Title
Longitudinal assessment of white matter pathology in the injured mouse spinal cord through ultra-high field (16.4T) in vivo diffusion tensor imaging

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Abstract

This study examined the sensitivity of ultra-high field (16.4T) diffusion tensor imaging (DTI; 70μm in-plane resolution, 1mm slice thickness) to evaluate the spatiotemporal development of severe mid-thoracic contusive spinal cord injury (SCI) in mice. *In vivo* imaging was performed prior to SCI, then again at 2 hours, 1 day, 3 days, 7 days, and 30 days post-SCI using a Bruker 16.4T small animal nuclear magnetic resonance spectrometer. Cross-sectional spinal cord areas were measured in transverse slices and various DTI parameters, i.e. fractional anisotropy (FA), mean diffusivity (MD), axial diffusivity (λ∥) and radial diffusivity (λ⊥), were calculated for total spared white matter (WM), ventral funiculi (VF), lateral funiculi (LF) and the dorsal columns (DC). Cross-sectional area measurements revealed significant atrophy (32% reduction) of the injured spinal cord at the lesion epicentre in the chronic phase of injury. Analysis of diffusion tensor parameters further showed that tissue integrity was most severely affected in the DC, i.e. the site of immediate impact, which demonstrated a rapid and persistent decrease in FA and λ∥. In contrast, DTI parameters for the ventrolateral white matter changed more gradually with time, suggesting that these regions are undergoing more delayed degeneration in a manner that may be amenable to therapeutic intervention. Of all the DTI parameters, λ⊥ was most closely correlated to myelin content whereas changes in FA and λ∥ appeared more indicative of axonal integrity, Wallerian degeneration and associated presence of macrophages. We conclude that longitudinal DTI at 16.4T provides a clinically relevant, objective measure for assessing white matter pathology following contusive SCI in mice that may aid the translation of putative neuroprotective strategies into the clinic.
Keywords
Magnetic resonance imaging, neurotrauma, spinal cord injury, tractography, demyelination, Wallerian degeneration.

Abbreviations
Axial diffusivity ($\lambda_\parallel$), diffusion tensor imaging (DTI), dorsal columns (DCs), fractional anisotropy (FA), lateral funiculi (LF), magnetic resonance imaging (MRI), mean diffusivity (MD), radial diffusivity ($\lambda_\perp$), spinal cord injury (SCI), Tesla (T), ventral funiculi (VF), white matter (WM)
1.0 Introduction

The adult mammalian spinal cord has very limited inherent capacity for repair. Neurotraumatic events that affect this part of the central nervous system (CNS) thus lead to permanent motor, sensory and autonomic impairments, with the degree of physical debilitation determined by the anatomical level and severity the spinal cord injury (SCI) (Bunge et al., 1993; Gonzalez et al., 2003; Park et al., 2004). CNS neurons that survived the initial mechanical insult become increasingly exposed during the acute and post-acute phase to an ischemic and toxic microenvironment that includes increased levels of excitatory amino acids and ions (excitotoxicity), reactive oxygen species (oxidative stress) and potent pro-inflammatory mediators. This further compounds damage by propagating the loss of neural beyond the initial impact site, a process collectively known as secondary injury (Dumont et al., 2001; Donnelly et al., 2008).

As most SCIs are anatomically incomplete contusion injuries, involving crushed or fractured vertebrae that bruise and/or compress the spinal cord (Bunge et al., 1993; Norenberg et al., 2004), the process of secondary injury is highly amenable to therapeutic intervention, and several putative therapies targeting these sequelae are currently being developed in animal models of SCI or undergoing clinical evaluation (Hawryluk et al., 2008; Kwon et al., 2010; Cadotte et al., 2011; Gensel et al., 2011). The simultaneous development of non-invasive imaging methods to quantitatively assess the effectiveness of such therapeutic interventions will be an important step for successful translation of candidate therapies from the laboratory into the clinic. In particular, the incorporation of advanced magnetic resonance imaging (MRI) techniques in preclinical studies offers an objective and independent accompaniment that could be used alongside functional testing. Whilst informative, functional improvements seen in animal models, particularly rodents, may not be directly transferable to the human context because of differences in anatomical
organization of the spinal cord, and the amount of supraspinal control that is required for quadrupedal versus bipedal locomotion (Courtine et al., 2007); MRI is not hampered by these limitations.

In the present study, we used diffusion tensor imaging (DTI) to chronologically assess SCI pathology in live mice at a microscopic scale (Le Bihan et al., 2001). In uninjured spinal white matter, water molecules predominantly diffuse along the longitudinal plane (the ‘axial diffusivity’ \( \lambda || \)). Conversely, the Brownian motion at right angles to the direction of white matter tracts (the ‘radial diffusivity’ \( \lambda \perp \)) is more restricted by both axonal membranes and myelin sheaths (Stroman et al., 2012). The overall degree of directionality (anisotropy) of water diffusion in tissues can be measured as fractional anisotropy (FA) (Beaulieu et al., 1994). Tissues with a high degree of diffusion directionality, such as spinal white matter, are anisotropic and have FA values of ~0.8 (Underwood et al., 2011). Conversely, more isotropic tissues in which water diffusion is much less restricted, e.g. grey matter or cerebrospinal fluid (CSF), have an FA value of ~0.2 or lower (Sundgren et al., 2004; Mori et al., 2006). The mean diffusivity (MD) provides an index of the overall magnitude of water molecular diffusion independent of direction. Combined, these quantitative parameters enable the detection of subtle and/or progressive changes in the integrity of spinal white matter as a result of pathology that cannot be detected through conventional MRI techniques (Huisman et al., 2001; Schwartz et al., 2003; Hesseltine et al., 2006; Shanmuganathan et al., 2008; Yin et al., 2010; Rajasekaran et al., 2010; Chang et al., 2010; Mulcahey et al., 2011). Indeed, DTI measures have statistically stronger correlations with clinical examination data (Chang et al., 2010; Mulcahey et al., 2011). A previous report by Kim et al. (2005) showed that DTI at a field strength of 4.7 Tesla (T) can also reveal and quantify developing pathology in mice. In the present study, we used ultra-high field (16.4T) DTI to longitudinally assess gross pathology and region-specific changes in the various diffusion parameters for up to 30 days
post-SCI, which was then followed by a detailed assessment of the histopathological correlate.

