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14. ABSTRACT
During year 2 of this funding support, we have developed more tools to demonstrate that DBSI is the future of diffusion MRI and neuroimaging modality to effectively detect underlying white matter tract pathologies noninvasively. We developed an objective quantitative histology analysis approach that allows an exact match between histology images and MRI maps. For situations where co-registered MRI-histology is not available due to data acquisition difficulties, the objective quantitative analysis of histology remains readily applicable. Current preliminary results suggest that DBSI detected axonal injury, demyelination, and inflammation closely correlated with histology findings. Most interestingly, results from the shorter time course of DBSI of optic nerve from EAE mice during acute ON indicated that visual impairment did not reflect underlying severity of optic nerve damage. DBSI detects all pathologies in optic nerves during ON.

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Multiple sclerosis, diffusion basis spectrum imaging, diffusion tensor imaging, EAE, inflammation, axonal injury, curizone, demyelination

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Introduction
Acute neurological impairments in MS are associated with the combined effect of the underlying inflammation, axonal injury, and demyelination, while long-term MS disability is due to the extent of permanent axonal damage, may or may not be dependent on the frequency or severity of relapses. In the current proposal, in vivo diffusion basis spectrum imaging (DBSI) has been employed to simultaneously quantify axonal injury, demyelination, and inflammation in CNS white matter, correlating with postmortem immunohistochemical staining, in experimental autoimmune encephalomyelitis (EAE) and cuprizone treated mice.

Keywords
Multiple sclerosis, diffusion basis spectrum imaging, diffusion tensor imaging, EAE, inflammation, axonal injury, cuprizone, demyelination

Overall project Summary
The primary goal of this project is to quantitatively validate diffusion basis spectrum imaging (DBSI) derived metrics of axonal injury, demyelination, and inflammation with post-MRI histology. Our work during this funding period (1) support our hypothesis that DBSI reflects underlying white matter pathologies, (2), DBSI derived white matter pathological metrics reflect acute neurological impairments, and (3) acute neurological impairments do not correlate with underlying white matter pathologies. In the following, we summarize the progress of the proposed studies in the current funding period.

1. Longitudinal and cross-sectional DBSI of corpus callosum (CC) from cuprizone treated mice
The approved statement of work for the year one covers all tasks proposed in specific aim 1 “Longitudinal DBSI evaluation of evolving pathology of corpus callosum from mice treated with cuprizone for 0, 4, and 8 weeks followed by 4, and 8 weeks of recovery. Cross sectional DBSI studies will be performed on the same longitudinal time points for histology validation of DBSI findings. (Months 1 – 14)”. At present time, we have completed the proposed longitudinal (Table 1) and cross-sectional (Table 2) DBSI-histology studies on CC from cuprizone treated mice.

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Previously, we have demonstrated that DBSI derived axial diffusivity (AD) accurately reflected axonal injury in CC from mice after 4 weeks of cuprizone feeding by correctly modeling increased cellularity resulting from microglial activation. In the present study, we examined CC at the chronic stage of cuprizone feeding. Our finding suggested that there is minimal axonal injury as previously reported using DTI. Our results also indicated that at the chronic stage, microglial activation subsided and both DTI and DBSI derived AD and radial diffusivity (RD) was capable to reflect axon and myelin integrity (data are being organized and correlated with quantitative histology for potential publication). Most interestingly, increased inter-axonal and extracellular space seen at chronic stage by previous electron micrograph (EM) examinations was also seen by DBSI derived non-restricted isotropic diffusion tensor component fraction in current study.

2. New approach for quantitative validation of DBSI derived pathological metrics
To validate in vivo DBSI findings with quantitative histology, we have developed a new approach to automatically quantify histology slides at high resolution after histology-DBSI image co-registration. Herein, we report the development of this method using autopsy human MS spinal cord specimens as the example.

Cervical spinal cord specimens were obtained following autopsy from three MS patients. The spinal cord and other tissues were fixed in 10% formalin in phosphate buffered saline (PBS) at room temperature at time of autopsy. A segment of the three fixed MS cervical spinal cord tissues was placed in a 3-ml syringe with 10% formalin and was imaged using a custom-made solenoid coil on an Agilent DirectDrive console equipped with a 4.7 T magnet and a 15-cm inner diameter, actively shielded Magnex gradient coil (60 G/cm, 270 μs rise time). The data acquisition parameters are: repetition time (TR) 2s, spin echo time (TE) 39 ms, time between...
application of gradient pulse 20 ms, diffusion gradient on time 8 ms, slice thickness 0.5 mm, number of slices 5, field-of-view $2.4 \times 2.4$ cm$^2$, data matrix $96 \times 96$. Diffusion sensitizing gradients were applied in 99 directions with max b-value = 3200 s/mm$^2$. In-plane resolution was $250 \times 250$ µm$^2$. Following MR imaging, the formalin fixed MS spinal cord was embedded in paraffin and sectioned using a sliding microtome at 5-µm thick. Sections were individually stained with Bielschowsky’s silver, Hematoxylin and Eosin (H&E), and Luxol Fast Blue-Periodic Acid Schiff (LFB-PAS) stains. Images were acquired with a Hamamatsu NanoZoomer 2.0-HT System (Hamamatsu, Japan) with a 40× objectives.

ImageJ (http://rsbweb.nih.gov/ij/) with NDPI Tools plugin was employed to process the full-view raw histology images. At 40× objective, the raw histology image had 0.23 µm/pixel spatial resolution. One MRI image voxel (250 µm$^2$ in-plane) corresponded to 1087×1087 histology image pixels. NDPI Tools plugin was employed to split the large raw histology images into small square pixels containing 1087×1087 pixels, equivalent to a single MRI imaging voxel. Each small RGB color histology image pixel was converted to indexed color image using minimum variance quantization method. Minimum variance quantization allocates fewer entries to colors that appear infrequently. The accuracy of the colors is higher than with uniform color quantization for a given number of colors. In this example, twenty indexed colors for each type of histologic images were employed. The color indices corresponding to positive staining were selected and consistently applied for all small square histologic image blocks of the same stain type to segment the positive staining regions. The ratio between positive stained pixel number and the total pixel number (1087×1087 = 1181569) was calculated as the quantitative measure of positive staining (Fig. 1).

ImageJ (http://rsbweb.nih.gov/ij/) with an in-house developed image registration plugin was employed to conduct the rigid registration between quantitative histology images and DBSI images using a modification of previously described method. Twelve corresponding pairs of landmarks along the perimeter of the cord were manually placed on both quantitative histology images and DBSI indices image to compute the transformation matrix of rigid registration. The computed transformation matrix was then used to convert the quantitative histology image to the orientation of DBSI images. The success of the newly developed quantification of histology slides can be demonstrated (Figs. 2 – 4).
Cross-sectional DBSI examined mouse brains have been perfusion fixed, sectioned, and stained using Bielschowsky’s silver, LFB-PAS, and H&E. High resolution 40× images have been captured and are currently being processed for quantification. Although the co-registered images would not be doable since we had to image mice at a different view to improve signal-to-noise ratio (SNR), we will be able to correlate quantitative histology measurements of the entire CC with in vivo DBSI metrics.

3. In vivo DBSI of optic nerve at the onset of optic neuritis (ON)

In the previously approved statements of work, year 2 focused on performing tasks related to Aim 2: “Longitudinal DBSI measurements will be performed on the optic nerve from experimental autoimmune encephalomyelitis (EAE) induced by active immunization of C57BL/6 mice using MOG_{35-55} peptide at baseline, onset of optic neuritis (assessed by decreased visual acuity), and chronic state of EAE (4 weeks after immunization). Cross sectional DBSI will be performed on the same time points with matched histology validation of DBSI findings. (Months 13 – 26)”. We imaged 10 mice at baseline before immunization. All mice then undergone MOG-EAE induction and daily visual acuity (VA) was performed. At the day when VA ≤ 0.25 cycles/degree mice were imaged and daily VA assessment continued. In this cohort of mice, unilateral ON was seen in every mouse. In vivo DBSI was performed again when the VA of the initially unaffected eye was lower than 0.25 cycles/degree. After the third DBSI examination, all mice were perfusion fixed and optic nerves excised, paraffin-embedded, sectioned, and Immunohistochemical staining performed. Our results show that in vivo DBSI detected underlying inflammation, demyelination, and axonal injury in the optic nerves at the onset of ON (Fig. 5). The corresponding Immunohistochemical staining results were quantitatively analyzed to count the SMI-31 positive axons, MBP positive myelin, and DAPI positive nuclei. Quantitative IHC results were consistent with in vivo DBSI findings suggested that acute VA impairment during ON is primarily...
caused by the significantly increased edema resulting from inflammation (Fig. 6). Group-averaged time course of DBSI derived pathological metrics (Fig. 7) suggested that the severity of optic nerve pathologies between eye-1 (earlier ON onset) and eye-2 (later ON onset) was not apparent. This is consistent with the notion that neurological impairments do not reflect the underlying nerve damages in MS.

4. *In vivo* DBSI may be used to assess treatment efficacy

Preliminary studies were performed where Oral FTY720 (1 mg/kg) or saline was given on the day of immunization to EAE mice (n = 5 each group). The onset of ON was defined operationally as VA ≤ 0.25 c/d. VA was normal in FTY720 treated and naïve control mice. After the VA of saline-treated EAE mice returned to normal for two consecutive days (~2 - 3 weeks after immunization), *in vivo* DBSI was performed on EAE mice accompanied by one randomly chosen animal from the FTY720-treated EAE and naïve control mice (Fig. 8). FTY720 effectively prevented inflammation (normal appearing restricted and hindered diffusion fractions) and axon/myelin injury (normal appearing λ∥ and λ⊥).

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Figure 6 Correlations between quantitative IHC and DBSI-metrics. Results support that DBSI-AD correlated with SMI-31 positive axon counts, DBSI-RD correlated with MBP positive area, and DBSI-restricted isotropic diffusion fraction correlated with DAPI positive nuclear counts.

Figure 7 Box-plots of group-averaged DBSI metrics for eye 1 (earlier ON onset) and eye 2 (later ON onset) at baseline, time 1 (ON onset of eye 1), and time 2 (ON onset of eye 2) suggested that optic nerve axonal injury and demyelination occurred early in EAE, at the onset of ON. However, the most significant changes resulted from inflammation associated increase in cellularity (DBSI restricted isotropic diffusion fraction) and edema (DBSI non-restricted isotropic diffusion fraction). The timing of ON onset does not seem to be the primary factor of optic nerve damage.

Figure 8 Inflammation and axon/myelin injury were assessed using DBSI derived λ∥, λ⊥, and fractions of restricted (~cellularity) and hindered (~edema) diffusion tensors. Without treatment (saline), optic nerves exhibited heterogeneous inflammation (↑ fractions of restricted and hindered diffusion) and axon/myelin damage (↓λ∥ and ↑λ⊥). FTY720 effectively prevented inflammation (normal appearing restricted and hindered diffusion fractions) and axon/myelin injury (normal appearing λ∥ and λ⊥).
although it prevented myelin injury (Fig. 9) and suppressed inflammation (normalized fractions of restricted and hindered diffusion and DAPI counts; data not shown). A very preliminary therapeutic FTY720 treatment starting at the onset of ON for 4 weeks suggested that FTY720 did not effectively prevent ON, protect axon/myelin, or suppress inflammation as seen in prophylactic treatment. This strongly supports our experimental design using FTY720 treatments to vary the severity of inflammation and axon/myelin injury to test the sensitivity and specificity of DBSI. All indications so far support DBSI as a versatile outcome measure for assessing drug trials in EAE mice. By combining DBSI assessment of pre-treatment axonal pathologies with FTY720 administration at the onset of the disease axon/myelin preservation efficacy of FTY720 will be elucidated.

Key Research Accomplishments
1. Developed a novel quantitative analysis approach dealing with histology that is independent of signal intensity threshold allowing the generation of quantitative histology counts in a map mode. Once published, this approach will change the practice of conventional manual counting of histology slides.
2. In vivo DBSI was able to reveal previously detected inter-axonal and extracellular space increase using EM in CC from chronic cuprizone fed mice.
3. Correlations between DBSI pathological metrics and quantitative histology findings strongly support the feasibility of the goal of this project. The future validation of DBSI in live human MS would significantly facilitate the development of new therapy by offering a more effective outcome measure.

Conclusion
We have been productive under the current support. However, the proposed aims have not been easy to complete due to many technical developments necessary for the execution of the proposed work. Up to now, we have completed majority of work associated with Aim 1 with the exception of quantitative histological analysis. To this end, a novel objective quantitative analysis approach has been developed and will be applied to all histology slides from the cross-sectional studies. The development itself will result in a publication that will teach fellow researchers in the field changing the labor intensive manual counting of histology slides.

