Fig. 2D).

Diffusion tensor imaging after spinal cord injury

The DTI measures were evaluated in three regions of white matter: dorsal, ventral, and lateral. The values of FA, λ_L , and λ_T in injured animals showed signs of changes in all regions and at all time points: FA and λ_L were both decreased compared to controls, while λ_T was increased. An example of injured and sham FA, λ_L , and λ_T values taken from the lateral white matter on day 56 are listed in Table 1. The dorsal values of injured animals were very similar to those obtained in the lateral region, while differences between the injured and sham animals were slightly less pronounced in the ventral region. In all white matter regions the FA values were significantly smaller in injured animals compared to sham controls. Figure 3A shows that the FA values close to the site of injury

were lower than the values 1 cm away, indicating increased disruption of the fiber tracts with proximity to the epicenter ($p < 0.00001$). Similarly, λ_L values were generally lower in the white matter regions of the injured group, though only significantly less so at region C1 ($p < 0.0007$; Fig. 3B). The transverse diffusivity, λ_T , was significantly higher in all white matter regions in injured animals compared to sham animals, and peaked at the epicenter ($p < 0.00001$; Fig. 3C).

Significant differences were observed between injured and sham tissue in regions that were 1 cm from epicenter (in regions R3 and C3), where the tissue appears normal in conventional MRI images. FA values from lateral white matter in the C3 region were significantly lower in injured animals at all time points ($p < 0.0009$; Fig. 4A). In addition, the lateral λ_T values were significantly higher in the injured animals at days 28 ($p = 0.0005$), 42 ($p = 0.0012$), and 56 ($p < 0.00001$; Fig. 4C), and the lateral λ_L values were significantly decreased at days 7 ($p = 0.0014$), and 28 ($p < 0.00001$; Fig. 4B). Similar significant differences were also seen in the dorsal and ventral white matter regions away from the epicenter.

Neurobehavioral and sensory tests

The results for the BBB, inclined plane, and grid walk assays are shown in Table 2. As expected, all injured animals showed severely diminished BBB scores immediately after SCI, followed by an improvement in performance over time. Injured animals showed an average BBB score of 0.7 ± 0.82 on day 3, and a maximum score of 14.6 ± 2.76 on day 56. The sham animals maintained a score of 21 throughout the study. At all time points, BBB scores for the injured animals were significantly lower than those of sham animals ($p < 0.00001$).

On day 14, injured animals could maintain their balance at an angle of $44.7 \pm 6.20^\circ$, while sham animals were at $51.4 \pm 2.58^\circ$. By day 56, the injured animals could remain on the plane up to an angle of $53.5 \pm 2.46^\circ$, and the sham animals were at $54.9 \pm 2.76^\circ$. While the injured animals did show slight recovery over time, they continued to maintain their balance at significantly less-steeply inclined angles ($p < 0.0159$) than the sham animals throughout the course of the study.

Similarly to the results seen with the inclined plane test, the injured animals recovered and improved their performance on the grid walk assay with time, but they had significantly more hindlimb foot falls than sham controls ($p < 0.0071$). At day 14 injured animals had 12.0 ± 5.45 foot falls, while sham

TABLE 2. PERFORMANCE OF INJURED AND SHAM ANIMALS ON NEUROBEHAVIORAL ASSAYS

	BBB			Inclined plane			Grid walk		
	Injured	Sham	p Value	Injured	Sham	p Value	Injured	Sham	p Value
Day 3	0.7 ± 0.82	21.0 ± 0.00	<0.00001						
Day 7	7.1 ± 2.21	21.0 ± 0.00	<0.00001						
Day 14	10.8 ± 2.93	21.0 ± 0.00	<0.00001	$44.7 \pm 6.20^\circ$	$53.5 \pm 2.46^\circ$	0.0005	12.0 ± 5.45	3.8 ± 2.04	0.0021
Day 28	13.1 ± 2.70	21.0 ± 0.00	<0.00001	$48.4 \pm 4.37^\circ$	$55.9 \pm 1.91^\circ$	0.0001	12.4 ± 6.20	3.5 ± 1.51	0.0021
Day 42	14.1 ± 2.79	21.0 ± 0.00	0.0008	$49.3 \pm 3.02^\circ$	$55.2 \pm 1.64^\circ$	0.0005	9.4 ± 4.38	1.9 ± 1.45	0.0004
Day 56	14.6 ± 2.76	21.0 ± 0.00	<0.0001	$51.6 \pm 2.57^\circ$	$54.9 \pm 2.76^\circ$	0.0159	8.0 ± 4.69	1.8 ± 0.67	0.0071

