High resolution diffusion tensor imaging of axonal damage in focal inflammatory and demyelinating lesions in rat spinal cord

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Inflammation, demyelination, gliosis and axonal degeneration are pathological hallmarks of multiple sclerosis (MS) and experimental autoimmune encephalomyelitis. Axonal damage is thought to contribute to irreversible damage and functional impairment, but is difficult to quantify. Conventional MRI has been used to assess the inflammatory and demyelinating aspects of MS lesions, but more sensitive and specific methods are needed to identify axonal damage to monitor disease progression and to determine efficacy of putative neuroprotective agents. We used high resolution diffusion tensor imaging (DTI) and fibre tracking to examine the spinal cord in rats with focal dorsal column inflammatory or demyelinating lesions to determine whether DTI measures can be used to detect pathology at the site of the focal lesion and to measure axonal damage in tracts distal to the focal lesion. Distant from the focal lesion, total axon counts, degenerating axon counts and SMI-31 staining, but not Luxol fast blue staining, were significantly correlated with fractional anisotropy, axial diffusivity and radial diffusivity, all of which are derived from the DTI data. These data suggest that high resolution DTI may be a more sensitive method than conventional imaging for detecting axonal damage at sites distant from inflammation.

Keywords: Demyelinating disease; Neuroimaging; Spinal cord; MRI; Axonal degeneration

Abbreviations: \(\lambda_1\) = axial diffusivity; \(\lambda_\perp\) = radial diffusivity; DTI = diffusion tensor imaging; FA = fractional anisotropy

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Introduction

The pathological hallmarks of multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE) include inflammation, demyelination, gliosis and axonal degeneration. Several lines of evidence suggest that axonal damage contributes to the irreversible disability and functional impairment associated with the progressive stage of the disease (Ferguson et al., 1997; Trapp et al., 1998; Bjartmar et al., 1999, 2003). While conventional MRI techniques are used to assess the inflammatory and demyelinating aspects of MS lesions, more sensitive techniques are needed to detect the extent of axonal damage to monitor disease progression and to determine efficacy of putative neuroprotective agents. In this report, we used high resolution diffusion tensor imaging (DTI) to image spinal cords in rat models with focal inflammatory and demyelinating lesions to determine whether DTI measures can detect pathology at the site of the focal lesion and measure axonal damage in tracts distal to the focal lesion.

DTI is a quantitative measure of the diffusion of water and provides information regarding the directionality of the diffusion that can be used to determine fibre orientation. DTI is more sensitive than conventional MRI because it accounts for the unequal or anisotropic diffusion of water, in three dimensions, which makes this method applicable to biological structures such as axons that restrict water diffusion (Basser, 1995). Both axonal membranes and myelin have been suggested to restrict water diffusion in white matter,
(Beaulieu and Allen, 1994) which may thereby contribute to greater anisotropy (unequal diffusion) within the axon tracts.

Diffusion tensor imaging uses a mathematical model to calculate fractional anisotropy (FA), which is a measure of the degree of diffusion anisotropy. An FA value close to 1 indicates the highest diffusion anisotropy, as found in white matter tracts, which restricts water diffusion to one prominent direction. An FA value close to 0 indicates that the diffusion within the tissue is isotropic, in which the direction of water diffusion is not restricted and therefore equal, as is found in CSF and to a lesser degree in grey matter (Mori and Zhang, 2006). In addition to FA, other DTI measures include axial diffusivity (\(\lambda_a\)) which indicates a rate for the diffusion of water parallel to an axon tract, and radial diffusivity (\(\lambda_r\)) which indicates the rate of the diffusion of water perpendicular to an axon tract. In spinal cord white matter, water diffusion is mostly parallel to axon tracts because there is less restriction in this direction in contrast to the perpendicular direction relative to the tracts. Additionally, 3D axon tracts can be reconstructed from these DTI measures using a method called tractography, or fibre tracking (Xue et al., 1999; Mori and van Zijl, 2002). With this method DTI measurements at a lesion site can be compared to axons within the same tract at a distal site.

The importance and potential application of DTI has been demonstrated in recent reports in which this method has been used to detect lesions as well as cerebellar cortical atrophy in mice with EAE (Ahrens et al., 1998; Kim et al., 2006; MacKenzie-Graham et al., 2006). While it is apparent that lesions can be detected with DTI in EAE, it is still not clear what each DTI measurement signifies in terms of the existing pathology. In the cuprizone model of corpus callosal demyelination increased \(\lambda_r\) was correlated with demyelination and decreased \(\lambda_a\) was seen during remyelination (Song et al., 2005). Alternatively in an optic nerve ischaemia model with axon damage it was reported that \(\lambda_a\) was most sensitive for axonal damage (Song et al., 2003). DTI of post-mortem MS tissues revealed strong correlations between myelin content and FA, although not surprisingly, myelin and axonal counts were also significantly associated in this study (Schmierer et al., 2007). Herein, we use recent advances in fibre tracking DTI technology and both new and well-established animal models exhibiting a pathological range of focal spinal cord lesions, similar to those found in MS, to test the hypothesis that FA, \(\lambda_a\) and \(\lambda_r\) can detect axonal damage in the spinal cord distant to the lesion site. We report that axon counts, degenerating axon counts, and SMI-31, but not Luxol fast blue (LFB), staining are significantly correlated with FA, \(\lambda_a\) and \(\lambda_r\). These data suggest that high resolution DTI can be used as a more sensitive method than conventional imaging to detect axonal damage at sites distal to the primary injury.