2.0 Methods

2.1 Animals

All experiments were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, with approval from The University of Queensland Animal Ethics Committee. Female C57BL6/J mice (n=4) were used in this study; animals were 12-16 weeks of age at the time of the pre-injury scan. Experimental mice were housed individually under conventional conditions, with a 12 hour light-dark cycle and ad libitum access to food and water.

2.2 Experimental Spinal Cord Injury

Mice were anaesthetised with xylazine (10 mg/kg i.p.) and zolazepam (50 mg/kg i.p.) prior to undergoing surgical laminectomy and severe (70 kdyne) contusive SCI. This involved making a dorsal incision along the midline of the lower thoracic region. Next, the underlying paravertebral muscles were carefully split and dorsal laminectomy performed at thoracic (T) vertebral level T9 to expose the animal’s intact dura overlying the spinal cord. The vertebral column was stabilized by rostral and caudal placement of Adson microforceps clamps, after which the mice were subjected to severe force-controlled contusion SCI using the Infinite Horizon Impactor with 1.3 mm diameter probe (Precision Systems and Instrumentation, Fairfax Station, VA, USA) as described previously (Scheff et al., 2003). The average applied force was 71.3±1.03 kdyne (mean ± SEM) and the associated tissue
displacement was 608±51.1 μm (mean ± SEM). Following impact, muscles and skin were sutured closed. Post-operative care involved a single dose of buprenorphine (0.05 mg/kg) diluted in 1ml of Hartmann’s solution, gentamicin (1 mg/kg) daily for 5 days post-injury, and twice-daily bladder expression for the duration of the experiment.

2.3 In vivo DTI

All data was collected at the Centre for Advanced Imaging (CAI, The University of Queensland, Australia) using a 16.4T nuclear magnetic resonance scanner with a 89 mm vertical bore magnet (Bruker BioSpin, Karlsruhe, Germany) and a transmit/receive linear surface coil (1.5 x 3.0 cm). Gradients were maintained at 30°C. Animals were anaesthetised using an isoflurane/oxygen mixture (0.5-1.5%). Animal respiration rate was monitored using a Biotrig Animal Monitoring System (M2M imaging, Brisbane, Australia). Mice heads were held in position via a tooth bar and nose/head cone; they were supported from underneath by a foam pad (see Supp. Fig. 1A). In this position, their dorsum was situated parallel to the coil. For each imaging session, the hypo-intense lesion core was identified and centred in pilot axial, sagittal and horizontal MR images that were acquired with the b-value set to ‘0’ (b₀; see Suppl. Fig. 1B). Slice angles were always set perpendicular to the long axis of the spinal cord. Vertebral landmarks (Harrison et al., 2013) were also used to further identify the relative position of the lesion core and to confirm that laminectomy was indeed performed at the level of T9. Images were acquired using respiratory gating, a DTI spin-echo sequence with interleaved slice acquisition using TR/TE=2400/21ms. Diffusion sensitising gradients were applied in 12 non-coplanar, uniformly distributed directions with the gradient strength b=1500s/mm², 2ms diffusion encoding and 14ms diffusion separation and 1 b₀ image. The acquisition matrix was 128x170 over the field of view 9x12mm to produce final in-plane resolution 70x70 μm, with 1.0 mm slice thickness. The number of excitations and the partial
Fourier encoding acceleration were both 2, and the total acquisition time was 2 hours. Ten transverse images covering part of the thoracic and lumbar spine were acquired as previously reported (Underwood et al., 2011). Live imaging was performed prior to injury, then again at 2 hours, and 1, 3, 7 and 30 days after injury.

The diffusion tensor eigenvalues ($\lambda_1$, $\lambda_2$, $\lambda_3$) were computed using the Paravision 5.0 (Bruker Biospin, Karlsruhe, Germany) diffusion tensor calculation module. The axial diffusivity ($\lambda_||$) is a measure of the diffusivity along the principal axis of the diffusion tensor ($\lambda_1=\lambda_||$). The radial diffusivity ($\lambda_\perp$) was calculated using the two minor diffusion axes ($\lambda_\perp=(\lambda_2+\lambda_3)/2$). The fractional anisotropy (FA) was calculated using the equation: $FA=\sqrt{1/2(\sqrt{(\lambda_1 - \lambda_2)^2+(\lambda_1 - \lambda_3)^2+(\lambda_2 - \lambda_3)^2})}/\sqrt{\lambda_1^2+\lambda_2^2+\lambda_3^2})$.

Paravision 5.0 (Bruker Biospin) was used for all image analysis. The cross-sectional areas of the spinal cord was defined by manually outlining the circumference of the spinal cord on transverse b0 and FA scans for each time point. Regions of interest (ROI), specifically total spared white matter (WM), the ventral funiculi (VF), lateral funiculi (LF) and dorsal columns (DCs), were manually outlined on transverse DTI FA images as shown in Suppl. Fig. 2.