BBB performance was assessed at all time points following surgery; injured animals had consistently lower scores than sham animals. Inclined plane was incorporated once the animals could maintain a weight-supported stance, and grid walk was added once the animals could take weight-supported steps. All values are expressed as group mean $\pm$ standard deviation.

BBB, Basso-Beattie-Bresnahan scale.

animals had 3.8 ± 2.04 foot falls. By day 56 the injured animals improved to 8.0 ± 2.04 foot falls, while sham animals had 1.8 ± 0.67 hindlimb foot falls.

The animals were tested in the activity box at post-injury days 14, 28, 42, and 56. For these time points there were no significant differences between injured and sham animals for any measures.

In these studies, 8% of injured rats experienced allodynia as indicated by the von Frey filament test, while none of the uninjured control animals demonstrated hyperresponsiveness to the stimulus. This difference between groups was not significant ($p = 0.1972$).

FIG. 5. Representative images of sham and injured tissue approximately 5 mm rostral to the epicenter. (A and B) The images were first obtained at 10 $\times$ (the images show SMI-31-stained tissue, and the arrows indicate the dorsal columns; scale bar = 500 μ m). (C–H) The dorsal, lateral, and ventral regions were individually assessed at 20 $\times$ (scale bar = 200 μ m). Tissue was stained for neurofilament (C and D), astrocytes (E and F), and for myelin and oligodendrocytes (G and H; GFAP, GFAP, glial fibrillary acidic protein).

Histology

An example of representative immunofluorescence images from injured and uninjured animals is shown in Figure 5. Injured animals had significantly less intense SMI-31 and MAB 328 labeling compared to sham controls, while GFAP was increased. In the injured animals, the quantitative results of the percentage expression of 200-kD neurofilament indicated that the staining intensity was significantly lower ($p < 0.0001$) in the dorsal, lateral, and ventral white matter regions of injured animals, signifying axonal damage throughout the spinal cord (Fig. 6). The MAB 328 staining

FIG. 6. Quantitative analysis of neurofilament staining. Staining with the SMI-31 antibody was significantly decreased in injured animals in the (A) dorsal, (B) lateral, and (C) ventral regions at 56 days post-injury.

was also significantly less intense ($p < 0.0001$) in the same tissue regions of injured animals, indicating a loss of myelin following SCI (Fig. 7). The GFAP labeling indicated that the injured tissue had significantly increased intensity in dorsal, lateral, and ventral white matter, both rostral and caudal to the epicenter compared to control tissues (Fig. 8).

Correlations with MRI measures

Results of the GEE analysis of DTI data are summarized in Table 3. In the injured group, the strongest correlations be-

tween DTI measures and neurobehavioral data were observed in the rostral region of the spinal cord. In the rostral section, the inclined plane assay was significantly linked to the λ_T measures in the dorsal, lateral, and ventral columns ($p = 0.009$). The inclined plane performance was also significantly correlated with λ_T in the dorsal and lateral regions within the epicenter ($p = 0.002$). The BBB and grid walk tests as well as rearing counts also correlated with various DTI measures in the rostral and epicenter regions of the spinal cord, but there were no correlations with the Von Frey analysis. There were only two significant correlations in the caudal

FIG. 7. Quantitative analysis of myelin and oligodendrocyte staining. MAB 328 staining was significantly decreased in the (A) dorsal, (B) lateral, and (C) ventral regions of injured spinal cord at 56 days post-injury.

FIG. 8. Quantitative analysis of astrocyte staining. Glial fibrillary acidic protein was significantly upregulated in the (A) dorsal, (B) lateral, and (C) ventral regions of injured tissue at 56 days post-injury compared to sham animals.