On the longitudinal DBSI examination of optic nerve during the course of ON, we were able to complete one shorter time course (same numbers of scans but all before reaching peak EAE) than planned due to the experience gained from preliminary “practice” to learn the limitation of the proposed plan. We learned that after ON onset and multiple (typically three) in vivo DBSI examinations increased and unpredictable mortality of EAE mice at the peak of the disease preventing the completion of a time course extending to chronic stage as initially planned. Thus, we performed a time course study monitoring the onset of ON of both eyes. Mice were perfusion fixed after the 3rd scan without allowing mouse to experience the peak of EAE. The current finding has provided interesting insights on the utility of DBSI and the interrelationship between underlying optic nerve pathologies and visual function. A publication is being prepared based on the preliminary results. Another time course is being planned to only follow the ON course of one eye reducing the number of scans to extend the time course to chronic stage for an opportunity to assess whether acute DBSI is capable of predicting outcome.

Publications

Inventions, Patents and Licenses

None

Reportable Outcomes

None

Other Achievements

None

References

Quantifying white matter tract diffusion parameters in the presence of increased extra-fibercellularity and vasogenic edema

Chia-Wen Chianga,1, Yong Wangb,f,1, Peng Sunb, Tsen-Hsuan Lin c, Kathryn Trinkaus d, Anne H. Cross c,f, Sheng-Kwei Song b,1

Abstract

The effect of extra-fiber structural and pathological components confounding diffusion tensor imaging (DTI) computation was quantitatively investigated using data generated by both Monte-Carlo simulations and tissue phantoms. Increased extent of vasogenic edema, by addition of various amount of gel to fixed normal mouse trigeminal nerves or by increasing non-restricted isotropic diffusion tensor components in Monte-Carlo simulations, significantly decreased fractional anisotropy (FA) and increased radial diffusivity, while less significantly increased axial diffusivity derived by DTI. Increased cellularity, mimicked by graded increase of the restricted isotropic diffusion tensor component in Monte-Carlo simulations, significantly decreased FA and axial diffusivity with limited impact on radial diffusivity derived by DTI. The MC simulation and tissue phantom data were also analyzed by the recently developed diffusion basis spectrum imaging (DBSI) to simultaneously distinguish and quantify the axon/myelin integrity and extra-fiber diffusion components. Results showed that increased cellularity or vasogenic edema did not affect the DBSI-derived fiber FA, axial or radial diffusivity. Importantly, the extent of extra-fiber cellularity and edema estimated by DBSI correlated with experimentally added gel and Monte-Carlo simulations. We also examined the feasibility of applying 25-direction diffusion encoding scheme for DBSI analysis on coherent white matter tracts. Results from both phantom experiments and simulations suggested that the 25-direction diffusion scheme provided comparable DBSI estimation of both fiber diffusion parameters and extra-fiber cellularity/edema extent as those by 99-direction scheme. An in vivo 25-direction DBSI analysis was performed on experimental autoimmune encephalomyelitis (EAE, an animal model of multiple sclerosis) optic nerve as an example to examine the validity of derived DBSI parameters with post-imaging immunohistochemistry verification. Results support that in vivo DBSI using 25-direction diffusion scheme correctly reflect the underlying axonal injury, demyelination, and inflammation of optic nerves in EAE mice.

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Introduction

Diffusion tensor imaging (DTI) successfully detects axon and myelin injury through decreased axial diffusivity ($\lambda_\parallel$, parallel to white matter tract) and increased radial diffusivity ($\lambda_\perp$, perpendicular to white matter tract) in animal models of central nervous system (CNS) diseases and injuries (DeBoy et al., 2007; Kim et al., 2006; Song et al., 2002; Sun et al., 2006). Although decreased DTI-derived fractional anisotropic (FA) has been demonstrated to reflect myelin damage in multiple sclerosis (MS) (Schmierer et al., 2007), it is not a marker specific to myelin damage since other pathological components may also contribute to diffusion anisotropy change (Assaf et al., 2002; Werring et al., 1999). For example, inflammation associated vasogenic edema has been recognized to increase apparent diffusion coefficient (ADC) and underestimate the DTI-derived FA of fiber tracts (Naismith et al., 2010; Pasternak et al., 2009). Increased cellularity has been demonstrated to decrease DTI-derived ADC (Anderson et al., 2000). However, its impact on diffusion anisotropy remains unclear. An in vivo experiment of white matter inflammation in rats has suggested the association of changes in DTI-derived ADC with the evolution of pathology.
It is clear that both axon/myelin and extracellular pathological changes can impact DTI-derived metrics. DTI assumes that diffusion of water molecules in the CNS white matter follows mono-exponential diffusion weighted signal decay (typically at b-value < 1000 s/mm²), and was modeled by a single anisotropic tensor. Thus, diffusion anisotropy of white matter tracts in the presence of multiple structural and pathological compartments poses significant challenges in DTI analysis of white matter tracts since non-Gaussian models or multiple diffusion tensors are needed to reflect the tissue and pathological complexity. Various diffusion techniques have been proposed to overcome the limitation of DTI by non-Gaussian modeling of both parametric (model-based) or non-parametric (model-free) approaches. For instance, diffusion spectrum imaging (DSI) resolves crossing or branching fibers by direct evaluation of diffusion displacement probability density function which is the inverse Fourier transform of the diffusion weighted signals, but typically requires a large number of measurements with extensive diffusion weighting (Wedeen et al., 2005); diffusion kurtosis imaging (DKI) quantifies the non-Gaussian diffusion by estimating apparent diffusion kurtosis of diffusion displacement probability distribution (Jensen et al., 2005); generalized diffusion tensor imaging (gDTI) models the white matter tract via higher order tensors (Liu et al., 2004); composite hindered and restricted model of diffusion (CHARMED) evaluates an extra-cellular compartment (assigned to hindered diffusion resulting from extra-axonal diffusion weighted signal) and intra-cellular compartments (assigned to restricted diffusion in a cylinder representing individual intra-axonal space) employing a comprehensive diffusion weighting scheme (Assaf and Basser, 2005). Recently, Scherrer et al. proposed multiple fascicle models (MFM) to model an isotropic compartment (assigned to free water diffusion) and multiple anisotropic compartments (assigned to single fascicle) using a cube and sphere (CISP) acquisition scheme (Scherrer and Warfield, 2012). Zhang et al. proposed neurite orientation dispersion and density imaging (NODDI) to model tissue components. Using high-angular-resolution diffusion imaging (HARDI) acquisition scheme, NODDI assesses intra-cellular (assigned to space within neurites), extracellular (assigned to space around the neurites but occupied by glial cells), and CSF compartments for deriving neurite density and orientation dispersion (Zhang et al., 2012). Although these approaches resolve possible fiber orientations and free water diffusion contaminations confounding DTI in the CNS, the restricted water diffusion outside fiber tracts affecting DTI measurements was less commonly dealt with. Glial cells have been modeled as a highly restricted isotropic component in an analytical model (Stanisz et al., 1997). A four-tensor model was proposed to include the restricted isotropic diffusion resulting from cell and the extracellular water components (Alexander et al., 2010), and most recently, restricted isotropic diffusion component has been included as one type of isotropic restricted compartment model in a taxonomic comparison study (Panagiotaki et al., 2012).

The recently-developed diffusion basis spectrum imaging (DBSI) approach models white matter diffusion as the linear combination of multiple discrete anisotropic diffusion tensors describing axonal tracts and a spectrum of isotropic diffusion tensors describing restricted (reflecting cells), and non-restricted (reflecting extra-axonal and extracellular space) diffusion components outside of axonal tracts (Wang et al., 2011). Employing a 99-direction diffusion-encoding scheme, DBSI has shown promise to accurately detect and quantify crossing fibers, axonal injury, demyelination, and inflammation-associated cell infiltration and edema in both ex vivo phantom and in vivo mouse brain. Although the effect of increased cell infiltration and edema on DTI-derived indices has been demonstrated previously using cuprizone treated mouse model and mouse trigeminal nerve phantoms (Wang et al., 2011), a more comprehensive study was needed to investigate the effect of increased cellularity and vasogenic edema associated with inflammation. In this study, diffusion weighted signals derived from Monte-Carlo simulations and acquired from tissue phantoms of fixed mouse trigeminal nerve and 2% agar gel were employed to demonstrate how cellularity and edema change DTI indices. The accuracy of DBSI to resolve the complexity of inflammation was also examined. To image the coherent white matter tracts without fiber crossing, such as optic nerve and spinal cord, a simplified DBSI with one anisotropic diffusion tensor component and a spectrum of isotropic diffusion tensors would be sufficient requiring less diffusion encoding directions. A reduced scanning time can be achieved by significantly reducing the number of diffusion weighted images. Thus, we adopted a 25-direction diffusion encoding scheme (Batchelor et al., 2003) on both fixed tissue phantoms and Monte-Carlo (MC) simulation. Comparisons between 25- and 99-direction DBSI results on both experimental tissue phantom and simulated data were conducted to examine the accuracy of DBSI analysis using the 25-direction diffusion encoding scheme. To further demonstrate the feasibility of the 25-direction scheme for DBSI analysis, in vivo DBSI was performed on a group of experimental autoimmune encephalomyelitis (EAE) affected mice at the onset of optic neuritis comparing with the age and gender matched sham control mice. The in vivo diffusion MRI data were analyzed using DBSI and verified by post-imaging immunohistochemistry.

**Materials and methods**

**Diffusion-encoding schemes**

Both 99- (Wang et al., 2011) and 25-direction (Batchelor et al., 2003) diffusion-encoding schemes were employed for DBSI analysis in this study. The 99 diffusion-encoding directions were selected as prescribed in diffusion spectrum imaging (DSI) where the position vectors are the entire grid points (q₁, q₂, q₃) over the 3-D q-space under the requirement that (q₁² + q₂² + q₃²) ≤ r², where r = 3 for DBSI (Kuo et al., 2008; Wang et al., 2011; Wedeen et al., 2005). The cosahedral 25-direction sampling scheme was as prescribed by Batchelor et al. with the addition of one extra non-diffusion (b = 0) weighted image. See Appendix A and B for the 99- and 25-direction diffusion-encoding schemes.

**Fixed trigeminal nerve phantom**

Trigeminal nerves (~4 mm long) were dissected from adult female normal C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) after perfusion fixation with 4% paraformaldehyde in 0.01 M phosphate buffered saline (PBS) followed by immersion fixation for 24 h and kept in 0.01 M PBS solution at 4 °C. Twenty trigeminal nerves were employed to generate phantoms of a single trigeminal nerve only (n = 7) and a single nerve juxtaposed with different amount of 2% aqueous agar gel to mimic vasogenic edema (n = 13). All phantoms were prepared with a nerve gently blotted using Kimwipes® tissue to remove extra solution. Nerves were placed on a microscope slide with an identifier, with or without agar gel, covering with plastic wrap to avoid dehydration.

**Diffusion-weighted spectroscopy of fixed trigeminal nerve phantom**

Diffusion-weighted data were collected immediately after nerve phantom preparation using a 6-mm inner diameter single-turn surface coil. Diffusion-weighted spin–echo spectroscopy was performed with the following acquisition parameters: repetition time (TR), 2 s; echo time (TE), 32 ms; time between application of gradient pulses (Δ), 16 ms; gradient pulse duration (δ), 8 ms; number of average, 1; the maximum diffusion-weighting factor (b-value), 3200 s/mm² for both 99- and 25-direction diffusion weighting schemes at a single setting. Total acquisition time was 4 min 15 s.

**Monte-Carlo simulation**

Monte-Carlo simulations were performed to evaluate the effect of vasogenic edema and cellularity on DTI and DBSI indices in a computer generated geometric model, mimicking the trigeminal nerve tissue.
phantoms. The simulation was performed by allowing water molecules (2.5 × 10^5, randomly distributed) to undergo random walk within a 90-μm diameter sphere (light blue, Fig. 1A) at 20 °C. Diffusivity for free water was set to be 2.02 μm^2/ms (at 20 °C). The time-step for simulated random walk was 0.2 ms. The computer generated model of the trigeminal nerve phantom for simulation were composed of (1) a coherent axon fiber bundle modeled by uniformly oriented, and tightly packed cylindrical tubes (green cylinders in Fig. 1A) with a diameter of 2 μm; (2) cellular components modeled as isotropic spheres (blue in Fig. 1A) with a diameter of 6 μm randomly placed surrounding the axonal fiber bundle; (3) extra-axonal and extracellular space occupied by water molecules distributed outside of axonal bundle and cellular components (Fig. 1A). The size of axonal cylinder and cells was adapted according to literature reports (Stanisz et al., 1997; Stolzenburg et al., 1989). The imaging voxel (50 × 50 × 50 μm^3, pink cube in Fig. 1A) was placed in the center of the sphere defined for random walk (light blue outer sphere in Fig. 1A). At the boundary of axon and cell components, the water spin reflected elastically (Liu et al., 2004). During the simulation, water spins were allowed to walk in or out of the imaging voxel without constrains to reflect the real physical condition (Liu et al., 2004). The boundaries of the axon and cell components were assumed to be impermeable. The random walk trajectory of each water spin was recorded and saved during the simulation.