2.4 Tissue preparation for ex vivo DTI

At 30 days post-injury, animals were deeply anaesthetised with sodium pentobarbital (150 mg/kg i.p.) and transcardially perfused with 20 ml of 0.9% saline solution containing 0.1% heparin and 2% NaNO2, followed by 30ml fixative (4% paraformaldehyde in phosphate-buffered saline (PBS: 1.7 mM NaH2PO4, 9 mM Na2HPO4, 0.15 M NaCl, pH 7.4). The vertebral column was dissected out and submerged in fixative for 48 hours at 4°C. Next, samples were washed in PBS containing gadolinium contrast agent over 2 weeks (0.2% Magnevist, Bayer).
2.5 *Ex vivo DTI and tractography*

Fixed specimens of the lower thoracic vertebral spine were trimmed in such a way that they fitted tightly into a glass tube and immersed in fomblin oil. The tube was placed inside a 15mm SAW linear coil (M2M Imaging). Spinal cords were imaged using a 3D DTI spin echo sequence with the parameters: TR/TE = 400/20.8 ms, 2.5 ms diffusion encoding and 10 ms diffusion separating, 12 non-collinear directions, 2 b₀ images, and the gradient strength b=3000 s/mm². The data was acquired with 1 excitation and 1.2 phase encoding acceleration factor. The sweep width was 50 KHz over a field of view of 3.07 x 0.48 x 0.54 mm with 512 x 80 x 90 matrix to produce 60μm isotropic resolution. The total scan time was 12 hours and 40 mins. Diffusion data were processed using the DTI module of Diffusion Toolkit software (v. 0.6.2.1).

Fibertracks were visualised using Trackvis (v. 0.5.2.1) by drawing ROIs (as shown in Suppl. Fig. 2) on transverse slices (*ex vivo* b₀ maps), 1mm rostral and caudal to the lesion epicentre; FA and angle thresholds were 0.1 and 45 degrees, respectively. A three-dimensional reconstruction of the lesion site was produced by manually outlining the hypo-intense regions of the lesion core on transverse and sagittal b₀ slices in the same program.

2.6 *Tissue preparation for histological analysis*

Following the conclusion of imaging, spinal cords were dissected out of the vertebral column and washed extensively in PBS. Tissue specimens were cryoprotected through subsequent overnight incubations in PBS containing 10% and 30% sucrose, respectively. Spinal cords were then snap-frozen in dry ice-cooled isopentane and stored at -80°C until further processing. Samples were cut into 20 μm thick transverse sections using a Leica
cryostat CM3050-S and collected in 1:5 series on Superfrost Plus slides (Menzel- Gläser). Slides were air-dried for 2 hours and stored at -80°C until further processing.

2.7 Assessment and quantification of histopathology

For quantification of myelin content, spinal cord sections were stained with FluoroMyelin™ Red (1:150; Molecular Probes, Invitrogen Detection Technologies) in combination with immunofluorescent labelling of astrocytes (rabbit anti-GFAP, 1:1000; Dako) as described previously (Blomster et al., 2012). Images were captured using Olympus SZX12 Research Fluorescence Stereo Microscope (Spectra Services) with NIS-Elements 3.0 software (Nikon Instruments Inc.) and analysed using the Java-based program ImageJ v1.43. The total section area and myelin-containing regions in spared white matter were outlined using the freehand selection tool. The image was converted to an 8-bit file and the level of FluoroMyelin signal in the white matter determined using the threshold function. Myelin content was calculated by dividing the FluoroMyelin-positive area by the total area of the section. Similar procedures were followed for the analysis of GFAP staining in the various ROIs. A separate set of sections was stained for neurofilament (NF200) (rabbit anti-NF200, 1:200; Sigma-Aldrich) in combination with FluoroMyelin™ Red and Hoechst nuclear dye (1:1000; Sigma-Aldrich). A third set of sections from each animal was stained for ionised calcium binding adaptor protein 1 (Iba1) (rabbit anti-Iba1, 1:500; Wako) and CD68 (rat anti-CD68, 1:200, Serotec), to visualise activated microglia/macrophages, and Hoechst nuclear dye. Stained sections were captured using an Axio Imager Azure (Zeiss). Immunopositive areas for NF200 and Iba1 were measured through thresholding in ImageJ for the relevant ROIs, i.e. the DCs and ventrolateral white matter.
2.8 Statistical analysis

Data was analysed using GraphPad Prism 4.0 software (GraphPad Software Inc., La Jolla, CA, USA). Two-way ANOVA with Bonferroni post-hoc was used to analyse the changes in cross-sectional area of the spinal cord after injury, with the ‘within group factor’ being the slice number and the ‘between group factor’ being time. One-way ANOVA with Newman-Keuls post-hoc analysis was used to compare lesion core data with time. Two-way ANOVA with Bonferroni post-hoc was used to analyse DTI parameters along the injured spinal cord for detection of Wallerian degeneration. Datasets were considered to be significantly different at p<0.05. All experimental data is presented as mean ± standard error of the mean (SEM).

3.0 Results

3.1 A reduction in spinal cord area in the chronic phase of injury indicates tissue atrophy

Longitudinal assessment of lesion development showed shrinkage of cross-sectional area (atrophy) at 30 days post-injury. Compared to pre-SCI, significant decreases in cord area were observed for up to 2 mm away from the lesion epicentre at the end point (Fig. 1A, B).