TABLE 3. CORRELATIONS BETWEEN DTI MEASURES AND NEUROBEHAVIORAL ASSAYS

	Rostral		Epicerter		Caudal	
	Injured	Sham	Injured	Sham	Injured	Sham
	p Value	p Value	p Value	p Value	p Value	p Value
Dorsal	λ_L - BBB λ_T - IP	λ_L - GW $p < 0.0001$	FA - BBB λ_L - BBB λ_L - IP λ_T - BBB λ_T - IP λ_T - Rearing	$p < 0.0001$ $p < 0.0001$ $p = 0.003$ $p < 0.0001$ $p = 0.001$ $p = 0.009$	λ_L - GW $p = 0.002$ λ_L - IP $p = 0.010$	$p = 0.001$
Lateral	FA - IP FA - Rearing λ_L - BBB λ_T - GW λ_T - IP	λ_L - GW $p = 0.006$	λ_L - GW λ_L - IP λ_T - GW λ_T - IP	$p < 0.0001$ $p = 0.005$ $p = 0.002$ $p = 0.002$	λ_L - GW λ_L - VF	$p < 0.0001$ $p < 0.0001$
Ventral	FA - BBB FA - IP FA - Rearing λ_T - BBB λ_T - IP λ_T - Rearing	$p = 0.008$ $p = 0.010$ $p < 0.0001$ $p = 0.001$ $p = 0.009$ $p = 0.001$	FA - IP	$p < 0.0001$	FA - GW λ_T - GW	$p = 0.007$ $p = 0.005$ $p < 0.0001$

GEE analysis was used to correlate behavioral assay data with DTI measures in the dorsal, lateral, and ventral areas of the spinal cord located in the rostral, caudal, and epicenter regions. BBB, Basso-Beattie-Bresnahan score; IP, inclined plane; GW, grid walk analysis; FA, fractional anisotropy; λ_L , longitudinal diffusivity; λ_T , transverse diffusivity; GEE, generalized estimating equation; DTI, diffusion tensor imaging.

TABLE 4. CORRELATIONS BETWEEN LESION VOLUME AND NEUROBEHAVIORAL ASSAYS

Injured	BBB	GW	IP	VF	Rearing
Hypo volume	$p = 0.001$	-	-	-	-
Hyper volume	$p < 0.0001$	$p < 0.0001$	$P < 0.0001$	-	$p < 0.0001$
Sham	BBB	GW	IP	VF	Rearing
Hypo volume	-	-	-	-	-
Hyper volume	-	$p = 0.006$	-	-	-

GEE analysis was used to correlate the longitudinal lesion volume values with behavioral assay data.

BBB, Basso-Beattie-Bresnahan score; IP, inclined plane; GW, grid walk analysis; VF= von Frey filament test; GEE, generalized estimating equation; Hypo, hypointense; Hyper, hyperintense.

region: FA and grid walk ($p = 0.007$) and λ_T and grid walk ($p = 0.005$). In all spinal cord regions (rostral, epicenter, and caudal) the injured animals had few correlations with the dorsal column DTI data. The sham animals had few correlations in the regions measured.

The results of the GEE analysis of lesion volume are listed in Table 4. In the injured animals there were significant correlations between the performance on nearly all neurobehavioral assays and the hyperintense lesion volume. The hypointense lesion correlated only with the BBB scores ($p = 0.001$).

The Pearson's correlations between end-point histology with neurobehavioral, DTI, and lesion volume data from day 56 are summarized in Table 5. In the injured animals, the percentage of MAB 328 staining in the ventral columns of the spinal cord significantly correlated with BBB scores ($p = 0.0230$), and the inclined plane test ($p = 0.0022$); SMI-31 staining correlated with BBB scores ($p = 0.0027$), grid walk scores ($p = 0.0243$), and rearing scores ($p = 0.0001$); GFAP staining in the dorsal columns correlated with the grid walk scores ($p = 0.0009$) and von Frey analysis ($p = 0.0003$). When the data were separated and analyzed as measures from the rostral and caudal areas of the cord, a significant correlation between SMI-31 in the caudal region of the lateral column was observed with λ_T ($R = 0.8656$, $p = 0.0002$), and myelin correlated with FA in the dorsal column of the caudal zone ($R = -0.8235$, $p = 0.0382$). In the sham animals, there were

many correlations between the DTI measure λ_L and histology data, as well as correlations between histology and rearing. The other behavioral measures in the sham group did not correlate with histology.