Based on previous fixed mouse trigeminal nerve phantom study (Wang et al., 2011), baseline simulation model (model # 1, Fig. 1B), mimicking nerve only mouse trigeminal nerve without added gel, consisted of 12% cells (133 spheres), 68% axonal fibers (529 cylinders), and 20% extra-axonal/extracellular space. The tissue phantom of single nerve plus gel was simulated by increasing the size of imaging voxel of baseline simulation model (model # 1, Fig. 1B), thereby increasing the extra-axonal/extracellular space (model # 2–11, Fig. 1B), to mimic vasogenic edema. In model # 2–11, the cell fraction ranged from 7% to 15%, the axonal fiber fraction ranged from 15% to 57%, and the extra-axonal/extracellular space fraction ranged from 27% to 78% (Fig. 1B). It is very challenging if at all possible to control the amount of cellular components in the experimental trigeminal nerve tissue phantom. However, the cellular components can be readily adjusted and simulated by increasing the number of spheres (cells) inside the simulated imaging voxel. In model # 12–21, the cell volume fraction ranged from 14% to 80%, the axon fiber fraction ranged from 12% to 63%, and the extra-axonal/extracellular space fraction ranged from 8% to 22%.

A simple spin echo sequence with diffusion gradients was implemented to simulate the diffusion weighted MRI signals from the simulated imaging voxel. The simulated phase of each water spin within TE was calculated based on the random walk trajectory (Liu et al., 2004). The overall measured diffusion MRI signal was simulated as the summation of signals contributed from all water spins, ending within the voxel of imaging at the time of TE. The effects of T1 and T2 decay for all spin trajectories were neglected (Liu et al., 2004). Both 99- and 25-direction schemes were employed to acquire the diffusion weighted signals. Other key MRI acquisition parameters included TE 32 ms, Δ 16 ms, δ 8 ms, maximum b-value 3200 s/mm^2 were kept the same intentionally with the fixed trigeminal nerve phantom experiment above. Rician noise was added to the simulated diffusion weighted signals from model # 12 mimicking mild cell infiltration (Fig. 1B) to examine the effect of signal-to-noise ratio (SNR) on DBSI-derived indices obtained using 25-direction diffusion scheme at SNR = 10, 20, 30, 40, and 100 of the non-diffusion weighted signal. The simulation was repeated 20 times for each SNR level.

**Acute optic neuritis of EAE**

Five adult female 8-week-old C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME, USA) were immunized using 50 μg myelin oligodendrocyte glycoprotein peptide (MOG35–55) emulsified in incomplete Freund’s adjuvant with 50 μg Mycobacterium tuberculosis. Five age-matched female mice received only incomplete Freund’s adjuvant and Mycobacterium tuberculosis in the absence of MOG35–55 served as the sham control. For EAE-immunized mice, the adjuvant pertussis toxin (300 ng; PTX, List Laboratories, Campbell, CA) was injected intravenously on the day of immunization and two days later. Transverse view of mouse optic nerve was imaged at the onset of acute EAE optic neuritis (Chiang et al., 2011). Matched sham control mice were randomly selected for in vivo DBSI acquisition whenever an EAE mouse was imaged.

**In vivo magnetic resonance imaging**

In vivo diffusion MRI experiments were performed on a 4.7 T Agilent DirectDrive™ small-animal MRI system (Agilent Technologies, Santa Clara, CA) equipped with Magnex/Agilent HD imaging gradient coil (Magnex/Agilent, Oxford, UK) with pulse gradient strength up to 58 G/cm and a gradient rise time ≤ 295 μs. After anesthetized using 1%
isoflurane/oxygen, mice were placed in a custom-made head holder. The rate of respiration and rectal temperature, at 37 °C, was monitored and controlled by a small animal physiological monitoring and control unit (SAIL Inc., NY). An actively-decoupled volume (transmit)/surface (receive) coil pair was used for MR excitation and signal reception. All image slices were acquired based on previously reported procedures (Sun et al., 2008) to plan for the final-targeted slice showing a transverse view of mouse brain with two optic nerves, as nearly as orthogonal to image slice as possible. A multi-echo spin-echo diffusion-weighted sequence (Tu et al., 2010) and a 25-direction diffusion-encoding scheme combined with one b = 0 were employed and MR acquisition parameters were TR 1.5 s, TE 37 ms, Δ 18 ms, δ 6 ms, max. b-value 2200 s/mm², slice thickness 0.8 mm, and in-plane resolution 117 μm × 117 μm (before zero-filled). The total acquisition was approximately 2 h, 20 min.

**Immunochemistry**

Mice were perfusion fixed with 0.01 M phosphate-buffered saline (PBS, pH = 7.4) followed by 4% paraformaldehyde in 0.01 M PBS immediately after imaging. Brains were excised and fixed for 24 h, and then transferred to 0.01 M PBS for storage at 4 °C until histological analysis. Fixed optic nerves were embedded in 2% agar blocks (Blewitt et al., 1982) before being embedded in paraffin. Five-μm thick transverse slices were sectioned, deparaffinized, rehydrated, and blocked using a 1:1 mixture of 10% normal goat serum and 2% bovine serum albumin in PBS for 20 min at room temperature to prevent nonspecific binding of goat secondary antibody. Sections were incubated in monoclonal anti-phosphorylated neurofilament primary antibody (SMI-31; 1:1000, Covance, US) to stain non-injured axons, and in rabbit anti-myelin basic protein (MBP) primary antibody (1:1000, Sigma Inc., MO) to stain myelin sheath at 4 °C overnight. After several rinses, secondary goat anti-mouse IgG and goat anti-rabbit IgG conjugated Alexa 488 (1:800, Invitrogen) were applied, respectively, to visualize immunoreactivity of materials at room temperature for 1 h. After washing, slides were covered using Vectashield Mounting Medium with 4′-6-diamidino-2-phenylindole (DAPI) (Vector Laboratory, Inc., Burlingame, CA). Histological images were acquired at 20× and 60× (water objective) magnifications on Nikon Eclipse 80i fluorescence microscope using MetaMorph software (Universal Imaging Corporation, Sunnyvale, CA).

**Data analysis**

Experimental and simulated data obtained using 99- and 25-direction diffusion encoding schemes were examined by DBSI multitemporal model analysis package developed in-house with Matlab® (MathWorks) (Wang et al., 2011) and conventional DTI single-tensor model analysis. Briefly, Eq. (1) was first solved by fitting total number k diffusion signals using a linear combination of diffusion basis sets consisting of cylindrically symmetric diffusion tensors with the freedom to vary λ⊥ and λ∥ to estimate the number of anisotropic diffusion tensor components (N_aniso) and the associated principal directions. After N_aniso was computed, isotropic diffusion tensor components were further analyzed using nonnegative least-squares (NNLS) technique. For coherent white matter tract in this study, N_aniso = 1. The global nonlinear optimization was conducted employing direct pattern search to solve Eq. (1). Sk is the kth measured diffusion weighted signals (k = 1, 2, ..., 99; or 25 in this study), fi and f(D) are signal intensity fractions of ith anisotropic diffusion components and a spectrum of isotropic diffusion components from a to b (diffusivity, typically from 0 to 3 μm²/ms), respectively.

\[
S_k = \sum_{i=1}^{N_{aniso}} f_i e^{-b_i (h_i - h_{0,i} - \lambda_{\perp,i}) / 2} \int_{a}^{b} f(D) e^{-b_i (h_i - h_{0,i} - \lambda_{\perp,i}) / 2} dD \quad (k = 1, 2, 3, \ldots)
\]

(1)

To the first approximation, the intracellular water may be modeled as restricted isotropic diffusion due to the hindrance of cell membranes and subcellular structures. Based on our previous experimental findings, the restricted isotropic diffusion fraction reflecting cellularity is derived by the summation of \( f(D) \) at 0 ≤ ADC ≤ 0.3 μm²/ms. The summation of the remaining \( f(D) \) at 3 > ADC > 0.3 μm²/ms was assigned to non-restricted isotropic diffusion reflecting vasogenic edema and CSF water.

**Statistical analysis**

All data were expressed as mean ± standard deviation or median and interquartile range (IQR). The comparison between DTI and DBSI derived diffusion parameters was performed on data obtained from trigeminal nerve phantoms and from Monte-Carlo simulations using t-tests or linear regression models with repeated measures where more than one observation per subject was included. A nonparametric Wilcoxon signed rank test has been performed to test equivalence of 99- and 25-direction DBSI-derived parameters including λ⊥, λ∥, and FA, as well as the fractions of different diffusion tensor components. Nonparametric Spearman’s rank correlations were used to compare DBSI/DTI and IHC. The tests were repeated for data from all simulation models, i.e., changing proportion of extra-axonal/extracellular space and for those with a changing proportion of cells. All statistical analyses were performed using SAS V9.3 (SAS Inc., Cary, NC).

**Results**

**Effect of vasogenic edema on DTI/DBSI-derived parameters**

For 99-direction diffusion weighted data (SNR = 40) from fixed trigeminal nerve only phantoms (N = 7), DTI derived λ⊥ = 0.77 ± 0.05 μm²/ms, λ∥ = 0.18 ± 0.02 μm²/ms, and FA = 0.72 ± 0.04 were compared with those derived using DBSI where λ⊥ = 1.09 ± 0.06 μm²/ms, λ∥ = 0.15 ± 0.02 μm²/ms, and FA = 0.84 ± 0.02 (n = 7, mean ± standard deviation). DBSI estimated axial fiber fraction to be 67 ± 3%, cellularity to be 14 ± 2%, and extra-axonal/extracellular space to be 20 ± 3% of the total signal intensity. This finding explains the difference of axial diffusivities derived by DTI and DBSI, and is consistent with our previously published results supported by immunochemistry (Wang et al., 2011). With the addition of 2% agar gel, DTI derived λ∥ or FA was significantly affected while DBSI derived parameters remained unchanged (Fig. 2A). A comparable observation was seen in MC simulation data where DTI derived FA decreased while λ∥ and λ⊥ increased with increasing vasogenic edema (i.e., extra-axonal/extracellular space, non-restricted isotropic diffusion component; Fig. 2B) with relatively stable DBSI derived FA, λ∥ and λ⊥.

**Effect of increased cellularity on DTI/DBSI-derived parameters**

Since cellularity is difficult to change experimentally using the fixed nerve, MC simulation was performed with various extents of extra-fiber cells (i.e., spheres, restricted isotropic diffusion component) to mimic cellularity changes. Diffusion parameters derived by DTI were significantly affected with increasing cellularity, i.e., DTI derived λ∥ and FA significantly decreased with increasing cellularity while DBSI derived parameters were largely unaffected (Fig. 3). DTI derived λ⊥ was minimally affected in contrast to the more profound changes seen in λ∥.

**Close correspondence between 25- and 99-direction DBSI-derived parameters**

There was no discernible difference between 25- vs. 99-direction diffusion encoding scheme in DBSI derived diffusion parameters on data
Effects of noise on 25-direction DBSI-derived parameters

MC simulation of 25-direction diffusion weighted data with SNR of non-diffusion weighted image at 10, 20, 30, 40 and 100 was performed on a mild cell infiltration model (model # 12). The noise affected the bias and precision of the 25-direction DBSI-derived axial diffusivity (Fig. 5A), radial diffusivity (Fig. 5B), restricted isotropic diffusion tensor fraction (Fig. 5C), and non-restricted isotropic diffusion tensor fraction (Fig. 5D). At SNR = 100, DBSI axial diffusivity, radial diffusivity, restricted isotropic diffusion and non-restricted isotropic diffusion fractions were all estimated with high precision indicated by a small interquartile range and absence of outliers (Fig. 5). With decreasing SNR, both bias and precision worsened (Fig. 5). At the SNR range readily achieved on typical animal and clinical scanner (SNR = 20–40), 25-direction DBSI-derived axial diffusivity was well-estimated (i.e., falls within the middle half of values at SNR = 100) at SNR ≥ 30 while slightly underestimated at SNR = 20 (Fig. 5A); DBSI-radial diffusivity was well-estimated at SNR ≥ 20 (Fig. 5B); the restricted diffusion fraction was well-estimated at SNR ≥ 40 while overestimated at SNR ≤ 30 (Fig. 5C); and non-restricted diffusion fraction was well-estimated at SNR ≥ 30 while underestimated at SNR = 20 (Fig. 5D).