The mean cross-sectional areas of the spinal cord, at and around the lesion epicentre, were slightly higher in the acute period (2 hours post-SCI) compared to pre-injury data, suggesting some minor tissue swelling but this did not reach statistical significance. Nonetheless, the MD of identified white matter (WM) was measured to determine the possible effect of oedema on injured spinal cord anatomy (Fig. 1C). For total WM, a significant reduction in MD was observed at the 2 hour, 1 day, 3 day and 7 day time points compared to pre-injury measurements, indicating the possible presence of some intracellular
and axonal oedema. At 30 days post-injury, MD values were similar to pre-injury values, which would be consistent with the view that tissue oedema should have largely resolved in the more chronic phase of SCI (Norenberg et al., 2004). Regional analysis showed that the decline in MD in the acute phase was most pronounced in the more dorsal aspects of the spinal cord, that is, the immediate site of impact. No significant change in MD was observed for other specific ROIs, i.e. the ventral funiculi (VF) and lateral funiculi (LF) of the spinal cord, although values were generally lower during the first week post-SCI (data not shown). We must note, however, that the changes in MD appeared to be mostly driven by a reduced $\lambda_\parallel$ and no clear reductions in $\lambda_\perp$ were observed for the various ROIs during the acute phase (see also below), which thus appears to argue against a significant presence of cytotoxic oedema in the various white matter regions analysed.

### 3.2 FA values at the lesion epicentre progressively decrease with time

Fractional anisotropy (FA) values were calculated from diffusion tensor datasets for the total WM, VF, LF and the DCs as outlined in Supplementary Figure 2. A progressive decrease in FA was observed at the lesion epicentre as time progressed for total WM, VF and LF following contusive SCI (Fig. 2). To best illustrate the progressive deterioration of FA values over time, we compared all earlier data points against 30 days post-SCI. FA values for each of the aforementioned regions were indeed significantly higher before injury as well as at 2 hours, 1 day and 3 days after injury as compared to the experimental endpoint of 30 days post-SCI (Table 1). The DCs, on the other hand, showed the most dramatic and rapid decline in FA over time. Specifically, although the FA in this region was still significantly higher before SCI and 2 hours after injury compared to endpoint values, values were already maximally diminished at 1 day post-injury for this ROI. Hence, there was no significant difference between the FA measured at 1, 3 and 7 days post-SCI compared to 30 days post-
injury for the DCs (Table 1 and Fig. 2D). These findings suggest that the VF and LF undergo more gradual degeneration and/or tissue remodelling after injury, which may be amenable for therapeutic intervention. In contrast, the rapid changes in diffusivity parameters for the DCs, i.e. within the first day of contusive SCI, indicates severe and likely irreversible damage.

3.3 SCI-induced changes in $\lambda_\parallel$ and $\lambda_\perp$ at the lesion epicentre

To gain a better understanding of what factors might be driving the progressive decline in FA values after SCI, changes in diffusion eigenvalues were subsequently examined. Overall, the axial diffusivity $\lambda_\parallel$, generally considered an indicator for axonal integrity (Kim et al., 2006; Budde et al., 2009), showed a profound and significant decline (~40%; $p<0.05$) for identified WM at 2 hours post-injury, with no obvious change thereafter (Fig. 3A and Table 1). Region-specific analysis showed that the $\lambda_\parallel$ was also significantly decreased for each of the respective ROIs, i.e. VF, LF and DC, at 2 hours post-SCI compared to pre-injury baseline (Fig. 3B-D and Table 1). This decline was most marked for the DCs ($p<0.001$), followed by the VF and LF ($p<0.01$). The $\lambda_\parallel$ remained significantly below pre-injury values at all other time points analysed, i.e. up until 30 days post-SCI for each ROI ($p<0.01$). Together, these findings indicate that DTI is able to detect acute axonal damage as a result of SCI. However, since the $\lambda_\parallel$ did not significantly change further as a function of post-injury time, the chosen imaging protocol appeared not sensitive enough to detect the more subtle loss of axons, if occurring, as part of secondary degeneration. All data points were therefore also compared to pre-injury measurements for this parameter.

In contrast to $\lambda_\parallel$, the radial diffusivity $\lambda_\perp$, generally considered to be an indicator of myelination status (Song et al., 2002; Song et al., 2005) and possibly gliosis (Harsan et al., 2007), progressively increased as a function of time at the lesion epicentre (Fig. 4 and Table 1). As a result, this parameter was significantly higher at 30 days post-SCI for each ROI.
compared to all previous time points, including pre-injury (p<0.05). Statistical comparisons here were again made against the endpoint, i.e. 30 days post-SCI, to best illustrate the progressive increase in $\lambda_\perp$ over time. Subsequent post-mortem histological analysis confirmed decreased myelin content as well as astrogliosis at and in close proximity to the lesion epicentre (Fig. 5). The loss of myelin corresponded well with the drop in FA values in WM at the lesion site (Fig. 5A) while an inverse relationship was observed between myelin content and $\lambda_\perp$ as anticipated (Fig. 5B). GFAP immunoreactivity, used as an indicator of astrogliosis, was prominently elevated at the lesion epicentre, in close correspondence with transverse slices that showed the lowest FA values (Fig. 5C).