**Methods**

**Animals**

Female Lewis rats (160–200 g) were purchased from Charles River. No manipulations were performed until animals were given a week to acclimate. Animals were housed in groups under a 12-h light/dark cycle with food and water _ad libitum_. All experiments were done in accordance with NIH guidelines for animal care and use.

**Animal models**

To assess the relationship between neuropathological events and various MRI measurements, focal lesions were induced by either the intraspinal injection of cytokines or lipopolysaccharide (LPS) into MOG<sub>1-125</sub>-immunized (‘MOG-sensitized’) rats (\(N=11\)) or the intraspinal injection of lyssolecithin (\(N=14\)). The former (focal myelitis) is an experimental autoimmune encephalomyelitis (EAE)-like disease resulting in both demyelination and axon loss (Kerschensteiner et al., 2004), while the latter (lysolecithin injection) has been reported to result in a predominantly demyelinating lesion with little axonal loss (limited to centre of lesion) (Blakemore et al., 1977).

**Focal myelitis**

Focal EAE/myelitis was induced in the dorsal column of rats as described (Kerschensteiner et al., 2004) with minor modifications. Briefly, 100 \(\mu\)l of 37.5 \(\mu\)g of MOG<sub>1-125</sub> (kind gift of Dr Sha Mi, Biogen-Idec) emulsified in a 1:1 mixture of PBS and incomplete Freund’s adjuvant (Pierce) was injected subcutaneously in the back of each rat near the base of the tail. Eighteen to twenty-two days after sensitization, rats were anaesthetized with avertin and the spinal cord was exposed by performing a dorsal laminectomy at T10-L1. A micropipette (Humangen, Charlottesville, VA) was used to inject 2 \(\mu\)l of PBS containing either a mixture of TNF-\(\alpha\) (500 ng; R&D systems) and IFN-\(\gamma\) (300 U; Peprotech, Rocky Hill, NJ) or, to induce a more destructive lesion, 200 ng of lipopolysaccharide (LPS; Sigma) into the right medial dorsal column at a depth of 0.7 mm and at a rate of 1 \(\mu\)l/min. Rats were sacrificed 9 to 21 days (MOG-sensitized rats) after the intraspinal injection. Control animals (\(N=11\)) received either no manipulation, were sensitized to MOG and intraspinally injected with PBS, or were not sensitized but injected intraspinally with cytokines or LPS only. While LPS injection alone (without MOG-immunization) does induce a focal demyelinating lesion, the pathological effects are much less severe and there can be extensive remyelination at day 21, which minimizes axonal loss (Felts et al., 2005). Thus, the LPS-injected animals (\(N=4\)) were excluded from group analyses comparing differences between models since they were the only subset of ‘controls’ that had recognizable pathology.

**Lyssolecithin**

Lyssolecithin (Sigma, St Louis, MO) (1.5 \(\mu\)l) was injected into the dorsal column of spinal cords of rats receiving laminectomy as described previously (Blakemore et al., 1977; Bambakidis et al., 2003). Rats were sacrificed at 4, 7, 14 and 21 days after lyssolecithin injection. Day 4 rats were excluded from group comparisons since at this early time point post-surgical
changes partly obfuscated pathology at the lesion site and statistical analysis confirmed this subset to be outliers from the rest of the Lysolecithin animals.

**Tissue collection for pathology and ex vivo MRI imaging**

The same rats used for MRI imaging were also used for pathological studies (electron microscopy, axon counts and histology). Rats were deeply anaesthetized and perfused through the heart with cold PBS followed by freshly prepared 4% paraformaldehyde. Brain and spinal cord tissues were immersed in the same fixative overnight at 4°C. After rinsing in PBS, the spinal cord was removed from the bone and used for ex vivo MRI imaging (described later). Following imaging, a small segment of tissue from T3 and the L1 areas were further fixed for 2 h at 4°C with 4% paraformaldehyde + 2.5% glutaraldehyde, post-fixed in 1% OsO4 and embedded in Epon812 resin for axon counts (1 µm toluidine blue-stained sections) and electron microscopy (see later). The area of the spinal cord that MRI imaging identified as the lesion epicentre and a region in the T2–T3 region (adjacent to the region used for axon counting and electron microscopy) was embedded in paraffin for immunohistochemistry.