In vivo DBSI of EAE mice at the onset of optic neuritis

A close examination of the 25-direction DTI and DBSI parameter maps of the representative sham (Figs. 6A–F) and EAE-affected (Figs. 6A–F) mice suggested the presence of axonal injury revealed by the decreased $\lambda_\perp$ derived by both DTI (Fig. 6a) and DBSI (Fig. 6b), and demyelination indicated by the increased $\lambda_\parallel$ derived by DTI (Fig. 6c) and DBSI (Fig. 6d), and inflammation implicated by the increased restricted (Fig. 6e) and non-restricted (Fig. 6f) isotropic diffusion fraction in the EAE-affected optic nerve. All diffusion MRI detected pathologies in the EAE optic nerve exhibited a heterogeneous distribution in the cross-section of the nerve (Figs. 6–F). Apparent heterogeneity of cell infiltration was also seen, consistent with a heterogeneous increase in DAPI-positive cell nuclei staining (Figs. 6K and k). Qualitatively, axonal injury (decreased $\lambda_\perp$) and demyelination (increased $\lambda_\parallel$) occurred within the regions where increased cellularity (increased restricted isotropic diffusion fraction) was present (Figs. 6b, d, and e).

Results from quantitative analyses showed significantly decreased $\lambda_\perp$ and increased $\lambda_\parallel$ (mean ± standard deviation; EAE vs. Sham) in both DBSI and DTI: DBSI $\lambda_\parallel = 1.73 \pm 0.22$ vs. $2.10 \pm 0.04 \mu$m$^2$/ms ($p = 0.020$); DTI $\lambda_\parallel = 1.34 \pm 0.37$ vs. $1.89 \pm 0.09 \mu$m$^2$/ms ($p = 0.028$); DBSI $\lambda_\perp = 0.15 \pm 0.03$ vs. $0.11 \pm 0.01 \mu$m$^2$/ms ($p = 0.047$); and DTI $\lambda_\perp = 0.21 \pm 0.04$ vs. $0.11 \pm 0.01 \mu$m$^2$/ms ($p = 0.0046$); DBSI derived restricted isotropic diffusion fraction (i.e., the putative cellularity) in EAE optic nerves was significantly higher than that of the control ($0.10 \pm 0.09$ vs. $0.03 \pm 0.01$, $p = 0.022$). The non-restricted isotropic diffusion fraction (Fig. 5D) at SNR = 100, DBSI axial diffusivity, radial diffusivity, restricted isotropic diffusion and non-restricted isotropic diffusion fractions were all estimated with high precision indicated by a small interquartile range and absence of outliers (Fig. 5). With decreasing SNR, both bias and precision worsened (Fig. 5). At the SNR range readily achieved on typical animal and clinical scanner (SNR = 20–40), 25-direction DBSI-derived axial diffusivity was well-estimated (i.e., falls within the middle half of values at SNR = 100) at SNR ≥ 30 while slightly underestimated at SNR = 20 (Fig. 5A); DBSI-radial diffusivity was well-estimated at SNR ≥ 20 (Fig. 5B); the restricted diffusion fraction was well-estimated at SNR ≥ 40 while overestimated at SNR ≤ 30 (Fig. 5C); and non-restricted diffusion fraction was well-estimated at SNR ≥ 30 while underestimated at SNR = 20 (Fig. 5D).
diffusion fraction (i.e., the putative vasogenic edema) was also significantly higher in EAE optic nerves than that of the control (0.15 ± 0.03 vs. 0.03 ± 0.01, p < 0.0001). The increased sum of restricted and non-restricted isotropic diffusion fractions may be a good indicator of inflammation, 0.24 ± 0.12 vs. 0.06 ± 0.02 (EAE vs. Sham, p = 0.024).

Quantitative immunohistochemical analyses revealed that DTI/DBSI detected axonal injury and demyelination was also seen in SMI-31-positive axon counts (5.3 × 10^5 ± 1.8 × 10^5 vs. 7.4 × 10^5 ± 0.2 × 10^5 counts/mm^2; p = 0.03; Figs. 7A and C) and MBP-positive staining area (37% ± 5% vs. 45% ± 3%; p = 0.01) exhibiting statistically significant correlations (Figs. 7B and D). The DAPI-positive nuclear counts increased in EAE optic nerves (6.6 × 10^3 ± 5.0 × 10^3 vs. 3.5 × 10^3 ± 0.7 × 10^3 counts/mm^2) without reaching a statistical significance (p = 0.2). DAPI-positive nuclear counts correlated with DBSI estimated cellularity (r = 0.65, p = 0.042; Fig. 7E).

Discussion

This study investigated the effect of increased cellularity and vasogenic edema, commonly seen in CNS injuries, on the DTI derived
diffusion parameters of white matter tracts. Specifically, increased cellularity, as evidenced by increased isotropic restricted diffusion, decreased the DTI derived $\lambda_\parallel$ and $\lambda_\perp$ while having a limited impact on $\lambda_\perp$. Increased vasogenic edema, as evidenced by increased non-restricted isotropic diffusion, also decreased the DTI derived $\lambda_\parallel$ but increased $\lambda_\perp$ with the extent of impact on $\lambda_\perp$ depending on the ADC value of edema relative to $\lambda_\parallel$ and $\lambda_\perp$ of axon. These confounding effects were resolved and quantified by the recently developed DBSI analysis (Wang et al., 2011), as demonstrated herein using data generated by both Monte-Carlo simulation and tissue phantoms. Our prior study used 99-direction diffusion encoding (Wang et al., 2011). A 25-direction diffusion encoding scheme (Batchelor et al., 2003) was assessed in this study to demonstrate its adequacy for performing DBSI analysis on optic nerves from the mouse at the onset of acute optic neuritis.

The partial volume effect of CSF, i.e., non-restricted (free) isotropic diffusion, on DTI indices has long been recognized, and limits DTI capability in regions abutting CSF, such as periventricular regions that are commonly affected by MS. The inclusion of the isotropic component of free diffusion improved fiber diffusion measurements (Alexander et al., 2001; Papadakis et al., 2002). However, such modification is insufficient to deal with coexisting CNS inflammation (increased cellularity and vasogenic edema) or tissue loss that may be seen in diseased tissues (Horsfield and Jones, 2002). To properly model the effect of CNS inflammation and tissue loss, allowing a more accurate evaluation of the diffusion properties of white matter fiber tracts, multiple extra-fiber diffusion components (both restricted and non-restricted isotropic diffusion components) must be identified and quantified. DBSI considers a spectrum of isotropic diffusion components of a wide range of ADC to account for the extra-fiber tissue and pathological components. The current results from fixed normal tissues and gel phantoms, and our previous in vivo mouse brain data (Wang et al., 2011) suggest that the DBSI approach allowed for an accurate estimation of white matter tract diffusion properties in the presence of increased cellularity and vasogenic edema.

The close agreement between the experimental measurements of fixed trigeminal nerve plus gel phantoms and the results from Monte-Carlo simulations using restricted and non-restricted isotropic diffusion components to mimic cells and vasogenic edema supported our approach of including a linear combination of isotropic diffusion spectra to account for the effect of CNS inflammation and tissue loss. One advantage of using DBSI is its capability to resolve crossing fibers while still quantitatively deriving fiber associated anisotropic diffusion tensors to assess the axon and myelin integrity (Wang et al., 2011). Previously, to resolve crossing fibers and quantifying extra-fiber cellularity and edema a 99-direction diffusion encoding scheme was employed.
In the case where crossing fibers are not of concern such as when studying optic nerve and spinal cord white matter tracts, a reduced data acquisition scheme may be sufficient for DBSI analysis. In the present study, the 25-direction diffusion scheme was assessed and proved to be adequate for DBSI analysis of coherent white matter tracts to quantify the extent of cellularity and edema. Thus, existing diffusion weighted data acquired using 25- or other multiple-direction encoding schemes with multiple b-values may be suitable for DBSI analysis without reacquiring new data to examine the single coherent fiber tracts.

Similar to previous results on corpus callosum from cuprizone treated mice (Wang et al., 2011), in vivo DBSI derived restricted isotropic diffusion fraction map of optic nerve from EAE mice revealed a heterogeneous increase in cellularity, closely matching the pattern of DAPI-positive cell nuclei staining of the same nerve (Figs. 6e, k, and l). Note that “cellularity” used in this context is not an equivalent of cell density. Instead, it is a measure of T1, T2, and proton density weighted signal fraction corresponding to restricted isotropic diffusion components within each image voxel (Eq. (1)). Restricted isotropic diffusion fractions have been assigned as “cellularity” fractions because our phantom and animal studies demonstrated a linear correlation between this restricted isotropic diffusion fraction and DAPI nuclei counts.

Various MR techniques have been used to assess inflammation of optic neuritis, but rarely demonstrated the ability to image cellularity changes non-invasively. For example, gadolinium-enhanced TW images reflect the leakage of blood–brain barrier (Guy et al., 1990; Hickman et al., 2004b; Qi et al., 2007) without information regarding the cellular infiltration. Iron-oxide particles have been used to allow in vivo visualization of macrophages, but this method is insensitive to non-phagocytic cells involved in inflammation (Vellinga et al., 2008). An increased optic nerve area (swelling) has also been considered to reflect the severity of inflammation in acute optic neuritis (Boretius et al., 2008; Hickman et al., 2004a). Unfortunately, this method is non-specific, as a changed nerve cross-sectional size may reflect cell infiltration and/or edema and is further confounded by any tissue loss that may be present (Hickman et al., 2004a).

In the present study, both DTI and DBSI derived axial and radial diffusivity correctly detected axonal injury and demyelination in the optic nerve at the onset of optic neuritis. This suggests that DTI works well on these mice probably due to the counteraction of increased restricted and hindered/free diffusion tensor components. When examined using DBSI, the co-localization of axonal injury and myelin damage with cell infiltration was seen in the EAE-affected optic nerve. Interestingly, the region of increased cellularity did not always correspond to visible nerve damage (Figs. 6a, b, and e). This is consistent with the pathogenesis of optic neuritis in EAE, i.e., nerve injury originates from the induced CNS inflammation but may not always occur with inflammation, and when it does occur axon injury may extend well outside the region of inflammation (Gold et al., 2006; Iglesias et al., 2001). Such pathological profile can now be detected using DBSI noninvasively.

MRI greatly aids CNS disease diagnosis, but current methods do not assess specific elements of CNS pathology. Gadolinium contrast enhancement (Gd +) can detect a compromised blood–CNS-barrier (BBB), which is useful to screen potential new anti-inflammatory disease modifying drugs. However, Gd + MRI is not sensitive to small inflammatory lesions or inflammation behind the intact BBB, underscoring the need to improve imaging sensitivity and specificity to identify new beneficial therapies. Without injecting Gadolinium contrast agent, DBSI provides a noninvasive imaging biomarker of inflammation, which could potentially enhance our capability to more accurately evaluate neuro-inflammation in CNS disease. In addition, DBSI-derived indices hold the promise to reflect the various specific components of the CNS pathology to identify new beneficial therapies that affect more than just inflammation. To be most useful, a new technique should be feasible on present clinical scanners. With fast diffusion-weighted EPI sequence available on 3.0 T clinical scanners,
99-direction human whole brain DBSI scans can be performed within 15 min, making DBSI protocol feasible for routine clinical scanning of patients.

DBSI in its current form does not consider the effect of water exchange between intracellular and extracellular space, which can potentially affect the accuracy of DBSI metrics, especially under pathological conditions where the permeability of cell membranes may be altered. In addition, DBSI was developed to quantify the effect of inflammation on diffusion MRI determined axonal injury and demyelination in white matter tracts. Further generalization of DBSI model is needed to expand its use in quantifying changes in gray matter pathology commonly seen in CNS disorders.

In summary, we have demonstrated that DBSI removes confounding effects of increased cellularity and vasogenic edema on DTI derived white matter tracts. Further generalization of DBSI model is needed to et al., 2011).

Acknowledgments

The authors thank Robert Mikess for his assistance with the immunization of mice to induce EAE. This study was supported in part by the grants from the National Institute of Health R01-NS047592 (S.-K.S.), P01-NS059560 (A.H.C.), National Multiple Sclerosis Society (NMSS) RG 4549A4/1 (S.-K.S.), and Department of Defense Ideal (S.-K.S.), P01-NS059560 (A.H.C.), National Multiple Sclerosis Society (NMSS) RG 4549A4/1 (S.-K.S.), and Department of Defense Ideal Award W81XWH-12-1-0457 (S.-K.S.).