3.4 Distal changes in FA and $\lambda_\parallel$ as indicators of Wallerian degeneration

Region-specific analysis of FA along the injured spinal cord segment showed a significant rostrocaudal asymmetry, which was most pronounced for the DC area (Suppl. Fig. 3). Here, FA values returned to normal levels at a distance of ~3 mm caudal to the lesion epicentre. Rostral to the injury site, however, FA values remained significantly below baseline for a distance of at least up to 5mm (Suppl. Fig. 3A; p<0.05). We theorised that this rostrocaudal asymmetry may have resulted from Wallerian degeneration as this region mostly contains ascending sensory fibres. Therefore, severed axons that originated from dorsal root ganglia below the level of the spinal injury would no longer be connected to the neuronal cell body and thus undergo degeneration. Consistent with this hypothesis, a similar degree of asymmetry was observed for $\lambda_\parallel$ in the DCs (Suppl. Fig. 3D; p<0.01). The opposite was observed for the ventrolateral white matter where descending tracts dominate. Here, FA values were similar to pre-injury baseline measurements at ≥2mm rostral but they did not return to normal for up to 4mm caudal to the lesion epicentre (Suppl. Fig. 3B, C; p<0.05).
loss of axonal integrity, resulting from degeneration of the distal portion of severed axons,
appeared again evident based on a significantly decreased $\lambda_\parallel$ for at least 5mm caudal to the
lesion epicentre (Suppl. Fig. 3E, F; p<0.05).

A comparison with the relative density of neurofilament staining confirmed that the
observed rostrocaudal asymmetry for the various ROIs was indeed reflective of Wallerian
degeneration (Fig. 6). Specifically, the pattern of axonal loss for the DCs was most
pronounced at and rostral to the lesion epicentre (Fig. 6A, D-F), closely correlating with the
observed rostrocaudal asymmetry in FA (Fig. 6A) and $\lambda_\parallel$ (Fig. 6B). For ventrolateral white
matter, rostrocaudal asymmetry in the relative density of neurofilament staining again closely
matched the pathology detected by DTI, with reduced FA values detected for up to 4mm
caudal to the injury site (Fig. 6C, D-F). Immunofluorescent staining for Iba1 and CD68
confirmed a prominent presence of macrophages at and around the lesion epicentre as
expected (Fig. 7). However, a clear rostrocaudal asymmetry was again observed in identified
ROIs undergoing Wallerian degeneration, with Iba1 staining being strongest in more distant
areas where the greatest degree of axonal loss was observed based on neurofilament staining
(compare with Fig. 6). Specifically, in the DC, the immunoreactive area for Iba1 was greatest
close to the lesion epicentre, as expected. However, a significantly greater presence of
macrophages was observed in rostral direction, for up to at least 5 mm away from the lesion
epicentre as compared to caudal (Fig. 7E-H; p<0.05). In general, macrophage presence
correlated inversely with FA (Fig. 7A) and $\lambda_\parallel$ (Fig. 7B). For the ventrolateral white matter,
the Iba1 immunoreactive area was greater caudal to the lesion site compared to rostral as
would be anticipated based on the pattern of Wallerian degeneration and associated presence
of macrophages (Fig. 7E-H). The observed asymmetry here again correlated inversely with
FA data (Fig. 7C) and $\lambda_\parallel$ values (Fig. 7D). The varying degree of damage to ascending and
descending pathways in the DCs, VF and LF, along with the change in various DTI
parameters, was further confirmed and visualised through tractography (Fig. 8). Of note, the absolute values for $\lambda$, have changed here compared to the preceding \textit{in vivo} measurements due to tissue fixation.

4.0 Discussion

Conventional MRI is the standard method for evaluating human SCI (Bozzo et al., 2011). MRI has also proved a valuable tool in various animal models of SCI, including dogs (Boekhoff et al., 2012), cats (Takahashi et al., 1996), rats (Kozlowski et al., 2008; Sandner et al., 2009; Martirosyan et al., 2010; Scholtes et al., 2011) and mice (Gonzalez-Lara et al., 2009; Tatar et al., 2009), to identify tissue compression, oedema, haemorrhage and scarring (O'Beirne et al., 1993; Mhuircheartaigh et al., 2006; Blomster et al., 2012). We demonstrate here that macroscopic assessment of longitudinal MRI data can also reveal the progression of atrophy in mice, with a significant tissue shrinkage detected between 7 and 30 days post-SCI. These observations are consistent with findings in human patients where spinal cord atrophy also occurs (Freund et al., 2011).

Although conventional MRI allows for macroscopic assessment of pathology, it is not normally an accurate predictor of functional outcomes as this technique does not provide quantitative information on the overall integrity and/or partial sparing of white matter tracts (Sundgren et al., 2004). Such information can, however, be obtained through DTI, which indeed provides a better correlation with clinical outcomes in SCI patients (Chang et al., 2010; Mulcahey et al., 2011). Yet, this imaging technique is not commonly applied to animal models of SCI, particularly mice. Technical challenges posed by the small size of the spinal cord, along with physiological motion and susceptibility artefacts, can make the
acquisition of high-quality images difficult (Cohen-Adad et al., 2008). Despite these obstacles, longitudinal in vivo DTI has the potential to considerably improve our understanding of secondary SCI pathology and to also identify potentially salvageable tissue through repeated imaging of the same animal. We show here that longitudinal assessment of specific DTI parameters reveals several key abnormalities following SCI in mice.

MD values transiently declined during the acute period but returned to control levels at 30 days post-SCI. This change is not likely the result of vasogenic oedema, i.e. leakage of plasma fluid into the extracellular space due to blood-spinal cord barrier dysfunction, as this would normally lead to an increased MD. Qualitative inspection of b₀ (T₂-weighted) images post-injury also did not reveal any obvious signs of fluid accumulation (hyperintensity) for the ROIs. Our experimental data also suggest minimal presence of cytotoxic oedema for the various white matter ROIs. After CNS injury, a variety of pathophysiological changes, including ischemia, energy depletion, excitotoxicity and/or intracellular Ca²⁺ accumulation, can all contribute to a decreased extracellular and increased intracellular fluid content. The resulting cell swelling (i.e. cytotoxic oedema) has been associated with a decreased MD, particularly in grey matter (Loubinoux et al., 1997; Ellingson et al., 2008; for review, see Maas and Mukherjee, 2005). However, while a rapid decline in λ∥ was observed along with the drop in MD, we did not see a concomitant reduction in λ⊥, which thus appears to argue against this type of oedema being prominently present.