**MRI**

*Ex vivo* imaging of formaldehyde-fixed spinal cords was performed using an 11.7 Tesla spectrometer and a Micro2.5 gradient system with a maximum gradient strength of 120 Gauss/cm and 30 mm inner diameter (Bruker Biospin, Billerica, MA, USA). Previous reports comparing *in vivo* and *ex vivo* diffusion measurements in animal brains suggest that fixation causes either no change or reduced diffusion coefficient (mean, $\lambda_1$ and $\lambda_2$), while preserving the FA (Sun et al., 2003; D’Arceuil et al., 2007). A 15-mm diameter birdcage coil was used as the radio frequency signal transmitter and receiver. To place the thoracic and lumbar segments in the sensitive region of the coil, we folded the rat spinal cords near T10 into a U shape and kept them in 10 mm diameter NMR tubes. The tubes were filled with fomblin (Fomblin Proflludropolyether, Ausimont, Thorafare, NJ, USA) to prevent dehydration. Images were acquired using a 3D multiple spin echo sequence, with an echo train length (ETL) of 6, a repetition time (TR) of 900 ms, two signal averages and echo times (TE) of $0.08 \text{ mm}$. The total imaging time was $0.1 \text{ mm}$. Images reconstructed from the six echoes were used to calculate tissue $T_2$ and later summed together to form one diffusion weighted image. Eight such diffusion-weighted images were acquired, with two images of minimum $b$ value ($50 \text{s/mm}^2$) and the rest with maximum $b$ value (circa $800 \text{s/mm}^2$) and diffusion sensitizing gradients were applied along six different orientations: [0, 0.707, 0], [0.707, 0, 0.707], [0, 0.707, 0.707], [−0.707, 0, 0], [0, 0, −0.707], [0, 0, 0.707]. High resolution $T_2$-weighted images were also acquired with a fast spin echo sequence (TE/TR = 50/900 ms, flip angle $\alpha = 40^\circ$) with a native imaging resolution of $0.08 \times 0.08 \text{ mm}$. The total imaging time was $\sim 20 \text{ h}$.

The diffusion tensor was calculated using a log-linear fitting method (Basser et al., 1994). Voxel by voxel, we computed the primary direction of water diffusion ($v_i$), which was assumed to be the average orientation of axons in a pixel, and axial diffusivity ($\lambda_1$) and radial diffusivity ($\lambda_2$), which measure the extents of water diffusion along ($\lambda_1$) and transverse ($\lambda_2$) to the axons. For the quantification of diffusion anisotropy, fractional anisotropy (FA) was used.

For each spinal cord, two regions of interest (ROIs) were manually defined within the left and right fasciculus gracilis (just on the right for Lysolecithin animals, which had unilateral lesions) at the level of T3 to match the regions where axon counting was performed. We then collected the mean FA, $T_2$, $\lambda_1$ and $\lambda_2$ from the two ROIs.

**Fibre tracking methods**

Axonal pathways in the dorsal column white matter were approximated by streamlines generated from the MRI data using our fibre tracking software (DTIstudio). With a FA threshold of 0.3 set to distinguish spinal cord white matter (FA > 0.3) from grey matter (FA < 0.3), streamlines were selected by manually placing two ROIs in rostral dorsal column and caudal dorsal column white matter. FA, $T_2$, $\lambda_1$ and $\lambda_2$ along the selected streamlines were collected and organized in rostral-to-caudal order. DTIstudio calculates all possible streamlines in the entire image volume. We selected streamlines that pass through the two ROIs simultaneously. This is equivalent to tracking initiated in both directions, i.e., A to B and B to A. We separated the images into lumbar and thoracic parts. For the lumbar spinal cord, we selected streamlines that passed through one ROI that defined the dorsal column at L1 or T10 and another ROI that defined the dorsal column near L5. For the thoracic spinal cord, we selected streamlines that passed through ROIs that defined the dorsal column at approximately T3 and T8. These streamlines formed a representation of axonal pathways in the dorsal columns that stem from the caudal spinal cord and run rostrally.

For spinal cords with lesions localized to one side of the spinal cord (spinal cords injected with lysolecithin), we further separated the lumbar streamlines that pass through the left dorsal column from the streamlines on the right. This operation was performed by introducing two ROIs immediately caudal to the lesion, one covering the left dorsal column and the other covering the right dorsal column. The side with lesion was defined as the lesion-side, while the other side as the control-side. Whether a streamline belongs to the lesion-side or the control-side depends on which ROIs it passes through.

To perform fibre tracking on an even more specific group of fibres for which healthy fibres are excluded from the streamlines within the group of fibres identified on the lesion-side, we used another ROI that defined the lesion as seen in the $T_2$-weighted image as another condition to select only streamlines that pass through the lesion. The values of FA, $T_2$, $\lambda_1$ and $\lambda_2$ along these selected streamline groups were collected and organized from the site of the lesion to T10 and T3 to T8.

**Co-registration of pathological regions of interest and MRI data**

Upon completion of MRI studies, the lesion epicentre and a region near T3 (the region collected for axon counting and electron microscopy studies) were marked by tying thread around the tissue (placement was confirmed by a ‘quick scan’).
using the dorsal roots as a guide. A cross-section of tissue was cut at these points and paraffin-embedded. Serial 10 μm sections were cut and mounted on to SuperfrostPlus slides. Sections from regular intervals were stained with LFB (see later) to ascertain the fine location of the lesion; once located, intervening serial sections were used for immunohistochemistry (see later). By keeping track of the number of 10 μm sections cut from the area marked after MRI, the histological sections were paired with sections from MRI images based on calculated distances from specific dorsal roots. Although some error is introduced by tissue shrinkage during fixation and paraffin processing, by blade slippage/misplacement during cutting of tissue before embedding, and by some tissue lost during block facing before cutting of paraffin sections, our data compares primarily differences between very distant structures in the cord (T3 and T10-L1 regions being 3.5–4.0 cm in rats of this age) and these errors we estimate would lead to no more than 2–3 mm of difference between the MRI image and the calculated position estimate would lead to no more than 2–3 mm of difference between the MRI image and the calculated position.

