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Diffusion tensor imaging to assess axonal regeneration in peripheral nerves

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Abstract

Development of outcome measures to assess ongoing nerve regeneration in the living animal that can be translated to human can provide extremely useful tools for monitoring the effects of therapeutic interventions to promote nerve regeneration. Diffusion tensor imaging (DTI), a magnetic resonance based technique, provides image contrast for nerve tracts and can be applied serially on the same subject with potential to monitor nerve fiber content. In this study, we examined the use of *ex vivo* high-resolution DTI for imaging intact and regenerating peripheral nerves in mice and correlated the MRI findings with electrophysiology and histology. DTI was done on sciatic nerves with crush, without crush, and after complete transection in different mouse strains. DTI measures, including fractional anisotropy (FA), parallel diffusivity, and perpendicular diffusivity were acquired and compared in segments of uninjured and crushed/transected nerves and correlated with morphometry. A comparison of axon regeneration after sciatic nerve crush showed a comparable pattern of regeneration in different mice strains. FA values were significantly lower in completely denervated nerve segments compared to uninjured sciatic nerve and this signal was restored towards normal in regenerating nerve segments (crushed nerves). Histology data indicate that the FA values and the parallel diffusivity showed a positive correlation with the total number of regenerating axons. These studies suggest that DTI is a sensitive measure of axon regeneration in mouse models and provide basis for further development of imaging technology for application to living animals and humans.

Keywords

diffusion; nerve; MRI; DTI; regeneration

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Introduction

The need for objective and noninvasive diagnostic measures of axonal regeneration in peripheral nerves is increasingly realized as new therapeutic targets are identified and a large number of novel interventions are entering preclinical stage of drug development. In preclinical studies use of rodent models of nerve regeneration forms an important nodal point prior to advancing to clinical studies. With the availability of transgenic technology, mouse models have assumed importance in investigating the roles of specific therapeutic target genes/proteins in nerve regeneration. Morphological studies can provide definitive and quantitative measurement of regeneration in animal models, however, they are very labor intensive, cannot be used serially, and are unsuitable for high throughput screening studies. Detailed electrodiagnostic testing to measure regeneration is generally not feasible in animals. In humans nerve conduction studies are widely used but these studies are limited only to the distal parts of peripheral nerves and not suitable to measure regeneration early on and in response to proximal lesions (Kimura, 1999). Furthermore, after complete axonal injury, particularly as it relates to traumatic nerve injuries or proximal plexus lesions, the distal portions of nerves are often inexcitable, which limits electrodiagnostic testing's utility to assess the degree of regeneration in proximal nerve trunks.

In certain experimental and clinical settings, conventional MRI can be a useful technique to assess morphological changes after nerve injury (Bendszus and Stoll, 2005, Stoll and Bendszus, 2008). On T2-weighted (T2-w) images normal nerves in humans and animals usually appear isointense, and can be barely distinguished from surrounding fat and muscle tissue. In contrast, injured nerves are characterized by a prolongation of the T2-relaxation time, which results in a hyperintense signal (Bendszus and Stoll, 2005). If the damage is incomplete, the hyperintense signal eventually return to baseline level over weeks along with the regeneration of injured nerve fibers (Bendszus and Stoll, 2005). In humans, it is possible to differentiate peripheral nerves from surrounding fat tissue by use of fat-saturated heavily T2-w sequences, a technique which has been denoted as MR-neurography (Dailey, et al., 1997, Filler, et al., 1993, Filler, et al., 2004, Moore, et al., 2001). Although this technique allows the identification of injured nerves, based on changes in the hyperintensity on T2-w images, its utility is restricted due to several reasons including specificity for regeneration (Bendszus and Stoll, 2005). Some of these obstacles can be overcome by the application of a gadolinium-based MR contrast agent (gadofluorine M), which accumulates in degenerating nerve fibers and disappears along with the regrowth of nerve fibers (Bendszus and Stoll, 2005, Stoll, et al., 2006, Wessig, et al., 2008); however, because of toxicity concerns due to accumulation of this agent in injured nerves this contrast may not be suitable for use in humans.