A decrease in FA values at the lesion core became increasingly apparent over time for all ROIs. These findings are in line with clinical studies, which have reported reduced FA values near the injury site in SCI patients (Shanmuganathan et al., 2008; Rajasekaran et al., 2010; Kamble et al., 2011; Petersen et al., 2012). As the FA correlates well with both the clinical completeness of SCI and electrophysiological measures (Petersen et al., 2012), this
parameter thus likely also provides a relevant and translatable indicator to assess damage and/or treatment efficacy in experimental animal studies.

The drop in FA was most pronounced for the DCs. Given that this area was directly impacted and thus severely damaged, this dramatic decline in FA is perhaps not surprising. Indeed, tractography at 30 days post-injury failed to show any indications of pathway preservation in this region. Previous studies at 4.7T indicated that axonal damage following contusive SCI in rodents is reflected by a decrease in $\lambda_\parallel$ (Budde et al., 2007), which most likely results from increased barriers to diffusion due to the collapse of axonal membranes and the accumulation of myelin debris within areas of necrosis (Loy et al., 2007). We therefore used this parameter to further assess pathway integrity in our model at 16.4T.

Reductions in $\lambda_\parallel$ confirmed that contusive SCI inflicted immediate and irreversible axonal damage for all ROIs, but particularly the DCs where the decline was greatest. The injury-induced reduction in $\lambda_\parallel$ at 2 hours post-SCI was less pronounced for regions more distant to the site of impact, i.e. VF and LF and a considerable level of anisotropic tissue integrity indeed confirmed through tractography at the experimental endpoint, 30 days post-SCI. Of note, a slight increase in $\lambda_\parallel$ was observed here compared to the 7-day time point although values remained well below baseline in all cases. It remains to be determined if, and to what degree, axonal sprouting, gliosis and/or scarring contribute to this phenomenon. In this context, it is important to point out that the presumptive DC region (for the lesion epicentre only) had to be extrapolated based on proximal and distal slices for the subacute and late time points after injury as the normal tissue architecture was completely disrupted here; this may explain the greater level of variability in $\lambda_\parallel$ (relative to other ROIs) that was observed at the lesion epicentre. Thus, region-specific analysis of $\lambda_\parallel$ at 16.4T can provide information on pathway integrity in a mouse model of SCI and possibly detect more subtle loss of axons as part of ongoing secondary degeneration, at least up to 7 days post-injury. However, larger
cohorts of mice, along with customised imaging protocols (Hui et al., 2010), are likely required to gain enough statistical power to reliably detect these latter changes.

Interestingly, a more comprehensive spatial analysis of SCI-induced changes in FA and $\lambda_\parallel$ revealed an ROI-dependent rostrocaudal asymmetry at the experimental endpoint. Similar observations were previously made at 4.7T in mice (Kim et al., 2007) and, more recently, at 3T in cats (Cohen-Adad et al., 2011). Consistent with previous findings after dorsal root axotomy (Zhang et al., 2009), we believe these changes to be indicative of Wallerian degeneration based on the post-mortem histological correlate. Specifically, for the DCs, we observed reductions in FA and $\lambda_\parallel$ for up to at least 5mm away from the lesion epicentre in rostral but not caudal direction. As the dorsal funiculus mostly contains ascending sensory fibres, a drop in FA, $\lambda_\parallel$ and fibre density as a result of Wallerian degeneration would be most notable rostral to the injury site, which was indeed the case. Increased macrophage presence was also noted here. We observed the opposite for the ventral and lateral funiculi where descending motor pathways dominate. Assessment of rostrocaudal asymmetry in FA and $\lambda_\parallel$ thus provides a non-invasive means to assess damage to ascending and descending tracts in SCI through region specific analysis.

As the FA takes into account both $\lambda_\parallel$ and $\lambda_\perp$, any changes at the lesion site are likely not just reflective of axonal degeneration but also demyelination and possibly gliosis (Harsan et al., 2006; Harsan et al., 2007; Qin et al., 2012). The disruption of barriers to transverse diffusion, particularly myelin sheaths, would permit greater movement of water in the perpendicular plane over time. Consistent with this, a progressive increase in $\lambda_\perp$ was indeed observed at the lesion epicentre for all ROIs. These observations are in line with previous reports showing close association between increased $\lambda_\perp$ and demyelination in the diseased...
CNS (Song et al., 2002; Song et al., 2005; Harsan et al., 2006; Hofling et al., 2009). We confirmed the existence of a similarly inverse relationship between $\lambda_\perp$ values and myelin content for our spinal cord samples through post-mortem histological analysis although DTI appeared to be more sensitive for detecting subtle pathology at the lesion margins. The relatively quick spatial normalisation of $\lambda_\perp$ (within ~2 mm of the lesion epicentre) also suggests that the established presence of tissue macrophages in more distant areas undergoing Wallerian degeneration exerts a minimal influence over this parameter. Assessment of $\lambda_\perp$ thus provides important information on myelination status and, considering the close correlation between white matter preservation and functional deficits (Bresnahan et al., 1987; Noble et al., 1989), also likely yield translatable insights on the efficacy of targeted therapeutic interventions.