Pathology

Immunohistochemistry

Ten micron thick sections of paraffin-embedded tissues were deparaffinized with xylene and re-hydrated by serial dilutions in ethanol. The SMI-31 (phosphorylated neurofilament) and SMI-32 (hypophosphorylated neurofilament) epitopes were unmasked by boiling slides in 10 mM Tris (pH = 9.5) or 0.1 M sodium citrate, respectively, for 15 min. After cooling, endogenous peroxidase was quenched by incubating slides in 3% H2O2/PBS for 30 min. Following PBS washes, sections were blocked in 2% w/v casein in PBS. Primary antibody was applied overnight at 4°C (1:5000 for SMI31, 1:7500 for SMI32; both antibodies from Sternberger Monoclonals, Inc, Lutherville, MD, USA). The high dilution used for SMI-32 staining rarely stained axons in the white matter tracts of healthy animals but highlighted accumulations of non-phosphorylated neurofilament proteins, which are transported more rapidly than phosphorylated neurofilaments and accumulate in distal portions of damaged axons (Archer et al., 1994; Trapp et al., 1998). After washing, sections were incubated with secondary antibody for 1 h at room temperature (biotinylated goat anti-mouse IgG/IgM, 1:500; Chemicon, Temecula, CA). Following washes, avidin–biotin complex (Vector Laboratories, Burlingame, CA) was applied to sections and incubated at room temperature for 1 h. Slides were washed and immune complexes were visualized using 0.05% (w/v) 3,3′-diaminobenzidine (Sigma) + 0.03% H2O2 as the substrate. Sections were counterstained in Mayer’s haematoxylin, dehydrated and mounted with Vectamount (Vector Laboratories).

Luxol fast blue staining

Spinal cords sections (10 μm) were incubated in 0.1% LFB dissolved in 95% ethanol and 0.05% acetic acid at 60°C for 18 h. Stained sections were differentiated in 0.05% lithium carbonate and 70% ethanol. Sections were counterstained with eosinY dehydrated, and mounted with Vectamount.

Toluidine blue staining

After perfusion with 4% paraformaldehyde, spinal cords were fixed in 2% glutaraldehyde and 4% paraformaldehyde for 2 h, osmicated for 3 h (1% OsO4), dehydrated and embedded in plastic. Tissue was cut into 1 μm sections and stained with toluidine blue.

Axon quantification

Toluidine blue-stained axons from 1 μm plastic sections of spinal cord from T3 were counted within a defined region within the medial dorsal column (see Fig. 4D, inset). This region was defined by drawing a line from the posterior midline of the dorsal column to a distance of half of the length of the dorsal column from the posterior to the ventral edge. A second line was drawn perpendicular to the first in the mid dorsal column at a distance of one-fourth of the length from the midline to the lateral edge of the dorsal column. The angle for the third line was determined by aligning the central canal to the lateral end of the second line. Axons were only counted if they were completely surrounded by myelin and >1 μm in diameter; degenerating axons were not included in this count. In the same region defined for total (healthy) axon counts, as a separate quantity, degenerating axons were counted if the axoplasm was not visible and the myelin appeared to have rippled morphology and resembled a dark dense mass.

Pathological quantification

Digital images were acquired using a DP70 digital camera (Olympus) mounted on a BX41 Olympus microscope equipped with a 4× objective. Quantification of LFB- or SMI-31-stained areas was performed using ImageProPlus5.1 (Media Cybernetics, Silver Spring, MD, USA). The area of the whole dorsal column at T3 and the lesion was measured, followed by measuring the area of blue (LFB) or brown (SMI-31 immunoreactivity) only. Percent of the area stained was then calculated and used for correlative analyses.

Electron microscopy

Sixty to seventy-five nanometres Epon812-embedded spinal cord sections (see ‘Tissue collection for pathology and ex vivo MRI imaging’ section) were placed on to mesh copper grids and double stained with uranyl acetate and lead citrate. Sections were examined on a Hitachi H600 electron microscope for qualitative ultra-structural analysis of myelin and axons to confirm our pathological impressions from the SMI-31 and Toluidine blue stains.

Statistics

Pearson correlation coefficients were calculated to determine correlations between axons counts and radiological measures. Multiple linear regressions were done to determine the relative contributions of different variables as described in a recent similar study (Schmierer et al., 2007). Wilcoxon rank-sum test was used to compare groups since the histopathological data were not normally distributed. Statistical calculations were performed using Stata 9.0 software (StataCorp LP, College Station, TX).
**Results**

**Grouped histopathology and MR data from inflammatory and demyelinating models**

Although our initial intent in choosing the focal myelitis and Lysolecithin injury models was to examine differences between inflammatory lesions with demyelination and axonal injury as compared to pure demyelination, we observed a spectrum of different pathologies in both the models (Table 1). As expected the focal inflammatory model (N=11) had significantly reduced SMI-31 and LFB staining at the site of the lesion with concomitant significant changes in all DTI measures as compared to control animals (N=5). The focal demyelinating model (N=10) had significantly decreased LFB, and a trend to decreased SMI-31, which also resulted in significant changes by MRI at the site of the lesion as compared to controls. The data were skewed with a wide variety in pathologies in both models, but up to 86% of the focal injury animals (combined inflammatory and demyelinating) had MR signal changes beyond the extreme value of the control group.