Diffusion tensor imaging (DTI) is an emerging MR technique that allows the assessment of axonal integrity in neural tissues. DTI uses the random thermal motion of water molecules within tissues along different axes. These measurements are fitted into a 3D diffusion ellipsoid, called tensor model (Basser, et al., 1994, Beaulieu 2002, Le Bihan, 2003, Mori and Zhang, 2006), which allows the generation of several contrast parameters. Among these parameters are the fractional anisotropy (FA) and parallel and perpendicular diffusivities, which reflect the anisotropic diffusion of water molecules along and perpendicular to axon bundles, respectively. FA ranges from 0 to 1, with high FA values indicating highly anisotropic diffusion as in normal axonal tracts and low FA values more isotropic diffusion as in the CSF. Potential links between these DTI parameters and the nerve fiber integrity/content have been suggested by several experimental and clinical studies focusing on the measurement of axon degeneration. Stanisiz et al. (Stanisiz, et al., 2001) showed in *ex vivo* rat sciatic nerves that reduction in diffusion anisotropy can be observed at 7 days after crush or cut injuries in the sciatic nerves distal to the injury site. They also showed that diffusion

anisotropy increases as the axons regenerate in crushed sciatic nerve. A study published earlier this year shows the feasibility of DTI and associated tractography to measure axon regeneration in rat sciatic nerves (Takagi, et al., 2009). To assess the potential of DTI to measure regeneration in peripheral nerves, we compared DTI parameters with morphometric measures in a mouse model of peripheral nerve regeneration and used DTI-based tractography to visualize nerve regeneration.

Material and Methods

Study design

All experiments were carried out in accordance to the National Institute of Health Guide for the Care and Use of Laboratory Animals and local institutional review committee regulations. A total of 42 mice were used to compare DTI parameters in four different inbred mouse strains. Different strains were used to compare the broad applicability of the technique in mice. In 12- to 14-week-old C57BL/6 (n=4), PWK/PHj (n=10), WSB/EiJ (n=12), and DBA/2J (n=6) mice the left sciatic nerve was crushed 35 mm above the middle toe for 30 s with a fine forceps as described previously (Lehmann, et al., 2007). An epineurial 10-0 prolene suture was placed right above the crush site to exactly localize the position of the crush. On day 17, mice were perfused with 4% paraformaldehyde and the sciatic and tibial nerves were harvested. Changes in DTI parameters and morphology were also assessed 10 days after complete transection of sciatic nerves by imaging and histology in C57/B6 mice only (n=10). This set of studies was restricted to C57BL/6 mice because comparative studies indicated that all four strains had similar imaging/DTI characteristics. The imaging for this set of experiments was done on excised nerves. In separate studies DTI and tractography was performed on hind limbs (n=8) of normal uninjured C57BL/6 to determine whether normal peripheral nerves could be delineated from surrounding tissues in mice.

Electrophysiology

Compound muscle action potential (CMAP) amplitudes were recorded on day 9, 15 and 17 after nerve crush as described (Lehmann et al., 2007). Briefly, mice were anesthetized (1.5% isoflurane), and the sciatic nerves were stimulated at the sciatic notch (above the crush site) by needle electrodes. CMAPs were recorded in the hindpaw (sole) by needle electrodes using a PowerLab signal acquisition set-up (AD Instruments, Grand Junction, CO). Experiments were performed on a heating pad at 37°C to control the body temperature.