In summary, this is the first longitudinal study to report on the use of ultra-high field (16.4T) in vivo DTI to quantitatively monitor the progression of white matter damage following contusive SCI in mice for up to 30 days post-injury. We then conducted a detailed post-mortem microscopic analysis of key pathological changes, i.e. axonal loss, demyelination, astrogliosis and inflammation, to explain what factors might drive the observed changes in diffusion parameters after SCI. Collectively, our findings suggest that the acute changes in FA are mostly driven by reductions in $\lambda_\parallel$ due to the disruption of tissue integrity as a result of impact. As time goes by, a loss of myelin and the onset of glial scarring appears to contribute to a progressive increase in $\lambda_\perp$, which in turn leads to further reductions in FA. Additional studies are, however, required in which the change in diffusion parameters is directly correlated to histopathology early after SCI to more definitively prove this point. Only one other group has utilised in vivo DTI to chronologically examine SCI
pathology in mice although at a lower magnetic field strength (4.7T) and only for up to 14 days post-injury (Kim et al., 2007). Consistent with our data, these authors reported a decreased FA and $\lambda_\parallel$ for spared ventrolateral white matter in the injured spinal cord segment while $\lambda_\perp$ was increased (Kim et al., 2007; Tu et al., 2010); these SCI-induced changes reportedly plateaued between 7 and 14 days post-injury. In the present study, a continued increase in $\lambda_\perp$ was, however, observed up until the late survival point of 30 days post-SCI, indicating ongoing and progressive demyelination. Differences in field strength and/or a more optimal b-value to detect changes in $\lambda_\perp$ (Hui et al., 2010) likely account for this seeming discrepancy. Our findings are also in general agreement with other in vivo and ex vivo DTI studies of SCI in different species such as rats (Krzyzak et al., 2005; DeBoy et al., 2007; Ellingson et al., 2008; Ellingson et al., 2010a; Kim et al., 2012) and cats (Cohen-Adad et al., 2008; Ellingson et al., 2010b; Qin et al., 2012). Most importantly, the changes in DTI parameters in mice with SCI closely match those observed in human patients (Chang et al., 2010; Cheran et al., 2011; Mulcahey et al., 2011; Petersen et al., 2012; Bosma et al., 2012), highlighting its significance as a non-invasive, clinically relevant assessment tool to assess white matter integrity and/or treatment effects in translational research.

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References


**Figure Legends**

**Figure 1.** The change in appearance of the lesion core with time after injury indicates general tissue atrophy and early cytotoxic oedema. (A) *In vivo* serial images of the same mouse spinal cord lesion core, showing the FA, MD, $\lambda_\parallel$, $\lambda_\perp$, and $b_0$. For simplicity, only the pre-injury, 2 hour, 7 day and 30 day scans are shown. Grey and white matter of the spinal cord can still be clearly distinguished in the pre-injury scans as well as 2 hour after SCI but the normal anatomical appearance progressively deteriorates thereafter. Note that the cross-sectional area of the spinal cord decreases with time, which is particularly evident at 30 days post-SCI. Note the increased amount of fluid (CSF) around the spinal cord at this time point in the $b_0$ map as a result of this tissue shrinkage. (B) Quantification of the cross-sectional area of the spinal cord in transverse views confirmed the occurrence of spinal cord atrophy between 7 and 30 days post-injury. Of note, the rostrocaudal asymmetry that was observed at all time points results from a changing cross-sectional area between the lumbar enlargement and lower thoracic region of the spinal cord. (C) A significant reduction in MD values was observed during the first week post-SCI. Data points represent mean ± SEM (n=4 per group); *, p<0.05; **, p<0.01; ns, not significant (repeated measures two-way ANOVA with
Bonferroni post-hoc).

**Figure 2.** SCI-induced changes in FA for the various ROIs as a function of post-injury time. Note the gradual decline in FA for white matter (WM) (A), ventral funiculi (VF) (B) and lateral funiculi (LF) (C). FA values were still significantly higher at 3 day post-injury compared to the experimental endpoint, 30 days post-SCI. In contrast, a much more dramatic and rapid decline in FA values was observed for the dorsal columns (DCs) (D). FA values here already plateaued by 1 day post-injury. Data points represent mean ± SEM (n=4 per group); *, p<0.05; **, p<0.01; ***, p<0.001 (repeated measures one-way ANOVA with Newman-Keuls post-hoc).

**Figure 3.** SCI-induced changes in axial diffusivity ($\lambda_\parallel$) for white matter (WM) (A), ventral funiculi (VF) (B), lateral funiculi (LF) (C), and dorsal columns (DCs) (D) as a function of post-injury time. Note the persistent decrease in $\lambda_\parallel$ at the lesion core at all time points after SCI compared to pre-injury values. Data points represent mean ± SEM (n=4 per group); *, p<0.05; **, p<0.01; ***, p<0.001 (repeated measures one-way ANOVA with Newman-Keuls post-hoc).

**Figure 4.** SCI-induced changes in radial diffusivity ($\lambda_\perp$) for white matter (WM) (A), ventral funiculi (VF) (B), lateral funiculi (LF) (C), and dorsal columns (DCs) (D) as a function of post-injury time. Note the progressive increase in $\lambda_\perp$ after SCI, with a significantly greater values obtained for this parameter at 30 days post-injury compared to all other experimental time points (except for the DCs at 3 days post-injury). Data points represent mean ± SEM (n=4 per group); *, p<0.05; **, p<0.01; ***, p<0.001 (repeated measures one-way ANOVA
with Newman-Keuls post-hoc).