**FA, $\lambda_{||}$ and $\lambda_{\perp}$ correlate with axon counts and degenerating axon counts within the medial dorsal column at spinal cord level T3**

We next examined the distal (T3 level) pathology in both models and found a significant reduction in total axon counts and degenerating axons in the focal myelitis model, but not in the demyelinating model, as compared to controls (Table 1). There were no significant changes in LFB or SMI-31 as compared to controls. The distribution of MR measures at T3 was also generally not significantly different than controls in these small samples. However, when we examined the relationship between the MR parameters and histopathology in all 34 rat spinal cords, both axon counts and SMI-31 staining were highly significantly correlated with FA and $\lambda_{||}$ and to a lesser extent $\lambda_{\perp}$ and even T2 signal in some cases (Table 2 and Fig. 1). There was no relationship between LFB staining and MR measures except a borderline significant relationship between LFB and $\lambda_{\perp}$ by fibre tracking, but not the ROI method. The ROI method provided better correlations than using fibre tracking, although the patterns were similar for both methods. The correlations of FA and $\lambda_{||}$ with axons and degenerating axons are explained by correlations of axons and degenerating axons with SMI-31 staining (multiple regression analysis). Thus disruption in SMI-31 staining, and not LFB, is the factor that drives the MRI at the T3 level.

**FA and T2-weighted images correspond to changes in myelin loss and changes in axon phosphorylation at the lesion site and distal to the lesion**

Figure 2 shows examples of pathology and MR images from one animal with a focal inflammatory lesion and one animal with a focal demyelinating lesion. The figure illustrates extreme examples of pathologies, but group data reveal a spectrum of severity as described earlier. No radiological signal abnormalities or pathologic changes were detected in control animals (no injection or cytokine only). LPS controls (no MOG) had the expected

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**Table I** Group comparison between animal models

<table>
<thead>
<tr>
<th>Lesion</th>
<th>Controls</th>
<th>Focal myelitis</th>
<th>Demyelinating</th>
<th>Focal myelitis + demyelinating</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMI-31 staining (%)</td>
<td>94 (5)</td>
<td>57** (11)</td>
<td>88 (8)</td>
<td>80** (19) [67%]</td>
</tr>
<tr>
<td>LFB staining (%)</td>
<td>97 (6)</td>
<td>48** (10)</td>
<td>89* (9)</td>
<td>78** (19) [57%]</td>
</tr>
<tr>
<td>Fractional anisotropy</td>
<td>0.9 (5)</td>
<td>0.7** (9)</td>
<td>0.59* (10)</td>
<td>0.65** (21) [86%]</td>
</tr>
<tr>
<td>Axial diffusivity ($\lambda_{</td>
<td></td>
<td>}$) (×10^-6 cm²/s)</td>
<td>5.4 (5)</td>
<td>3.7 (11)</td>
</tr>
<tr>
<td>Radial diffusivity ($\lambda_{\perp}$) (×10^-6 cm²/s)</td>
<td>0.48 (5)</td>
<td>0.9** (11)</td>
<td>1.2 (10)</td>
<td>1.0** (21) [81%]</td>
</tr>
<tr>
<td>T2 (ms)</td>
<td>38 (5)</td>
<td>45 (11)</td>
<td>45 (10)</td>
<td>45 (21) [81%]</td>
</tr>
</tbody>
</table>

Note: Wilcoxon rank-sum tests: *P<0.05, **P<0.01. Values given are medians in each class. Values in parentheses are the number of animals in each class. Values in brackets are the percentages of lysolecithin- or MOG-injected animals that had values of each index below (SMI-31, LFB, axons, fractional anisotropy, parallel diffusivity) or above (degenerating axons, perpendicular diffusivity, T2) the extreme value among controls. Excludes lysolecithin day 4. Control group includes no injection and cytokine-only injection. MOG group includes MOG + LPS and MOG + cytokine. MRI variables are measured with ROI approach, not fibre tracking.
demyelination and some low-level axonal injury as described previously (Felts et al., 2005). Fibre tracking in the control dorsal columns was routinely performed to establish normative data (Fig. 2A). Both T2-weighted signal change and FA were sensitive to detecting focal inflammatory or demyelinating lesions, and the corresponding histopathology revealed a loss of SMI-31 staining and concomitant loss in myelin staining at the lesion sites.

### Table 2

Pearson correlation coefficients comparing MRI variables to histopathology variables in the upper thoracic spinal cord

<table>
<thead>
<tr>
<th></th>
<th>Fractional anisotropy</th>
<th>Axial diffusivity ($\lambda_\perp$)</th>
<th>Radial diffusivity ($\lambda_\parallel$)</th>
<th>T2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axons</td>
<td>ROI</td>
<td>0.69****</td>
<td>0.65****</td>
<td>−0.51**</td>
</tr>
<tr>
<td></td>
<td>FT</td>
<td>0.59****</td>
<td>0.51****</td>
<td>−0.49**</td>
</tr>
<tr>
<td>Degenerating axons</td>
<td>ROI</td>
<td>−0.79****</td>
<td>−0.66****</td>
<td>0.59***</td>
</tr>
<tr>
<td></td>
<td>FT</td>
<td>−0.61****</td>
<td>−0.47****</td>
<td>0.48**</td>
</tr>
<tr>
<td>SMI-31 staining</td>
<td>ROI</td>
<td>0.62****</td>
<td>0.65****</td>
<td>−0.41*</td>
</tr>
<tr>
<td></td>
<td>FT</td>
<td>0.31</td>
<td>0.45*</td>
<td>−0.26</td>
</tr>
<tr>
<td>LFB staining</td>
<td>ROI</td>
<td>0.15</td>
<td>0.33</td>
<td>−0.25</td>
</tr>
<tr>
<td></td>
<td>FT</td>
<td>0.34</td>
<td>0.33</td>
<td>−0.39*</td>
</tr>
</tbody>
</table>

**Note:** *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001*. ROI, region-of-interest approach (medial posterior dorsal column at T3); FT, fibre-tracking approach (entire dorsal column); LFB, luxol fast blue.