Appendix A. 99-Direction DBSI diffusion encoding scheme (Wang et al., 2011)

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Appendix B. 25-Direction icosahedral sampling scheme (Batchelor et al., 2003)

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References


Diffusion fMRI detects white-matter dysfunction in mice with acute optic neuritis

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INTRODUCTION

Optic neuritis is a frequent and early symptom of multiple sclerosis (MS). Conventional magnetic resonance (MR) techniques provide means to assess multiple MS-related pathologies, including axonal injury, demyelination, and inflammation. A method to directly and non-invasively probe white-matter function could further elucidate the interplay of underlying pathologies and functional impairments. Previously, we demonstrated a significant 27% activation-associated decrease in the apparent diffusion coefficient of water perpendicular to the axonal fibers (ADC⊥) in normal C57BL/6 mouse optic nerve with visual stimulation using diffusion fMRI. Here we apply this approach to explore the relationship between visual acuity, optic nerve pathology, and diffusion fMRI in the experimental autoimmune encephalomyelitis (EAE) mouse model of optic neuritis. Visual stimulation produced a significant 25% (vs. baseline) ADC⊥ decrease in sham EAE optic nerves, while only a 7% (vs. baseline) ADC⊥ decrease was seen in EAE mice with acute optic neuritis. The reduced activation-associated ADC⊥ response correlated with post-MRI immunohistochemistry determined pathologies (including inflammation, demyelination, and axonal injury). The negative correlation between activation-associated ADC⊥ response and visual acuity was also found when pooling EAE-affected and sham groups under our experimental criteria. Results suggest that reduction in diffusion fMRI directly reflects impaired axonal-activation in EAE mice with optic neuritis. Diffusion fMRI holds promise for directly gauging in vivo white-matter dysfunction or therapeutic responses in MS patients.

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ly separated into two groups: seven in the EAE group and seven in the housed under a 12-hour dark/light cycle for a week. Mice were random-

Experimental autoimmune encephalomyelitis (EAE) mouse model

Fourteen 7-week-old, female C57BL/6 mice were obtained from Jackson Laboratory (Bar Harbor, ME). Before immunization, mice were housed under a 12-hour dark/light cycle for a week. Mice were randomly separated into two groups: seven in the EAE group and seven in the sham group. The mice in the EAE group were immunized with 50 μg MOG35–55 peptide emulsified (1:1) in incomplete Freund’s adjuvant (IFA) and desiccated Mycobacterium tuberculosis. Pertussis toxin (300 ng; PTX, List Laboratories, Campbell, CA) was injected intravenous-

Animal anesthesia and set-up

For initial setup, mice were anesthetized with 1.5% isoflurane in O2. Anesthetized mice were placed in a custom-made stereotactic head holder. The experimental eye remained open to receive light stimulus. The contralateral eye was covered with Parafilm™ and two layers of black electrical tape to block out the flashing light (also identified as “blocked” eyes in the text). After careful setup the mouse and head holder were placed in the imaging cradle. Respiratory rate and body temperature were monitored during experiments using an MR-compatible animal-monitoring system (SA Instrument, Inc., Stony Brook, NY). The body temperature was maintained at 37 °C via a regulated circulating warm water pad underneath the mouse body and regulated warm air blown into the magnet bore. When mouse respiration was stable, a subcutaneous 0.3 mg/kg bolus of medetomidine was administered follow

Diffusion-weighted image (DWI) protocol

MRI experiments were performed on a 4.7-T Agilent DirectDrive™ small-animal MRI system (Agilent Technologies, Santa Clara, CA) equipped with a Magnex/Agilent HD imaging gradient coil (Magnex/Agilent, Oxford, UK) capable of pulsed-gradient strengths of up to 58 G/cm and a gradient rise time ≤ 295 μs. An actively-decoupled 1.7–cm Receive-only surface coil was positioned on top of the mouse head. Then, the animal holder assembly, including the receive coil was placed inside an 8-cm actively-decoupled volume transmit coil. DWI measurements were carried out as previously described (Spees et al., 2013). In brief, a multi-echo spin-echo diffusion-weighted imaging sequence (Tu et al., 2010) was employed to improve the signal-to-noise ratio (SNR) by averaging the extra echoes of the same k-space line, during the readout period after diffusion weighting, with negligible increase in imaging time comparing with the conventional spin-echo diffusion sequence. A train of three echoes were co-added to increase signal-to-noise in the summed MR image. The following acquisition parameters were used: TR = 1.5 s, TE = 37.1 ms, inter-echo delay = 23.6 ms, FOV = 20 × 20 mm², matrix size = 256 × 256 (zero-filled to 512 × 512), slice thickness = 1.3 mm. To obtain the final target image plane and minimize partial volume effects, a middle-sagittal DW image (diffusion gradient applied in the slice-select direction) was acquired based on axial scout images. The sagittal DWI encompassed optic nerves of both eyes longitudinally. An image plane parallel to the optic nerves was placed overlying the nerves. By rotating this image plane by 90°, the final targeted image slice plane was orthogonal to the optic nerves to minimize partial volume effects. Two diffusion-sensitizing factors (b values), 0.1 and 1.4 ms/mm², i.e., 100 and 1400 s/mm² (with b = 5 ms and Δ = 18 ms), were applied to generate a pair of DWIs (Figs. 1A and B).
with monoclonal anti-phosphorylated neurocell nuclei (Budde et al., 2009; Wang et al., 2011). Histological slides were stained with 2-phenylindole (DAPI, Vector Laboratory, Inc., Burlingame, CA) to stain myelin sheaths (Budde et al., 2009; Song et al., 2003; Sun et al., 2013; 1:1000, Covance, US) to stain non-injured axons or with rabbit anti-myelin basic protein (MBP) antibody (1:1000, Sigma Inc., MO, US) to stain non-specific binding. Slides were incubated overnight at 4 °C with 5% normal goat-serum solution for 20 min at room temperature to prevent non-specific binding. Sections were deparaffinized, rehydrated, and then blocked using 1% bovine serum albumin (BSA, Sigma Inc., MO, US) to block non-specific binding. The brain was excised and post-fixed in 4% paraformaldehyde in 0.01 M PBS. The brain was then transferred to 0.01 M PBS for storage at 4 °C until histological analysis. Mouse optic nerves were embedded in 2% agar blocks (Blewitt et al., 1982). Then, the agar block was embedded in paraffin wax and 5-μm thick transverse slices were sectioned for IHC staining. Sections were deparaffinized, rehydrated, and then blocked using 1% bovine serum albumin (BSA, Sigma Inc., MO, US) and 5% normal goat-serum solution for 20 min at room temperature to prevent non-specific binding. Slides were incubated overnight at 4 °C with monoclonal anti-phosphorylated neurofilament antibody (SMI-31; 1:1000, Covance, US) to stain non-injured axons or with rabbit anti-myelin basic protein (MBP) antibody (1:1000, Sigma Inc., MO, US) to stain myelin sheaths (Budde et al., 2009; Song et al., 2003; Sun et al., 2008). After rinsing, goat anti-mouse IgG or goat anti-rabbit IgG conjugated Alexa 488 (1:800) was applied to visualize immunoreactivity of phosphorylated neurofilament and MBP. Finally, slides were covered using Vectashield Mounting Medium with 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratory, Inc., Burlingame, CA) to stain cell nuclei (Budde et al., 2009; Wang et al., 2011). Histological slides were examined with a Nikon Eclipse 80i fluorescence microscope equipped with a 60 × water objective, and images from the center of optic nerve were captured with a black-and-white CCD camera with MetaMorph software (Universal Imaging Corporation, Sunnyvale, CA). The 60 × IHC images (0.018 mm²) covered ~40% of the whole optic nerve area (0.045 ± 0.013 mm², mean ± SD). Histological counts are reported for 7 EAE-affected and 6 sham optic nerves in this study (one sham optic nerve specimen was damaged during tissue processing). Whole-field SMI-31, MBP, and DAPI images at 60 × magnification were captured with the same fluorescence light intensity and exposure time. All captured images were converted to 8-bit gray scale and analyzed using threshold, particle analyzer and gray level watershed segmentation functions in ImageJ (http://bigwww.epfl.ch/sage/soft/watershed/).

**Statistical analysis**

ADCc changes (initial/baseline vs. stimulus on, stimulus on vs. stimulus off, etc.) were tested for statistical significance via a cluster linear repeated measures model, which took into account that there were three time points for each eye and two eyes per mouse. The p-values of visual function (VA), SMI-31, MBP, and DAPI were from nonparametric Wilcoxon two-sample tests for difference between sham and EAE groups. The correlation coefficients for VA with each of SMI-31, MBP, DAPI, and decreased ADCc were analyzed by Spearman’s rank correlation coefficient.

**Results**

**Impairment of visual function and reduced activation-associated ADCc response in EAE optic nerves**

For animals in the EAE group, the onset of optic neuritis, defined as VA ≤ 0.25 c/d (Chiang et al., 2012), occurred in the range from 8 to 13 days post-immunization (10.6 ± 1.7 days post-immunization, mean ± SD). Group-averaged visual acuities of the EAE-affected (optic neuritis), sham and blocked eyes (n = 7, 7, and 14, respectively) were 0.15 ± 0.08, 0.36 ± 0.02, and 0.36 ± 0.03 c/d (mean ± SD, Fig. 2A). Since all EAE-affected mice in this study developed unilateral visual impairment, the VA of blocked eyes (n = 14) were in the normal range. VA of EAE-affected eyes with optic neuritis showed a significant 58% decrease (p < 0.005, EAE vs. sham) in measurements just prior to diffusion fMRI. ADCc maps were generated from the diffusion-weighted image data (Fig. 1). The group-averaged baseline optic-nerve ADCc of affected acute-stage EAE optic neuritis eyes was higher than in sham eyes (Figs. 2B and C, Table 1). Compared to their own pre-stimulus baseline and stimulus off measurements, ADCc during visual stimulus of EAE-affected versus sham eyes showed no significant (7%, p = 0.45, vs. control) difference.
Axonal impairment was assessed by immunohistochemistry (IHC) staining

Previously, we have employed immunohistochemical (IHC) staining of phosphorylated neurofilaments (SMI-31, intact axons), myelin basic protein (MBP, myelin sheath), and 4′,6-diamidino-2-phenylindole (DAPI, cell nuclei) to reflect white-matter integrity (Song et al., 2003; Sun et al., 2007; Wang et al., 2011). Representative zoomed-in 60 × images (56% of the 60 × field of view) from EAE-affected (n = 7) optic nerves demonstrated axonal beading, with lower density of intact axons, less myelin positive area (with thinner myelin), and higher density of cell nuclei (Figs. 3A, D, and G) than sham optic nerves (n = 6).

Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>Stimulus on</th>
<th>Stimulus off</th>
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<tr>
<td>EAE (n=7)</td>
<td>0.200 ± 0.012</td>
<td>0.186 ± 0.015</td>
<td>0.207 ± 0.012</td>
</tr>
<tr>
<td>Sham (n=7)</td>
<td>0.171 ± 0.014</td>
<td>0.128 ± 0.008</td>
<td>0.164 ± 0.016</td>
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<tr>
<td>Blocked (n=14)</td>
<td>0.188 ± 0.006</td>
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ADC (μm²/ms or ×10⁻⁷ mm²/s): mean ± standard error of the mean.

Discussion

This is the first study applying diffusion fMRI to assess functional deficits of a white-matter tract in vivo. The results presented here demonstrate an attenuated neuronal-activation-associated ADC response (~7% non-significant reduction) in EAE mice at the onset of optic neuritis, compared to a significant decremental response in sham mice (~25% reduction). The results for EAE sham mice are comparable to our

**Fig. 2.** Group-averaged visual acuity was significantly decreased by 58% (p < 0.005, vs. sham) in affected EAE eyes (n = 7) compared with the normal vision of sham eyes (n = 7); the visual acuity of contralateral blocked (n = 14) eyes was in the normal range (A). In this mouse model of EAE optic neuritis, visual deficits typically develop asymmetrically, beginning in only one eye. An activation-associated ADC decrease was observed in both EAE and sham eyes but not in blocked eyes (B, C, and Table 1), suggesting that the decreased ADC in stimulated EAE and sham optic nerves was caused by axonal activity. When compared to its own baseline and stimulus-off, ADC significantly decreased in sham optic nerves by 25 and 22%, respectively (both p < 0.005, B, C, and Table 1), with visual stimulation. In contrast, only a slight and non-significant ADC decrease (7%, p = 0.45, vs. baseline and 10%, p = 0.13; vs. stimulus-off) was observed in EAE optic nerves (B, C, and Table 1). Group ADC maps of EAE (n = 7) and sham (n = 7) optic nerves were generated by averaging stacked ADC maps, which were interpolated to 1024 × 1024 (C). * indicates p < 0.005.