Discussion

RARE MRI scans reveal the evolution of lesion volume

To the best of our knowledge, these are the first comprehensive, longitudinal studies of experimental SCI using *in vivo* multi-modal MRI, multiple neurobehavioral assays, and histology. In this study, untreated SCI was evaluated over an 8-week time period using high-resolution anatomical imaging. Both hypointense and hyperintense lesion volumes were quantified for each time point to determine the temporal and spatial evolution of pathology.

The spinal cord microvasculature is damaged immediately following mechanical trauma and results in areas of hemorrhage that progress to hemorrhagic necrosis over time (Dumont et al., 2001; Tator and Fehlings, 1991). It has been previously shown that the initial hemorrhage starts to decline by 7 days post-injury (Noble and Wrathall, 1987). The MRI hypointense lesion at this early time point reflects a combination of both hemorrhage and necrosis, but transitions to necrosis by the chronic time points. In our study, the volume of the hypointense lesion did not significantly change in injured animals for any of the time points observed. While it appears that the hypointense lesion was stable, this does not imply pathology that is static. As hemorrhage begins to subside, the necrotic zone expands and replaces it with a growing lesion cavity (Tator and Fehlings, 1991). Additionally, the spinal cord tissue at and near the injury epicenter atrophies with time (Noble and Wrathall, 1985); therefore, a chronic lesion that appears to be of the same volume as at an earlier time point actually occupies a larger region of spinal cord.

Unlike the hypointense lesion, the hyperintense lesion volume underwent measurable changes with time. It was observed that the volume of the hyperintense lesion was highest on day 7 and significantly decreased in size over the course of the study. These results are similar to those described by Mihai and colleagues (2008), who also observed that the hyperintense lesion decreased over the initial 2-week

TABLE 5. CORRELATIONS BETWEEN HISTOLOGY, DTI MEASURES, AND NEUROBEHAVIORAL ASSAYS

Injured			Sham		
	Correlation (R)	p Value		Correlation (R)	p Value
Myelin _V - BBB	0.4539	0.0230	SMI _V - Rearing	0.5784	<0.00001
Myelin _V - IP	0.5306	0.0022	GFAP _V - Rearing	0.4153	0.0097
SMI _V - BBB	0.4519	0.0027	Myelin _L - λ_L	0.4925	0.0216
SMI _V - GW	-0.3862	0.0243	SMI _L - λ_L	-0.6237	<0.00001
SMI _L - Rearing	-0.5216	0.0001	Myelin _L - Rearing	0.6916	<0.00001
GFAP _D - GW	1.4839	0.0009	GFAP _L - Rearing	0.3955	0.0183
GFAP _D - VF	0.5133	0.0003	Myelin _D - λ_L	0.4799	0.0282
			SMI _D - λ_L	-0.5343	0.0003
			SMI _D - Rearing	0.6470	<0.00001

Pearson's correlation analysis was performed to correlate the end-point histology data with day 56 DTI and behavioral measures.

SMI, SMI-31, marker of neurofilament; Myelin, MAB 328, marker of myelin and oligodendrocytes; GFAP, glial fibrillary acidic protein, marker of astrocytes; V, ventral; L, lateral; D, dorsal; BBB, Basso-Beattie-Bresnahan scale; IP, inclined plane; GW, grid walk analysis; VF, Von Frey filament test; λ_L , longitudinal diffusivity; λ_T , transverse diffusivity; DTI, diffusion tensor imaging.

period post-SCI. In that study, Mihai and associates confirmed a causal relationship between cord swelling and edema, and additionally found that a reduction in the cord swelling mirrored the reduction in hyperintense lesion. They concluded that the reduction in the hyperintense lesion over this initial 2-week period corresponded with a gradual resolution of edema. Additionally, other studies have shown that edema persists up to 15 days post-injury (Demediuk et al., 1987; Nolan, 1969; Sharma, 2005). In our study it appears that the edema resolved in a similar time frame, but there was still evidence of a hyperintense lesion remaining at the later time points. Weirich and colleagues (1990) indicated that the hyperintense lesion reflects demyelination in the subacute and chronic phases of injury. Our histological measures confirm this fact, as myelin and oligodendrocytes were noticeably absent in the injured tissue.