**Fig. 1** Graphs showing axon counts from medial dorsal columns at spinal cord level T3 relative to (A, B) FA, (C, D) axial diffusivity $\lambda_\perp$, (E, F) radial diffusivity $\lambda_\parallel$, and (G, H) T2 imaging derived from (A, C, E, G) fibre tracking or (B, D, F, H) ROI corresponding to area used to count axons. (Focal myelitis model; n = 11, lysolecithin model; n = 14, controls; n = 9).
(Fig. 2B focal myelitis and Fig. 2C lysolecithin). The spinal cord depicted had an extremely destructive type focal myelitis lesion with areas of cystic tissue necrosis that had low signal on T2-weighted images. This interfered with fibre tracking and caused drop out of fibres that could be tracked distally (Fig. 2B). The lesions (as defined by abnormal T2 signal here) in the focal myelitis and lysolecithin models are shown by yellow fibres, while normal fibres appear in blue. The lysolecithin model results in a lesion on one side of the dorsal column and therefore fibre tracking from the side with the injection (red) can be compared to the contralateral side (blue).

Sections that were taken at T3, distal to the lesion area, showed SMI-31 and LFB abnormalities in the dorsal column tracts that passed through the sites of destructive lesions, but not in purely demyelinating lesions. While histopathological changes were generally not detected by SMI-31 or LFB distal to pure demyelinating lesions at T3, there were changes in the DTI signal in some of the animals.
DTI fibre tracking depicts changes compared to control values at the lesion site and distal to the lesion in focal models with extensive axon damage

Axial or parallel diffusivity ($\lambda_\parallel$) and radial or perpendicular diffusivity ($\lambda_\perp$) was quantified in the tracked dorsal columns for each focal model (Fig. 3). To determine the extent of axon damage distal to a focal lesion we first investigated how each DTI measure within the dorsal column compared at the site of the lesion origin and distal to the lesion within the same fibre tract (Fig. 3A and B). T2-weighted imaging is used to portray the lesion in the sagittal image for the focal myelitis and lysolecithin models.
Fig. 4 Photographs of Toluidine blue stained spinal cord sections (A–D), electron microscopy (E–J) and immunohistochemistry with SMI-32 (K, L) from rat spinal cord. Toluidine blue stained dorsal column from spinal cord sections (Mag: 100×, inset Mag: 10×) from MOG immunized rat injected with LPS, 21 dpi (A) at the lesion site and (B) distal to the lesion at T3 and from rat injected with lysolecithin, 21 dpi (C) at the lesion site and (D) distal to the lesion at T3. Arrows point to degenerating axons, (A,C) boxed areas in the lesion insets identify the location of the 100× regions, and (B,D) the polygons in the T3 insets identifies the region used for axon counting. Electron micrographs depict (E) a normal axon with intact myelin, Mag: 35 K, (F) an axon with an abnormally large number of mitochondria Mag: 17 K, (G) an unmyelinated axon, Mag: 15 K and (H) regions of remyelinating axons (yellow arrow heads) and degenerating axons (white arrows) from a dorsal column lesion in a MOG + LPS rat spinal cord and (I) a thinly myelinated axon (yellow arrow head) and unmyelinated axon (#) separated by gliosis (?) Mag: 25 K from a dorsal column lesion in lysolecithin-injected rat spinal cord and (J) degenerating axons Mag: 6 K, from a dorsal column rostral to a lesion in a lysolecithin-injected rat spinal cord. SMI-32 immunohistochemistry from spinal cord sections rostral to the injection site (Mag: 20×) from (K) a control rat injected with cytokine, without MOG immunization, (L) MOG immunized rat injected with LPS 21 dpi and (M) rat injected with lysolecithin 7 dpi. Arrows indicate areas with immunostaining.
(Fig. 3C and D). DTI measures in the focal myelitis model are compared to measures from a control animal receiving cytokine injection only, with no immunization. DTI measures in the lysolecithin model are compared to measures from the contralateral side of the injection site because the lesion generated from lysolecithin injection only affects one side of the dorsal column. FA and \( \lambda_\| \) decrease at the lesion and do not return to control values rostral to the lesion site in this case of focal myelitis as one can see in the images (Fig. 2 yellow arrows and Fig. 3A and C plots). In contrast, \( \lambda_\perp \) and T2 values increase at the lesion site but approach baseline values rostral to the lesion at T3 level (Fig. 3C).