**Figure 5.** Correlation between myelin content, GFAP immunoreactivity and select DTI parameters for spinal cord white matter (WM) at 30 days post-injury. (A) Note that the reduction in Fluoromyelin™ staining (red line) is closely associated with the decline in FA (grey line); both are maximally reduced at the lesion epicentre. (B) An inverse relationship was observed between myelin content (red line) and the radial diffusivity (λ⊥; grey line) across the injury site. (C) Quantification of GFAP immunoreactivity revealed an inverse relationship between astrogliosis and FA. Note that the maximum decrease in FA (grey line) corresponds with the peak of GFAP immunoreactivity (green line) in proximity to the epicentre. (D-F) Representative photomicrographs of the injured mouse spinal cord at 30 days post-SCI, showing relatively normal myelin staining (red) and low GFAP immunoreactivity (green) in more distant areas (D, F), but diminished myelin content and increased GFAP immunoreactivity at the lesion epicentre (E). Scale bar: 300μm.

**Figure 6.** Correlation between neurofilament staining and select DTI parameters. (A, B) Note that the pattern of axonal loss for the dorsal columns (DCs), as detected by neurofilament staining (NF200; green line), closely correlates with observed rostrocaudal asymmetry in FA (A) and λ⊥ (B) (grey lines). (C) For ventrolateral white matter (WM), the observed rostrocaudal asymmetry in DTI parameters again correlated well with the pattern of axonal loss as detected by NF200 staining and shown here for FA (grey line). (D-F) Representative photomicrographs of the injured mouse spinal cord at 30 days post-SCI, showing NF200 (green) and FluoroMyelin™ (red) staining; cell nuclei are shown in blue (Hoechst nuclear dye). Images on the right show a higher magnification of the boxed areas in the left hand image. Note the loss of NF200 staining in the DC area at (E) and rostral to the
lesion epicentre (D) as compared to caudal (F). For ventral white matter, NF200 staining was highest rostral to the lesion epicentre (D), diminishing at the injury site (E) and caudal to it (F). Scale bars: 300μm (D-F), 50μm (insets).

**Figure 7.** Relationship between macrophage presence and select DTI parameters in areas undergoing Wallerian degeneration. Note that immunoreactive area for Iba1 staining (blue line) is greater for regions with reduced FA and $\lambda_1$ (grey lines), in both the dorsal columns (DCs; A, B) and ventrolateral white matter (C, D). (E-H) Representative photomicrographs of the injured mouse spinal cord at 30 days post-SCI, showing Iba1 (green) and CD68 (red) staining; cell nuclei are shown in blue (Hoechst nuclear dye). Images on the right show a higher magnification of the boxed areas in the left hand image. Note that for the DCs, and consistent with the pattern of Wallerian degeneration, there is an increased abundance of Iba1⁺CD68⁺ macrophages rostral to the lesion epicentre (as compared to caudal compare E, F with H), which correlates inversely with the rostrocaudal asymmetry in FA and $\lambda_1$. The opposite pattern was observed for the ventrolateral white matter. Of note, in contrast to ventrolateral white matter, macrophage presence for the DC region peaks just rostral to the lesion epicentre as relatively fewer numbers of these cells occupy the fibrotic scar that replaces the severely damaged neural tissue over time (compare F₁ to G₁). Scale bars: 250μm (E-H), 30μm (insets).

**Figure 8.** Representative tractography images from *ex vivo* DTI data at 30 days-post-SCI. A three-dimensional reconstruction of the lesion core is shown in yellow for each panel. Streamlines are colour-coded for FA (left) and $\lambda_\perp$ (right), with blue and red representing the low- and high-end values of the scale, respectively. Note the dramatic loss of tissue anisotropy for the dorsal column (DC) area at the lesion site (A, B), indicating near complete
damage to white matter tracts through this ROI. For the lateral (C, D) and ventral (E, F) funiculi, track reconstructions still indicated substantial tissue damage although some preservation of fibers was still evident for both these ROIs. Note also that FA values are decreased close to the lesion epicentre while $\lambda_\perp$ is increased. The rostrocaudal asymmetry in FA and $\lambda_\perp$ can also be observed, most clearly for the DCs. Scale bar: 1mm.

**Supplementary Figure Legends**

**Supplementary Figure 1.** Set-up and images used to ascertain the spinal level to be imaged for repeated images. **A:** The mouse was placed parallel to the coil, supported by foam pads and head shield, and its breathing monitored using the Biotrig animal monitoring system. **B-C:** Pilot sagittal images of the injured mouse spinal cord at 1 day (B) and 30 days (C) post-SCI. Note that there is some scoliois apparent at the late time point, but the lesion site can be clearly identified based on the site of laminectomy and a localised hypointensity at the corresponding spinal level. **D-E:** Pilot transverse (D) and axial (E) images were also used to verify that the spinal cord was centered so that the correct anatomical region was scanned.

**Supplementary Figure 2.** Overview of the various regions of interest (ROIs) overlayed on FA maps of the intact mouse spinal cord. **A** White matter (WM), **B** ventral funiculi (VF), **C** lateral funiculi (LF), and **D** dorsal columns (DCs).

**Supplementary Figure 3.** Rostrocaudal asymmetry in FA and axial diffusivity ($\lambda_\parallel$) for the
dorsal column (DC) area (A, D), ventral funiculi (VF) (B, E), and lateral funiculi (LF) (C, F) at 30 days post-injury. Note the significant, persistent decrease for both FA and $\lambda_1$ in the DC area for up to 5mm in rostral (but not caudal) direction compared to pre-injury values (A, D). A similar asymmetry was observed for VF (B, E) and LF (C, F), however, FA and $\lambda_{||}$ values for these ROIs were persistently lower in caudal (but not rostral) direction for up to at least 4mm away from the lesion epicentre. Data points represent mean ± SEM (n=4 per group); *, p<0.05; **, p<0.01; ***, p<0.001 (repeated measures two-way ANOVA with Bonferroni post-hoc).