Diffusion tensor imaging (DTI) and image processing

Ex vivo imaging of excised sciatic nerves and hind limbs were performed on an 11.7 Tesla spectrometer equipped with a Micro2.5 gradient system (maximum gradient strength 120 Gauss/cm, Bruker Biospin, Billerica, MA, USA). During imaging, the excised nerves were embedded in 2% agarose gel and kept in a MR compatible plastic tube filled with fomblin (Fomblin Perfluoropolyether, Solvay Solexis, Inc., West Deptford, NJ, USA) to prevent dehydration. Hind limbs were kept in a MR compatible tube filled with fomblin. The temperature of the specimens was maintained at 37 °C via the spectrometer's temperature control system. Images were acquired using a 15-mm diameter birdcage coil as the radio frequency transmitter and signal receiver and a three dimensional diffusion weighted multiple spin echo sequence, with a repetition time (TR) of 600 ms, echo times (TE) of 27/37/47/57 ms (four spin echoes) and two signal averages. The native imaging resolution was 0.08 mm × 0.08 mm in the transverse plan and 0.1 mm along the nerve. Images reconstructed from the four spin echoes with 20% trapezoid apodization window were summed together to form one image. Two non-diffusion weighted images and six diffusion weighted images (*b* value = 1500 s/mm²) were acquired. The directions of diffusion

sensitizing gradients were the same as described (DeBoy, et al., 2007). The diffusion time was 15 ms, and the duration of the diffusion gradients was 6 ms. A T₂-weighted image was obtained by averaging the third and fourth echo images (TE = 47 ms and 57 ms respectively) acquired for the non-diffusion weighted images. An average diffusion weighted image (aDW) was obtained by averaging the six diffusion weighted images. The total imaging time was approximately 20 hours for each session. The signal-to-noise ratios measured in the non-diffusion weighted images was greater than 15 for all experiments. The diffusion tensor was calculated using a log-linear fitting method (Basser, et al., 1994, Basser and Pierpaoli, 1998). The fractional anisotropy (FA), parallel diffusivity ($\lambda_{||}$, the primary eigenvalue), and perpendicular diffusivity ($\lambda_{\perp}$, the average of the secondary and tertiary eigenvalues) were calculated on a voxel-by-voxel basis from diffusion tensor using DTIStudio (<http://www.mristudio.org>) (Jiang, et al., 2006). In the aDW images, the site of crush injury was first identified for each nerve. Regions of interest (ROIs) were placed at a 1 mm proximal to the crush site and 3 mm distal to the crush site. The mean FA, parallel diffusivity, and perpendicular diffusivity values in these ROIs were obtained. Tractography was performed using the DTIStudio software with a FA threshold of 0.5 (Xue, et al., 1999). For individual nerve, a seed ROI was manually placed in axial images at approximately 1 mm proximal to the crush site (or comparable location in control specimen) to reconstruct streamlines that pass through the seed ROI.

Morphometry

After imaging the nerves were immersion-fixed overnight and sciatic nerve segments ~3-5 mm distal to the crush site were embedded in Epon. One μ m cross sections were stained with toluidine blue as described previously (Lehmann, et al., 2007). For quantification, all myelinated axons in a single whole cross section of the nerve were counted at light level (40 $\times$) by using a motorized stage and stereotactic imaging software (Stereo Investigator, version 5).

Statistical analysis

For statistical analysis Kruskal-Wallis test with Dunn's multiple comparison test was used for multiple variables without Gaussian distribution. Nonparametric Spearman correlation test was used for correlation tests. $P < 0.05$ was considered statistically significant.

Results

We characterized axon regeneration after sciatic crush in 4 different inbred mice strains. Motor reinnervation was assessed by recording the CMAP amplitudes at the level of the hind paw. Electrophysiology showed a comparable pattern of CMAP amplitude recovery in all strains examined in this study, indicating a similar degree of motor axon regeneration. At day 9, no CMAP was detectable, confirming a complete crush with degeneration of all axons distal to the crush site, whereas at day 15 and day 17 reduced and dispersed CMAP amplitudes could be recorded in all animals, consistent with axon regeneration and reinnervation of muscles in the hind paw (Fig. 1). In crushed nerves conventional MRI and diffusion tensor measurements were obtained in sciatic nerve segments ~4 mm distal to the crush site. Epineurial sutures allowed accurate localization of the crush site. Nerve segments distal to the crush site were used for correlation of DTI and morphometry. A subset of nerves were transected and measured 10 days after transection. These two nerve injury paradigms were used for DTI and morphometry analysis because at day 10 after transection, all axons degenerate, whereas at day 17 after crush significant proportion of axons regenerate in the distal stump (Fig. 2A-C). We found that the average FA values were significantly lower in transected nerves compared to control nerves and crushed nerves (Fig. 2D).