**Table 2**

<table>
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ADC (μm²/ms or ×10⁻⁷ mm²/s): mean ± standard error of the mean.

**Reduced activation-associated ADC in EAE optic neuritis was correlated with impaired visual function and optic nerve pathologies**

The correlation between activation-associated ADC response and visual acuity was found when pooling EAE and sham groups under our experimental criteria (Fig. 4A, r = 0.76, p = 0.0015), axon density (Fig. 4B, r = 0.92, p < 0.0001), and myelin sheath area (Fig. 4C, r = 0.76, p = 0.0023) and, to a lesser degree, with cell nuclei density (Fig. 4D, r = 0.60, p = 0.03). The data suggest that diffusion fMRI can provide a means to non-invasively and directly assess axonal dysfunction associated with axonal injury, demyelination, and inflammation at optic neuritis onset in this EAE mouse model.
previously-reported ~27% ADC decrease in optic nerves of normal mice (Spees et al., 2013). Reduced activation-associated ADC decrease in association with impaired visual function (Fig. 4A) correlated with underlying optic-nerve axonal pathology (Fig. 3).

Visual signal conduction through the optic nerve depends on repeated cycles of membrane depolarization/action potential/regeneration and repolarization, which are accompanied by ion gradient changes (Nicholls et al., 2001; Zhang et al., 2006). Unlike BOLD fMRI, which is largely limited to gray matter (Gawryluk et al., 2009; Logothetis et al., 2001), we proposed that diffusion fMRI could detect the effect of functional activation of axons and further improve our understanding of CNS functionality. Extracellular space (ECS) shrinkage during electrical stimulation has been reported (Holthoff and Witte, 1996; Nicholson and Sykova, 1998; Ransom and Orkand, 1996; Ransom et al., 1985), such ECS shrinkage is highly associated with transient ionic gradient changes, osmotic shifts of water across cell membranes, or glial cell swelling associated with changes in size and shape (Bay and Butt, 2012; Flint et al., 2009; Kole et al., 2008; Le Bihan, 2007; Le Bihan and Johansen-Berg, 2012; Schwartzkroin et al., 1998; Waxman, 1977). We speculated that this may be due to that ADC decrease is likely to be affected more by the intra-axonal water than that in the ECS. Hence, diffusion fMRI measuring ADC of axonal bundles has the potential to directly probe axon functional integrity without confounding blood-flow effects.

For MS and its animal model, EAE, mitochondrial impairment reduces energy production due to increased nitric oxide and free radical/reactive oxygen species (Smith and Lassmann, 2002). Sodium channel redistribution in demyelinated axons is required to maintain signal propagation but this also increases energy consumption (Holthoff and Witte, 1996; Nicholson and Sykova, 1998; Ransom and Orkand, 1996; Ransom et al., 1985), such ECS shrinkage is highly associated with transient ionic gradient changes, osmotic shifts of water across cell membranes, or glial cell swelling associated with changes in size and shape (Bay and Butt, 2012; Flint et al., 2009; Kole et al., 2008; Le Bihan, 2007; Le Bihan and Johansen-Berg, 2012; Schwartzkroin et al., 1998; Waxman, 1977). We speculated that this may be due to that ADC decrease is likely to be affected more by the intra-axonal water than that in the ECS. Hence, diffusion fMRI measuring ADC of axonal bundles has the potential to directly probe axon functional integrity without confounding blood-flow effects.

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Table 2

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<th>SMI-31 (#/mm²)</th>
<th>MBP (area %)</th>
<th>DAPI (#/mm²)</th>
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<tr>
<td>EAE</td>
<td>5.3 ± 0.6 × 10⁵</td>
<td>36.2 ± 2.5</td>
<td>5.8 ± 1.2 × 10⁵</td>
</tr>
<tr>
<td>Sham</td>
<td>6.8 ± 0.5 × 10⁵</td>
<td>43.6 ± 2.1</td>
<td>4.3 ± 0.5 × 10⁵</td>
</tr>
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Mean ± standard deviation.
associated ADC decrease, which likely results from ECS shrinkage during axonal activation, was less in EAE-affected optic nerves than in sham mice optic nerves during visual stimulation.

In this study, visual acuity measurements in EAE and sham eyes represent overall function of the entire visual efferent pathway (Douglas et al., 2005; Prusky et al., 2004). If we accept that the VA measurement reflects overall electrophysiological competence as measured by VEP (Ridder and Nusinowitz, 2006), and that the optic nerve is the primary site of impaired visual function in this EAE animal model, then the VA measurement provides an indirect measure of optic nerve electrical competence. As expected, the VA in EAE optic neuritis eyes was significantly lower than that in sham eyes (Fig. 2A) in the current study. Subsequent histological results of these same optic nerves revealed axonal injury, demyelination, and inflammation (Figs. 3C, F, and I), suggesting that these optic-nerve-specific pathologies were a primary source of the impaired visual function in EAE mice.

Based on VEP measurement in the mouse visual system, the optimal visual stimulation condition in C57BL/6 mice has been determined (Ridder and Nusinowitz, 2006) and applied to this study. During diffusion measurement with visual stimulation, decreased ADC and unchanged ADC were observed in unblocked and blocked normal (sham) eyes, respectively (Figs. 2B and C), suggesting that the ADC directly revealed the effect of functional activation in optic nerves. For the unblocked eyes, the ADC change in EAE-affected optic nerves was less than that in sham optic nerves and provides some measure of the extent of axonal dysfunction (Figs. 2B and C).

Since contributions from retinal dysfunction at the early time point measured herein may (Quinn et al., 2011; Shindler et al., 2008) or may not (Meyer et al., 2001) be ruled out, the RGC (retinal ganglion cell) histological data of our own EAE model were considered before this study. The RGC counting results of sham and EAE-affected groups at these early time-points were no different, and suggested that RGC pathologies may occur later in the progression of optic neuritis (Chiang et al., unpublished). Therefore, the attenuated diffusion fMRI response (reduced ADC decrease with stimulation) is likely a direct result of axonal dysfunction. The somewhat elevated baseline ADC in EAE optic neuritis (Table 2) may result from acute demyelination or vasogenic edema since radial diffusivity or ADC increases reflecting those two pathologies have been reported in EAE mice and MS patients (Borelius et al., 2008; Castriota-Scanderbeg et al., 2002; Chiang et al., 2012; Hickman et al., 2004; Song et al., 2005; Sun et al., 2007).

Our results showed that the functional deficits in EAE-affected optic nerves using diffusion fMRI provide a unique perspective on the consequences of inflammatory demyelinating pathologies. Since the EAE optic neuritis nerves at the acute stage exhibit multiple coexisting pathologies, it is not possible to determine contributions of each specific type of tissue damage to the overall reduced activation-associated ADC response. Consequently, pre-onset study for EAE optic neuritis would be needed to understand if functional deficits precede morphological changes and the inter-relationship between mild injury and diffusion fMRI activation. In vivo diffusion fMRI is a promising tool for direct investigations of evolving pathology, therapeutic targets, and/or longitudinal assessment in white-matter tracts.

Acknowledgments

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Diffusion basis spectrum imaging detects and distinguishes coexisting subclinical inflammation, demyelination and axonal injury in experimental autoimmune encephalomyelitis mice

Xiaojie Wang\textsuperscript{a}, Matthew F. Cusick\textsuperscript{b}, Yong Wang\textsuperscript{c}, Peng Sun\textsuperscript{c}, Jane E. Libbey\textsuperscript{b}, Kathryn Trinkaus\textsuperscript{d}, Robert S. Fujinami\textsuperscript{b} and Sheng-Kwei Song\textsuperscript{c,e,*}

Clinicopathological paradox has hampered significantly the effective assessment of the efficacy of therapeutic intervention for multiple sclerosis. Neuroimaging biomarkers of tissue injury could guide more effective treatment by accurately reflecting the underlying subclinical pathologies. Diffusion tensor imaging-derived directional diffusivity and anisotropy indices have been applied to characterize white matter disorders. However, these biomarkers are sometimes confounded by the complex pathologies seen in multiple sclerosis and its animal models. Recently, a novel technique of diffusion basis spectrum imaging has been developed to quantitatively assess axonal injury, demyelination and inflammation in a mouse model of inflammatory demyelination. Lenaldekar, which inhibits T-cell expansion in a non-cytolytic manner, has been shown to suppress relapses and preserve white matter integrity in mice with experimental autoimmune encephalomyelitis. In this study, relapsing–remitting experimental autoimmune encephalomyelitis was induced through active immunization of SJL/J mice with a myelin proteolipid protein peptide. The therapeutic efficacy of Lenaldekar treatment was evaluated via daily clinical score, cross-sectional ex vivo diffusion basis spectrum imaging examination and histological analysis. Lenaldekar greatly reduced relapse severity and protected white matter integrity in these experimental autoimmune encephalomyelitis mice. Diffusion basis spectrum imaging-derived axial diffusivity, radial diffusivity and restricted diffusion tensor fraction accurately reflected axonal injury, myelin integrity and inflammation-associated cellularity change, respectively. These results support the potential use of diffusion basis spectrum imaging as an effective outcome measure for preclinical drug evaluation. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: multiple sclerosis; diffusion MRI; axonal injury; inflammation; demyelination; diffusion basis spectrum imaging (DBSI); Lenaldekar; experimental autoimmune encephalomyelitis (EAE)

INTRODUCTION

Multiple sclerosis is an inflammatory demyelinating disorder of the central nervous system (CNS) characterized by lymphocytic infiltration and axon and myelin injury [1]. Although inflammatory demyelination is the hallmark of white matter pathology, axonal injury is now widely accepted to play a significant role in irreversible neurological disability [1,2]. Currently, an efficacious therapy to stop the progressive neurodegeneration in multiple sclerosis is not available.

Myelin-specific autoreactive T cells mediate early lesion formation [3] and correlate with the extent of acute axonal injury in multiple sclerosis [4]. Thus, autoreactive T-cell modulation may be a potential therapeutic approach for human multiple sclerosis. Lenaldekar (LDK), 1H-indole-3-carbaldehyde quinolin-8-yl-hydrazone, is an anti-leukemia agent proven to effectively...
eliminate immature T cells (5). It has been shown to inhibit relapses and to reduce demyelination in mice with experimental autoimmune encephalomyelitis (EAE) (6).

With promising new therapies for multiple sclerosis on the horizon, novel neuroimaging modalities providing quantitative assessment of CNS axonal integrity are needed to non-invasively assess treatment efficacy. Conventional MRI has revolutionized the diagnosis of multiple sclerosis. Nevertheless, it is hardly quantitative and lacks specificity with regard to the differentiation of complicated pathologies. Advanced MR methods, such as myelin water imaging (7) and magnetization transfer ratio (8), have emerged to reflect myelin integrity in multiple sclerosis. Diffusion tensor imaging (DTI) has shown promise for the differentiation between axon and myelin pathologies through changes in axial diffusivity (λ∥ describing water diffusion parallel to axons) and radial diffusivity (λ⊥ describing water diffusion perpendicular to axons) (9,10). However, the interpretation of these DTI findings is confounded by the presence of inflammation (11,12), tissue loss (13), crossing fibers (14) and cerebrospinal fluid contamination (15,16). A novel diffusion basis spectrum imaging (DBSI) approach has been developed to resolve crossing fiber tracts, remove cerebrospinal fluid partial volume effects and quantitatively assess axonal injury, demyelination and inflammation in a mouse model of cuprizone-induced inflammatory demyelination (17).

In this study, the therapeutic efficacy of LDK treatment was assessed by treating EAE mice at the first relapse. Spinal cords from EAE and control mice were examined using diffusion MRI and immunohistochemistry (IHC). Significant preservation of myelin and axonal integrity was observed by DBSI-derived λ∥ and λ⊥ in LDK-treated mice, the extent of inflammation was also accurately reflected by DBSI-derived cellularity. DBSI derived λ∥, λ⊥ and cellularity correlated with IHC findings. In contrast, the DTI findings did not accurately reflect the treatment effect. Our studies indicate that DBSI has major advantages over DTI.