In the illustrated case from a rat injured with lysolecithin, FA and \( \lambda_\parallel \) decrease, while \( \lambda_\perp \) and T2 increase at the lesion site, but each measure returns to baseline rostral to the lesion (Fig. 3D). For this example (Fig. 3D) two methods of fibre tracking are employed to derive DTI measures. The standard method is shown in red, for which all fibres within the dorsal column are included. Therefore, both damaged and healthy axons contribute to the average DTI values since the lesion is unilateral. In contrast to the standard method, a second method is used in which healthy axons have been removed from the fibre tracking set, which therefore specifically identifies the lesion and fibre tracts that have been demyelinated (yellow) and illustrates more dramatic changes at the lesion site, but essentially no signal change rostrally consistent with absence of distally detected histopathology in this animal (Fig. 3C).

Focal myelitis results in axonal loss and degenerating axons distal to the lesion site

To determine the extent of axon loss, spinal cord sections at T3 were stained with toluidine blue (Fig. 4) and axons were counted in the medial dorsal column. Axon counts revealed extensive axon loss and an increased number of degenerating axons in the focal myelitis model compared to uninjured control rats. Additionally, some axon loss and degenerating axons were also found in the demyelinating lysolecithin model. To compare the lesion site to the T3 spinal cord level, we examined toluidine blue-stained sections at the site of the lesion. In both models we saw loss of myelin surrounding axons, axonal loss and degenerating axons. To further evaluate the ultrastructural characteristics of the axonal and myelin damage, we used electron microscopy (EM). In addition to degenerating axons, EM revealed unmyelinated and thinly myelinated axons in the focal myelitis model, which suggests demyelinated axons and remyelinating axons, respectively. EM also revealed axons with abnormally large numbers of mitochondria, suggestive of impairment in axonal transport. Similarly, in the lysolecithin model degenerating axons, unmyelinated and thinly myelinated axons were also observed with EM (Fig. 4).

To further investigate the extent of axon damage, we performed immunohistochemistry with SMI-32 to detect accumulations of non-phosphorylated neurofilaments. Compared to control animals, at approximately the same spinal cord level for which the axon counting was performed, there was extensive SMI-32 staining distal to the lesion site in the focal myelitis model and only minimal to moderate staining in the lysolecithin model (Fig. 4). These data suggested that in both the lysolecithin-induced demyelinating model and the inflammatory-mediated demyelinating model, non-phosphorylated neurofilament accumulates distal to the initial injury site within the dorsal column, suggestive of axonal dysfunction and transport impairment (Archer et al., 1994; Trapp et al., 1998).

Discussion

In this report, high resolution DTI was found to detect not only pathology at a lesion site within rat spinal cord dorsal column, but also axonal loss and degenerating fibres within the ascending dorsal column fibre tracts related to the lesion. We show that using fibre tracking with DTI, FA, axial diffusivity (\( \lambda_\parallel \)) and to some extent even radial diffusivity (\( \lambda_\perp \)) correlate with axon counts distal to dorsal column focal lesions.

We first demonstrate in this report that high resolution DTI can detect rat focal dorsal column lesions that correspond to histology depicting a loss of myelin and loss of phosphorylated neurofilament (Fig. 2). By applying fibre tracking, DTI can be used to compare measures within an axon tract both caudal and rostral to the lesion site. Fibre tracking based on DTI can illustrate and quantify differences in pathology in focal lesion models that exhibit a range in the extent of axonal damage (Fig. 3). These data indicate that FA and \( \lambda_\parallel \) values do not return to baseline rostral to the lesion in the dorsal column of the majority of focal myelitis lesion model animals in which extensive axon damage occurs, but that the same measures may return to baseline rostral to the lesion in the lysolecithin-induced demyelinating model in cases where axonal damage is limited.

While fibre tracking allows for the detection of DTI measures in axons rostral and caudal to a lesion, there are limitations inherent in the method which, if overcome would increase the sensitivity of detecting axonal damage. For instance, while fibre tracking uses similar FA values to identity axons that run together, severely damaged axons which fall below a specified FA threshold are excluded from the measure, and information from areas of complete loss of axons is also lost. Therefore, complete axonal drop-out is depicted at the centre of a lesion, which has severe axonal loss (Fig. 2B). Furthermore, although fibre tracking derived \( \lambda_\perp \) and T2 measures approached baseline values distal to the lesion (Fig. 3C), the image (Fig. 2B) revealed some hyperintense T2-weighted signal even at T3. This difference between the images and the DTI measures can be explained
because the fibre tracking data do not include severely damaged axons and therefore depict values only for the outer edge of the visible lesion.

In addition to reducing the sensitivity to detect damaged axons by excluding severely damaged fibres, fibre tracking also includes normal axons, which dilutes the signal change. Figure 3D depicts DTI measures based on a standard fibre tracking method and a modified method, in which only fibres that pass through the lesion are included and therefore excludes normal fibres. The modified fibre tracking method results in a greater difference in T2 values at the lesion site relative to control, but has little effect on FA values. This suggests that T2 and FA detect different fibres within the lesion. This difference in detection is also suggested by differences in the shape of the hypointense and hyperintense regions, defined by FA and T2, respectively, shown in Fig. 2C.