SCI influence on DTI measures

In this study, evaluation of the injury using DTI was performed in parallel to the anatomical imaging studies. Our DTI results indicated that FA, a general indicator of the state of white matter decreased, signifying that the tissue had become disrupted and the diffusion of water was no longer highly restricted. While FA gives a general measure of the tissue organization, it does not provide specific information about the underlying pathology. The measures of λ_T and λ_L provide more specific information about tissue pathology.

Several studies have found a correlation between λ_L and axonal integrity (Budde et al., 2007, 2009; DeBoy et al., 2007; Kozlowski et al., 2008; Song et al., 2002, 2005). In our study we found that λ_L was decreased at sites away from the epicenter, and remained lowered through day 56. However, at the epicenter there was not a significant difference between injured and sham animals. We would, however, like to point out that the loss of contrast between gray matter and white matter at the epicenter makes the estimation of the DTI measures of white matter in this region less reliable than those from regions further away from the epicenter. Additionally, the histological marker for heavy-chain neurofilament significantly decreased in all regions of the spinal cord in the SCI animals at day 56. In the injured animals we observed no statistically significant correlation between neurofilament expression and λ_L . It is possible that SCI influenced the DTI in a complex manner, such that the λ_L measures were not solely attributed to neuronal integrity.

DTI measures are also affected by demyelination, as intact myelin helps to restrict the diffusion of water perpendicular to the axons. Previous studies have also indicated that λ_T is influenced by myelin (Herrera et al., 2008; Kozlowski et al., 2008; Song et al., 2002, 2005; Wang et al., 2009; Zhang et al., 2009). Our results indicate that λ_T increased following SCI, meaning that water was able to diffuse more freely in the transverse direction (i.e., perpendicular to the fiber tracts). In our study, we found that a marker of both myelin and oligodendrocytes diminished following SCI. The loss of myelin therefore suggests a role in the transverse diffusion of water and in altering λ_T values following injury. However, we failed to find significant associations between histology and λ_T values in either injured or sham tissues, except for a single correlation between λ_T and myelin and oligodendrocytes in the lateral columns located caudal to the site of injury. Similarly to

the lack of association seen with λ_L values, it is possible that myelin is not the only contributor to λ_T values.

It has been suggested that astrocytes may affect the DTI measures, as they contain water channels and play a role in water homeostasis (Ellingson et al., 2008; Herrera et al., 2008; Wang et al., 2009). The relationship between astrocytes and DTI measures is not well understood, but some studies have shown that reactive gliosis, reflected by increased GFAP staining, significantly correlated with both λ_T and λ_L (Ellingson et al., 2008; Lu and Sun, 2003; Wang et al., 2009). We assessed the tissue using the GFAP marker and found that injured tissue had significantly more intense GFAP staining in all regions of the white matter. SCI also results in the upregulation of AQP-4, which regulates the flow of water (Nesic et al., 2006). Since these channels are found on astrocytes, and the number of astrocytes present in the tissue is increased after injury, the diffusion of water within the tissue is greatly affected by these changes, and therefore likely affects all DTI measures. We failed to find significant correlations between astrocytes and DTI measures, reinforcing the possibility that a complex interaction of multiple tissue components affects each DTI measure.

While we were unable to find significant correlations between λ_L , λ_T , and the histological markers for phosphorylated neurofilament and myelin, it is possible SCI caused other tissue components to affect the DTI measures. This is supported by the observation that significant correlations exist between λ_L and SMI-31 and myelin in the dorsal and lateral white matter of uninjured animals, but those correlations are lost after injury. A recently published study assessing Wallerian degeneration following dorsal root axotomy found links between λ_L and λ_T with axonal and myelin integrity, respectively (Zhang et al., 2009). In that study, the authors found a high degree of correlation between phosphorylated neurofilament and λ_L in the early phase of injury (up to 3 days post-injury), but in later phases (up to 30 days post-injury), the accumulation of nonphosphorylated neurofilament and amyloid precursor protein may affect λ_L . The authors postulated that the delayed clearance of axon debris accounted for the reduced λ_L at later time points after injury. In that same study, Luxol fast blue was used to stain myelin, and significant correlations with λ_T measures were found. However, we did not find correlations between λ_T and myelin, a finding similar to that reported by Budde and associates (2007).