MATERIALS AND METHODS

DBSI

The diffusion MRI signals were analyzed according to Equation [1] (17):

$$S_k = \sum_{i=1}^{N_{noise}} f_i e^{-|b_i|\Delta} e^{-|b_i|\left((\lambda_{\parallel}\lambda_{\perp})\cos^2\psi\right)}$$

$$+ \int_0^\infty f(D) e^{-|b_i|D} dD \quad (k = 1, 2, 3, ...)$$

In Equation [1], $S_k$ and $|b_i|$ are the diffusion-weighted MR signal and b value of the kth diffusion gradient, $N_{noise}$ is the number of anisotropic tensors (reflecting fibers), $\psi$ is the angle between the kth diffusion gradient and the principal direction of the kth anisotropic tensor, $\lambda_{\parallel}$ and $\lambda_{\perp}$ are the axial and radial diffusivities of the ith anisotropic tensor, $f_i$ is the signal intensity fraction for the ith anisotropic tensor, and a and b are the low and high diffusivity limits for the isotropic diffusion spectrum (reflecting cellularity and edema) f(D). In the present study, a single anisotropic tensor (i.e. $N_{Aniso} = 1$) fitted the spinal cord white matter well. Thus, the derived $\lambda_{\parallel}$ and $\lambda_{\perp}$ were interpreted similarly to those derived by DTI, but without extra-axon isotropic tensor components confounding the measurements. The isotropic diffusion spectrum was tentatively divided on the basis of the apparent diffusion coefficient (ADC) into three components representing cellularity (restricted diffusion), vasogenic edema (hindered diffusion) and cerebrospinal fluid or tissue loss (free diffusion).

Animal preparation

To induce EAE, 15 female SJL/J mice (Jackson Laboratory, Bar Harbor, ME, USA) were injected subcutaneously with 200 µg/mL of myelin proteolipid protein (PLP) peptide (PLP139-151) in complete Freund’s adjuvant (CFA) consisting of incomplete Freund’s adjuvant (Pierce Biotechnology, Rockford, IL, USA) containing Mycobacterium tuberculosis H37 Ra (2 mg/mL) (Difco Laboratories, Detroit, MI, USA). On days 0 and 2 following immunization, mice were injected intravenously with 100 µL of Bordetella pertussis with an initial concentration of 1.0 × 10¹¹ organisms/mL (Michigan Department of Public Health, Lansing, MI, USA). Five additional mice, which received only CFA in the absence of PLP139-151, served as age- and sex-matched controls (sham group). Mice were scored daily for clinical signs using a standard 0–5 scoring system: 1, limp tail; 2, hind limb weakness sufficient to impaire righting; 3, one limb paralyzed; 4, two limbs paralyzed; 5, three or more limbs paralyzed or the animal is moribund (mice were killed if they reached grade 5). At the first remission [clinical score (CS) ≤ 0], five mice were killed and subjected to intra-cardiac perfusion fixation using 0.01 M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1M PBS. Vertebral columns were excised and post-fixed in the same fixative overnight, and then transferred to 0.1M PBS. Starting at the first relapse (CS ≥ 0.5), the remaining ten EAE mice were injected intraperitoneally with 40 mg/kg/day of LDK (n = 5) or vehicle [dimethyl sulfoxide (DMSO), n = 5] until the study end point when all EAE mice were at the second remission (CS ≥ 0). All LDK- and vehicle-treated EAE and sham control mice were killed and perfusion fixed at the end point of the study.

MRI

For ex vivo MRI scans, excised vertebral columns were placed in 1-ml syringes filled with 0.01 M PBS. A solenoid coil of 8 mm in diameter and 25 mm in length was used for data acquisition. Ex vivo diffusion MRI examinations were performed on a 4.7-T Agilent DirectDrive small-animal MRI system (Agilent Technologies, Santa Clara, CA, USA) equipped with Magnex/Agilent HD imaging gradients (Magnex/Agilent, Oxford, UK). The magnet, gradient coil and gradient power supply were interfaced with an Agilent DirectDrive console (Agilent Technologies) controlled by a Linux workstation.

A sagittal image of the mouse vertebral column was acquired using a gradient echo sequence to visualize vertebral disks, as references to plan the target axial images. A diffusion-weighted, multi-echo spin-echo imaging sequence (18) was employed to acquire diffusion-weighted images of eight contiguous transverse slices covering T12 to L2 vertebrae. The acquisition parameters were as follows: field of view, 9 × 9 mm²; data matrix, 128 × 128 (resulting in voxel dimensions of 70 × 70 µm², zero filled to 35 × 35 µm²); TR = 1,0 s; TE = 38 ms; Δ = 20 ms; δ = 5 ms; slice thickness, 1.0 mm; 99 diffusion-encoding directions prescribed by placing the position vectors at the grid points ($q_\alpha, q_\beta, q_\gamma$) in the three-dimensional q space assuming that ($q_\alpha^2 + q_\beta^2 + q_\gamma^2$) ≤ r², where r = 3 (17). The maximum b value was 3000 s/mm². The total data acquisition time was 3 h and 31 min.
**Histological analysis**

Following *ex vivo* imaging, mouse vertebral columns were decalcified for 48 h and then embedded in paraffin. The embedded tissues were sectioned using a sliding microtome set at a thickness of 5 μm. The slides were then deparaffinized, rehydrated and blocked using 1% bovine serum albumin and 5% goat serum in 0.01 M PBS for 30 min at room temperature. Myelin and axonal integrity were assessed by incubating the processed slides with polyclonal anti-myelin basic protein (anti-MBP; 1:500 dilution; Sigma Chemical Company, St. Louis, MO, USA) and monoclonal anti-phosphorylated neurofilaments (SMI31; 1:1000 dilution; Convance, Emeryville, CA, USA) antibodies, respectively, at 4°C overnight. After rinsing in 0.01 M PBS for 30 min, the slides were incubated with Alexa Fluor 488 conjugated goat-anti-mouse IgG (H+L) (1:400 dilution; Invitrogen Co., Camarillo, CA, USA) antibody for 1 h at room temperature to visualize immunoreactive materials. After washing in 0.01 M PBS for 30 min, the slides were mounted in Vectashield medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratory, Burlingame, CA, USA). DAPI stains cell nuclei. It was used to validate the reliability of the DBSI-derived restricted isotropic diffusion component as a cellularity marker. Increased cellularity reflects the extent of inflammation as it is commonly seen in CNS inflammation. Images were acquired with a Hamamatsu NanoZoomer 2.0-HT System (Hamamatsu, Shizuoka Prefecture, Japan).

The whole field of each staining (SMI31, MBP or DAPI) image at 40× magnification was captured with the same fluorescence light intensity and exposure time. Using ImageJ [http://rsbweb.nih.gov/ij/], all captured images were converted to 8-bit gray scale and subjected to background subtraction followed by Gaussian blurring (DAPI images) or edge preservation (SMI31 and MBP images). Then, the DAPI and SMI31 images were segmented using the gray-scale watershed algorithm, quantified using the ‘analyze particles’ function following threshold. The MBP images, however, were quantified directly using the ‘analyze particles’ function following automatic threshold without segmentation.

**Data analysis**

The diffusion-weighted MR data were analyzed via a DTI/DBSI analysis package developed in house using MatLab* (MathWorks, Natick, MA, USA). The ventral–lateral white matter (VLWM) area was manually delineated as the region of interest (ROI) on the 0 (non-diffusion-weighted) image using ImageJ with customized tools. DTI-derived λ∥, λ⊥, and relative anisotropy (RA) maps were used as references whilst defining VLWM ROIs. Group-averaged DTI- and DBSI-derived parameters, SMI31-positive axon density, DAPI-stained cell density and MBP-positive region fraction were compared for the VLWM region between sham control mice, mice with EAE at first remission and mice with EAE at second remission with LDK and vehicle treatment. Wilcoxon rank-sum test was used to determine whether any differences existed among these groups, and a significance level of 0.05 was used for all tests. The p values were adjusted using the false discovery rate to account for the large number of tests. Spearman’s rank correlation was used to test for the presence of a monotonic increase or decrease between IHC results and DTI or DBSI parameters. All data are expressed as the mean ± standard deviation.

**RESULTS**

**LDK prevented EAE relapse**

The initial neurological disability associated with an acute attack of EAE in SJL/J mice was observed between days 10 and 12 post-immunization with PLP139–151; the first relapse occurred at days 25–29, and remitted at days 36–37 in vehicle-treated EAE mice (Fig. 1, filled squares). Five EAE mice were killed after the first remission (at day 21). LDK or vehicle treatment was initiated on the day of the observed EAE relapse (CS ≥ 0.5, day 26) and was continued daily thereafter. The treatment continued until the second remission of all EAE mice (day 37). LDK effectively ameliorated EAE relapse. The mean peak CS during the relapse of the LDK-treated group was 0.8 ± 0.8, whereas that of the vehicle-treated group was 2.8 ± 0.4. At day 30 post-immunization and

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**Figure 1.** Clinical score time course of Lenaldekar (LDK)- and vehicle-treated experimental autoimmune encephalomyelitis (EAE) mice. Female SJL/J mice were immunized with myelin proteolipid protein (PLP) peptide (PLP139–151) in complete Freund’s adjuvant (CFA). On EAE relapse at day 26, the mice were injected with LDK (40 mg/kg/day, open circles) or vehicle [dimethyl sulfoxide (DMSO), filled squares] once a day for days 26–37. The mean clinical score was lower in the LDK-treated group during EAE relapse. Data represent mean clinical scores ± standard derivation for groups of five mice.
beyond, none of the LDK-treated mice showed any clinical signs of disease (Fig. 1, open circles), whereas the vehicle-treated mice still showed significant clinical signs of disease (Fig. 1, filled squares).

**DTI detected axonal and myelin injury at remission**

Color-coded DTI maps of the VLWM region were overlaid on T2-weighted images (Fig. 2). White matter tract lesions were detected on representative DTI parameter maps at the T13 vertebral level of the spinal cord from EAE mice at the first and second remission (Fig. 2). At the first remission, before treatment, decreased $\lambda_1$ and RA, as well as increased $\lambda_\perp$, were visible in the VLWM regions (arrows, second column, Fig. 2). At the second remission following LDK treatment, normal-$\lambda_1$ of the LDK-treated mouse resulted in an apparent RA decrease in the same region (arrows, fourth column, Fig. 2).

**DBSI detected axon/myelin injury and inflammation at remission**

The VLWM of spinal cords from the same representative mice as shown in Fig. 2 was examined using DBSI. Compared with the sham control spinal cord (first column, Fig. 3), the EAE spinal cord exhibited moderately decreased $\lambda_1$ in the VLWM at the first remission (arrow, second column, Fig. 3). Increased $\lambda_\perp$ was also observed in the same representative spinal cord (arrows, second column, Fig. 3). At the second remission, increased patchy lesions can be seen in the VLWM of the vehicle-treated mouse, marked by significantly decreased $\lambda_1$ and increased $\lambda_\perp$ (arrows, third column, Fig. 3). In addition, a substantial increase in restricted and hindered diffusion fraction can be seen in the vehicle-treated mouse spinal cord at the second remission (arrows, third column, Fig. 3). With therapeutic LDK treatment (fourth column, Fig. 3), all DBSI parameters of the representative mouse spinal cord exhibited an overall improvement compared with those of the vehicle-treated mouse. Residual patches of lesions remained visible in the $\lambda_1$ map.

**LDK preserved axons, reduced inflammation and prevented demyelination**

In Fig. 4, SMI31 and DAPI double-stained images are shown for the same representative spinal cord from each group at the T13 vertebral level. At the first remission, massive meningeal and perivascular (arrows) cell infiltration was seen in the VLWM (Fig. 4b). At the second remission, a markedly increased and diffuse cell population was observed in the representative vehicle-treated spinal cord, coinciding with substantial loss of SMI31-positive axons (Fig. 4c). In contrast, the representative LDK-treated spinal cord at the second remission exhibited significantly decreased cellularity and increased SMI31-positive axon staining (Fig. 4d). Nevertheless, meningeal cell aggregation (arrows) was still seen in the LDK-treated spinal cord (Fig. 4d). MBP and DAPI double-stained images (Fig. 5) revealed moderate demyelination at the proximity of the meninges with massive cell aggregation at the first remission (Fig. 5b). At the second remission, progression of myelin damage was apparent in the vehicle-treated spinal cord (Fig. 5c). Myelin staining of the spinal

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**Figure 2.** Representative diffusion tensor imaging (DTI) maps. Color-coded relative anisotropy (RA), $\lambda_1$ and $\lambda_\perp$ maps of the ventral–lateral white matter (VLWM) region were overlaid on gray-scale T2-weighted images. One set of DTI maps is shown for one representative mouse spinal cord at the T13 level from each of the four groups. At the first remission, a decrease in RA was detected at the VLWM, especially in perivascular regions (arrows). Decreased $\lambda_1$ and increased $\lambda_\perp$ were also detected in the same areas. At the second remission, the vehicle-treated mouse exhibited markedly decreased RA and $\lambda_1$ as well as increased $\lambda_\perp$. The perivascular and meningeal white matter areas were most severely injured, which formed a ‘hypointense border’ lining the RA and $\lambda_1$ maps. In contrast, the Lenaldekar (LDK)-treated mouse exhibited normalized $\lambda_1$ at the second remission. However, slightly increased $\lambda_\perp$ was still seen at the VLWM, resulting in decreased RA (arrows). CS, clinical score.
cord appeared normal in the representative LDK-treated EAE mouse at the second remission, whereas perivascular and meningeal cell infiltration was still visible (arrows, Fig. 5d).