Despite limitations, fibre tracking is a practical way to compare pathology within axon tracts distal to a specific lesion. Furthermore, even with limitations that decrease sensitivity to changes in DTI values, these data indicated that axon loss and degenerating axons correlated with FA, \( \lambda_\parallel \) and \( \lambda_\perp \) when using fibre tracking. However, because of the discussed limitations with fibre tracking we also determined if correlations exist between axon counts at T3 and DTI measures within a defined ROI, which was defined to match the area used to count axons. For this method, values from all damaged fibres, even those below the specified FA threshold were included. Using the ROI method, axon loss and degenerating axons correlated even better with DTI measures. Additionally, when using this method, T2 was also found to correlate with axon loss and axon degeneration as has been suggested in fibre bands emanating from large lesions in MS brain (Simon et al., 2000, 2006). Thus our data suggest that while T2 can detect axonal damage in severe lesions, it is less apparent to the eye in mild lesions and that DTI measures are more sensitive. Although fibre tracking has limitations, it is more practical than using ROI to compare axonal damage that occurs distal to lesions.

Recent efforts have been made to determine if T2 and \( \lambda_\parallel \) define different pathology compared to FA and \( \lambda_\parallel \). Diffusion in the giant axon of squid was measured with MRI and it was suggested that axon membranes and myelin play a major role in limiting water diffusion but that neurofilaments do not (Beaulieu and Allen, 1994). More recently, it has been suggested that \( \lambda_\parallel \) is indicative of axon damage and \( \lambda_\perp \) is indicative of demyelination (Song et al., 2003, 2005; Kim et al., 2006). While data presented in this report suggest that \( \lambda_\parallel \) and \( \lambda_\perp \) detect differences in pathology in white matter, these data also suggest that axonal changes often accompany demyelination, and the two are difficult to separate as was recently shown in MS brain (Schmierer et al., 2007). Indeed, it is well known that demyelinated axons have loss of phosphorylated NF and that accumulations of non-phosphorylated NF indicate impaired transport in injured axons (Archer et al., 1994; Trapp et al., 1998; Peterson and Trapp, 2005). The lyssolecithin-induced demyelinating lesion caused loss of myelin accompanied by secondary axonal changes including loss of phosphorylated NF at the lesion site (Fig. 2C). Our results differ in some respects from data reported in the cuprizone model in that the lyssolecithin-induced demyelinating lesion caused changes in both \( \lambda_\parallel \) and \( \lambda_\perp \) (Song et al., 2005). This would be expected since there is both increased diffusion radially due to loss of the myelin membrane and decreased diffusion axially consistent with secondarily impaired axon transport and damage.

Distal to the lesion site in the focal myelitis model, extensive accumulations of non-phosphorylated NF were detected, suggestive of a decrease in axonal transport, which corresponds with a decrease in FA and \( \lambda_\parallel \) rostral to the lesion as well. These data are consistent with the possibility that accumulations of NF as well as other transported proteins could play a role in restricting water diffusion in the axial plane at great distances from damaged axons (Harsan et al., 2006); however, this should be confirmed in quantitative studies of SMI-32 and other relevant proteins. Secondary changes in radial diffusion may also occur as a result of ‘inside out’ myelin loss as has been described previously (Tsunoda and Fujinami, 2002).

While data in this report focus on high resolution DTI in rat spinal cord, advances have been made in MRI technology which allow for high resolution imaging in humans (Jeong et al., 2005; Papanikolaou et al., 2006; Smith et al., 2006). Fibre tracking based on DTI has been used to reconstruct the corticospinal tract in brain and detect abnormalities in relapsing-remitting MS (Reich et al., 2006) which has been an important contribution to enable detection and monitoring of MS lesions. Therefore, the data presented in this report are important to making advances in understanding and treating MS because of the need to better detect lesions and monitor tissue-specific pathological progression. It is likely that axons initially spared at a site of acute demyelination are more susceptible to secondary degeneration at a later time through increased exposure to toxic mediators as well as loss of trophic support. This may explain axon degeneration that occurs in progressive MS patients that do not have subsequent attacks (Arnold, 2005). Data presented in this report support the notion that high resolution DTI can detect axonal damage that occurs distal to an initial lesion. While the mechanisms of axonal degeneration temporally and spatially distinct from a lesion site are incompletely defined, these data suggest that high resolution DTI may be a useful method to study this process.

A limitation to the data presented in this report is that all data was collected \textit{ex vivo}. It will be important to extend this work in future studies to include \textit{in vivo} imaging. Additionally, while fibre tracking has limitations in that fibres are excluded that are severely damaged, it is still an occlusive.
efficient method to derive meaningful data distant to a lesion site. Developing an animal model that allows the separation of demyelination from axonal damage is challenging if not impossible due to the dynamic interactive relationship between the myelin membrane and axolemma. Nonetheless, concomitant changes in myelin and axons also occur in MS and thus understanding the relative contributions of each primary pathology to the radiological measures will further our ability to monitor disease in MS.

In conclusion, these data demonstrate the sensitivity of high resolution DTI in detecting a variety of pathologies in the spinal cord that are relevant to MS. DTI measures are more sensitive than conventional T2 measures in detecting axonal pathology distant to the site of injury. FA, $\lambda_{\parallel}$ and $\lambda_{\perp}$, and to a lesser extent, T2 significantly correlate with axon degeneration and loss. These data therefore suggest that high resolution DTI can be used to detect axonal damage, and may be most useful in detecting pathology in distant non-inflammatory sites.

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References


Basser PJ. Inferring microstructural features and the physiological state of tissues from diffusion-weighted images. NMR Biomed 1995; 8: 333–44.