We also found that white matter in regions away from the injury epicenter that appeared normal in the RARE images exhibited significant changes in DTI measures compared to controls. The high-resolution anatomical MRI images did not show hyperintense or hypointense pathology in those regions, yet DTI indicated that the white matter had undergone measurable changes. These results suggest that DTI is a more sensitive technique for assessing white matter damage after SCI, and that these changes in axonal and myelin integrity precede the changes that are observed on conventional MRI.

Neurobehavioral outcome

The functional recovery seen in these studies is very similar to that seen in other models of SCI (Basso et al., 1996; Behrmann et al., 1992; Bresnahan et al., 1987; Gensel et al., 2006). In terms of the BBB assay, the animals' recovery followed a typical three-phase pattern, with an early more severe deficit,

followed by recovery, and then a plateau (Gensel et al., 2006). When comparing BBB scores to DTI measures from the different white matter regions of injured animals, we found that BBB scores correlated with λ_L and FA measures from the dorsal, lateral, and ventral columns, and with λ_T measures in the dorsal and ventral regions. We also found a positive correlation between the histological markers for neurofilament and myelin in the ventral area and the BBB scores. This suggests that the integrity of axons and myelin within the spinal cord tracts can be measured with DTI, and that they significantly affect performance in assays of open-field locomotion.

More specialized assays such as the grid walk and inclined plane tests can provide better information about the nature of SCI. In our studies, injured animals showed significant deficits on both tasks immediately after injury, followed by some improvement with time. The outcome of the grid walk analysis appears to have some significant associations with λ_T and λ_L values in the lateral region of the injured spinal cord. We also observed a significant association between grid walk performance and FA and λ_T in the ventral region of the cord. When comparing grid walk scores and histology, we also found a significant correlation between grid walk performance and the intensity of neurofilament labeling in the ventral region. These results suggest that the degree of damage to the tracts in the ventral spinal cord can be evaluated using DTI and matched to performance on the grid walk task.

The inclined plane assay evaluates the animal's muscle strength and postural control (Gale et al., 1985; Rivlin and Tator, 1977; Sedy et al., 2008; von Euler et al., 1997), and performance on this task has been associated with the integrity of the rubrospinal, vestibulospinal, raphespinal, and reticulospinal tracts, but not the corticospinal tracts (Deliagina et al., 2006; Fehlings and Tator, 1995; Lyalka et al., 2005; Matsuyama and Drew, 2000; Sedy et al., 2008). The performance on the inclined plane assay significantly correlated with FA and λ_T measures in the ventral region of the cord. We also found that the ability to maintain balance on the inclined plane positively correlated with the amount of myelin and oligodendrocytes present in the ventral region of the cord. These results suggest that demyelination of the tracts within the ventral spinal cord can be observed with DTI and confirmed by performance on the inclined plane assay.

We chose to incorporate the von Frey assay for mechanical allodynia in these studies, as many SCI patients develop chronic pain (Dijkers et al., 2009). We found that a subset of the injured rats did exhibit hyperresponsiveness to the Von Frey stimulus consistently at days 28, 42, and 56, though it was not significantly increased compared to the sham group. We found no correlations between von Frey measures and DTI data or locomotor assays. Interestingly, we did find a link between the staining intensity of GFAP in the dorsal column and allodynia. Nescic and colleagues (2006) previously showed that increased astrocytic activation underlies the development of neuropathic pain-like behavior in contusive SCI. Our data are consistent with this, as we found that an increase in GFAP in the dorsal region was associated with an increase in chronic neuropathic pain.

Conclusion

These studies provide a comprehensive description of the evolution of untreated SCI in rats using multiple techniques

for assessing injury and recovery. MRI allowed us to non-invasively evaluate the changing lesion, as well as integrity of the white matter fiber tracts at the injury epicenter, as well as in regions up to 1 cm rostral or caudal to the injury. One-to-one correlations between DTI measures and histology could not be firmly established, suggesting that a complex interplay between multiple tissue components affects each of the DTI metrics. Extensive behavioral evaluations indicate that animals experience some functional recovery with time, but never fully recover to pre-SCI performance, and that damage to the tracts involved can be assessed using DTI.

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