**Group analysis: axonal injury**

Because of the exclusion of the isotropic restricted diffusion tensor component with lower apparent diffusion coefficient, the DBSI-derived $\lambda_||$ was systematically higher than that derived by conventional DTI (Fig. 6a, b). Axonal injury at the first remission was evidenced by the decreased $\lambda_||$ derived by DTI (Fig. 6a) and DBSI (Fig. 6b), as well as by the reduction in the SMI31-positive axon density (Fig. 6c), in mice with EAE. However, none of these differences reached statistical significance compared with control (sham). At the second remission, DTI/DBSI-derived $\lambda_||$ and SMI31-positive axon density decreased further in the vehicle-treated EAE mice. The difference between the first and second remission vehicle-treated groups was statistically significant for SMI31-positive axon density. Neither DTI- or DBSI-derived $\lambda_\perp$ in the LDK-treated group at the second remission decreased as seen in the vehicle-treated group. Higher DTI- and DBSI-derived $\lambda_\perp$ was seen in the LDK-treated group than in the vehicle-treated group at the second remission without reaching statistical significance. This is consistent with a significantly higher SMI31-positive axon density in the LDK-treated group compared with the vehicle-treated group at the second remission.

**Group analysis: demyelination**

Significantly increased $\lambda_\perp$ derived by both DTI and DBSI was seen in EAE mice at the first remission compared with the sham group (Fig. 6d, e). At the second remission, the vehicle-treated EAE mice showed comparable $\lambda_\perp$ relative to the mice at the first remission (Fig. 6d, e). However, mice treated with LDK showed a lower $\lambda_\perp$ at the second remission than non-treated EAE mice at the first remission and vehicle-treated EAE mice at the second remission. In line with these DTI- and DBSI-derived $\lambda_\perp$ findings, reduced MBP staining was seen in EAE groups at the first and second remission (Fig. 6f). LDK treatment improved the myelin integrity at the second remission compared with vehicle treatment, but this improvement did not reach statistical significance.

**Group analysis: inflammation**

The DBSI-derived fraction of the restricted isotropic diffusion tensor component was increased significantly in EAE mice without treatment at the first remission and in the vehicle-treated group at the second remission, compared with the sham group (Fig. 6g). In the LDK-treated EAE mice, the restricted isotropic diffusion tensor component fraction was decreased compared with that of the first and second remission vehicle-treated group, although statistical significance was not reached. This is consistent with the DAPI-positive nuclear staining results (Fig. 6h).

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**Figure 3.** Representative diffusion basis spectrum imaging (DBSI) maps. Color maps of $\lambda_||$, $\lambda_\perp$, and restricted and hindered diffusion fractions at the T13 level for the same representative mouse spinal cords as shown in Fig. 2. Compared with the sham mouse, the experimental autoimmune encephalomyelitis (EAE) mouse showed moderately decreased $\lambda_||$ in the ventral–lateral white matter (VLWM) at the first remission. An observed increase in $\lambda_\perp$ was detected in the representative spinal cord at this time point (arrows). At the second remission, a further decrease in $\lambda_||$ and increase in $\lambda_\perp$ are readily detected in the representative vehicle-treated mouse. Patchy lesions were seen at the periphery of the VLWM, marked by significantly decreased $\lambda_||$ and increased $\lambda_\perp$ (arrows). A substantially increased fraction of restricted isotropic diffusion was overwhelmingly seen in the left VLWM. The fraction of hindered isotropic diffusion increased in the peripheral VLWM. With therapeutic Lenaldekar (LDK) treatment, all DBSI parameters were improved compared with those of the vehicle-treated group. The $\lambda_||$ and restricted and hindered water ratios of LDK-treated mouse spinal cord were comparable with those of the sham control spinal cord, yet $\lambda_\perp$ was still elevated. CS, clinical score.
Group analysis: correlating diffusion MRI findings with histology

DBSI-derived $\lambda_\parallel$ and $\lambda_\perp$ showed strong correlation with SM31-positive axon density and MBP-positive myelin area, respectively (Fig. 7a, b). The DBSI-derived restricted isotropic diffusion tensor component fraction correlated with the DAPI density (Fig. 7c). The DTI-derived $\lambda_\parallel$ did not correlate with SM31-positive axon counts (Fig. 7d). The DTI-derived $\lambda_\perp$, however, demonstrated correlation with MBP-positive myelin area with lesser significance than that of DBSI.

DISCUSSION

In this study, we verified the previously reported therapeutic efficacy of LDK in relapsing–remitting EAE in mice (6) by performing a cross-sectional examination of white matter integrity of the spinal cord using both ex vivo diffusion MRI and IHC. Diffusion MRI findings supported the utility of the newly developed DBSI technique to not only more accurately reflect axonal and myelin integrity, but also to assess the extent of inflammation. All animals at the time of examination exhibited no hind limb disabilities (CS = 0, Fig. 1) with various degrees of white matter pathology reflected by diffusion MRI (Figs. 2 and 3) and IHC (Figs. 4 and 5), suggesting the need for a biomarker to assess disease progression and treatment efficacy, overcoming the clinico-pathological paradox.

The sensitivity of in vivo DTI-derived $\lambda_\parallel$ and $\lambda_\perp$ to reflect IHC-detected axonal and myelin injury in EAE mouse spinal cord white matter has been reported (19–22). Consistent with previous reports, current ex vivo DTI-derived $\lambda_\parallel$ and $\lambda_\perp$ correctly reflected lesions detected by SM31 and MBP staining (Figs. 2, 4 and 5). Perivascular and meningeal lesions were seen in SM31/MBP images, as well as in DTI-derived $\lambda_\parallel$ and $\lambda_\perp$ maps, reflecting the hallmark EAE pathologies. The loss of SM31 and MBP staining largely appeared at the regions with increased DAPI staining, yet not all SM31/MBP lesions co-localized with positive DAPI staining. Similar to DTI, DBSI-derived $\lambda_\parallel$ and $\lambda_\perp$ also reflected SM31- and MBP-detected lesions in EAE mice (Figs. 3–5), suggesting a potential role for diffusion MRI as an outcome measure for the assessment of EAE spinal cord pathologies.

It has been reported that the presence of high cellularity decreases the DTI-derived $\lambda_\parallel$ (23). Thus, an exaggerated decrease in DTI-derived $\lambda_\parallel$ might result from the region in which axonal injury occurs with increased DAPI staining (i.e. increased cellularity associated with inflammation). This could explain the over-estimated axonal injury by DTI in the first remission group compared with the sham group (Fig. 6a). To take this confounding factor into account, DBSI models diffusion MRI signals as a linear combination of anisotropic (discrete axonal fiber tracts) and isotropic (various inter-axonal components reflecting vasogenic edema and cellularity) diffusion tensors. By removing the confounding effect from isotropic diffusion associated with inflammation or tissue loss, DBSI-derived fiber directional diffusivity more accurately reflects the diffusion characters of axonal tracts. Thus, there was a closer trend between DBSI-derived $\lambda_\parallel$ and SM31-positive axon density (Figs. 6b, c) than between DTI-derived $\lambda_\parallel$ and SM31-positive axon density (Figs. 6a, c). In addition, DBSI
enables the estimation of increased cellularity (restricted diffusion) and vasogenic edema (hindered isotropic diffusion) associated with inflammation. Although there is no IHC equivalent of vasogenic edema reflected by hindered isotropic diffusion, the restricted diffusion component offers a putative surrogate marker reflecting the extent of increased DAPI-positive nuclear counting (Figs. 3–5).

It is thought that relapsing–remitting EAE is initiated via perivascular and meningeal lymphocyte and neutrophil infiltration, followed by resolution of the inflammatory infiltrate and progression of axon and myelin damage (24). At the first remission, a significant increase in the DBSI-estimated restricted isotropic diffusion tensor fraction (Fig. 6g) and cell density (Fig. 6h) was observed. Meanwhile, we saw a decreased \( \lambda_{||} \) and increased \( \lambda_{\perp} \), derived by DBSI in EAE mice at first remission relative to the sham control (Figs. 6b, e). These changes in directional diffusivity were consistent with the observed decrease in SMI31-positive axon density (Fig. 6c) and MBP-positive area fraction (Fig. 6f), respectively. Thus, pathologically, both DBSI and IHC results suggest that moderate axon/myelin damage and only partial resolution of inflammation occurred at the first remission, although full functional recovery was suggested by clinical signs (CS = 0, Fig. 1).

The underlying mechanisms of remission following the acute paralytic attack are not well understood. However, it has been demonstrated that the EAE relapse is mediated predominantly by T cells, specific for endogenous myelin epitopes, which are activated as a result of myelin debris generated from acute inflammatory demyelination, a phenomenon known as epitope spreading (25, 26). At the second remission, further damage to the axons was detected in the spinal cord from vehicle-treated EAE mice using both DBSI (significantly decreased \( \lambda_{||} \) compared with that of the sham group) and histology assessment (significantly decreased SMI31-positive axon density compared with that of the first remission) (Figs. 6b, c). Interestingly, the extent of myelin injury seen in the spinal cord from vehicle-treated EAE mice at the second remission was comparable with that at the first remission (Figs. 6e, f). This might be caused by a potential remyelination process occurring in these EAE mice. Meanwhile, further increased cellularity was also observed in the vehicle-treated EAE mouse spinal cord at the second remission (Fig. 6h). Both DBSI and histology results suggested an increased mean cellularity in the VLWM region relative to the first remission, which did not reach statistical significance (Figs. 6g, h).

LDK was first found to be a potent inhibitor of immature leukemic T cells, where it dephosphorylated members of the phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway and delayed sensitive cells in late mitosis (5). Later, the efficacy of LDK in treating relapsing–remitting EAE in mice was demonstrated, and the mechanism was thought to possibly involve the modulation of highly active T cells in a non-cytolytic manner (6). Here, the treatment efficacy of LDK in the same EAE model was reproduced (Fig. 1). The current findings further demonstrated that cellularity in the LDK-treated group decreased at the second remission compared with that in the vehicle-treated group (Figs. 6g, h). Thus, an overall inflammation suppression effect can be reasonably inferred. In addition, remyelination was also suggested by the improved
myelin integrity detected by diffusion MRI and IHC after LDK treatment at the onset of EAE relapse (Figs. 6d–f). The improvement in DBSI-derived $\lambda_{||}$ and SMI31-positive axon density (c), myelin basic protein (MBP)-positive area (f) and 4',6-diamidino-2-phenylindole (DAPI)-stained cell density (h) were compared for the VLWM region between sham control, experimental autoimmune encephalomyelitis (EAE) mice at first remission and EAE mice at second remission with Lenaldekar (LDK) and vehicle treatment (five mice per group), as described in the Materials and Methods. DBSI-derived $\lambda_{||}$ (b) was systematically higher than DTI-derived $\lambda_{||}$ (a). LDK-treated mice showed higher DBSI-derived $\lambda_{||}$ (b) and SMI31-positive axon density (c), consistent with the preservation of axons, compared with vehicle-treated mice at the second remission time point. LDK-treated mice showed lower DTI- (d) and DBSI- (without statistical significance) (e) derived $\lambda_{\perp}$ compared with vehicle-treated mice at the second remission time point and with mice at the first remission time point. LDK treatment also resulted in higher MBP staining, consistent with improved myelin integrity, compared with vehicle-treated mice at the second remission time point and mice at the first remission time point. Meanwhile, LDK-treated mice showed reduced restriction diffusion and cell density compared with vehicle-treated mice at the second remission time point and mice at the first remission time point.

In summary, DBSI offered more accurate estimation of white matter integrity relative to DTI in this EAE model, reflected by significantly higher correlation with IHC results. Meanwhile, the...
DBSI-derived cellularity marker successfully characterized the inflammatory feature of this model and reflected the anti-inflammatory effect of LDK. The excellent linear correlation between the restricted diffusion fraction and DAPI count supports its use as a surrogate marker for cellularity in EAE, multiple sclerosis and other neuroinflammatory disease.

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