The three eigenvalues λ_1 , λ_2 , and λ_3 were used to derive the parallel and perpendicular diffusivities. The primary eigenvalue λ_1 (or $\lambda_{||}$) refers to the extent of water diffusion parallel to the direction of axons, whereas the average of secondary (λ_2) and tertiary (λ_3) eigenvalues measure the extent of water diffusion perpendicular to the direction of axons ($\lambda_{\perp}$) (Basser, et al., 2000, Mori and Zhang, 2006, Xue, et al., 1999). Differences in the average $\lambda_{||}$ and $\lambda_{\perp}$ values did not reach statistical significance in crushed or transected nerves as compared to control nerves (data not shown).

Morphometry of the sciatic nerves showed that the numbers of regenerated myelinated axons were comparable between all studied strains, indicating that the regeneration of myelinated axons is similar in these strains. Comparison of morphometry and DTI showed a significant positive correlation between the FA value and the total number of myelinated axons at the examined time point, i.e., 17 days after crush (Fig. 3A). There was also a significant correlation between the overall number of myelinated axons in sciatic nerves and $\lambda_{||}$ values (Fig. 3B). No correlation was observed for axon counts and $\lambda_{\perp}$ values (Fig. 3C). Fiber tracking performed with a threshold value of mean FA in nerve transection group (FA = 0.5) allowed the tracking of longitudinally orientated fiber tracts in control and regenerating nerves, whereas in transected nerves the fiber tract orientation is not well aligned and disappear distal to the transection (Fig. 4). The signal in the initial segment of the nerve adjacent to the transection site likely reflects injury related changes at the cut site, which was tied with the suture and held with a forceps for dissection purposes.

In a subset of animals complete hindlimbs were imaged with conventional T2 and DTI to delineate peripheral nerves from surrounding tissues. Fig. 5 shows that compared to conventional T2 sequences DTI allows imaging of nerve tracts because of their high anisotropy. This provides much needed contrast that can differentiate peripheral nerves from surrounding tissues and delineates their location, size, and shape, as shown in Fig. 5. Despite the small size of rodent nerves, the largest sciatic nerve is ~ 500 μm in diameter; development of high-resolution DTI for delineation and three dimensional (3D) reconstructions of peripheral nerves by color map visualization allows delineation of nerves from surrounding tissue by the coherent color along its trajectory, which is based on the orientation of axons/nerve fibers (Fig. 6).

Discussion

In this study we evaluated the utility of DTI in assessing axon regeneration in mice after peripheral nerve injury. The spatial resolution achieved with DTI technology allowed us to measure axon regeneration and delineate peripheral nerves from surrounding tissues in mice. Among the three different DTI parameters used in this study FA was the most sensitive measure in distinguishing between normal uninjured, transected, and regenerating nerves (after crush injury). We found that compared to uninjured nerves transected nerves showed a significant decrease in FA and that FA returned towards normal with axonal regeneration in the nerve crush group. Notably, FA and $\lambda_{||}$ significantly correlated with the total number of axon counts. All mice strains showed a comparable pattern of axon regeneration and similar imaging characteristics, which emphasizes the utility of DTI as a measure of axon regeneration in mouse models with different genetic backgrounds. Overall, these findings support that DTI technology has the potential to measure axon degeneration and regeneration in peripheral nerves in both preclinical and clinical settings.

While the mouse models offer the opportunity to investigate the role of specific molecules/genes in axon degeneration and regeneration, the miniature size of the mouse nerves pose a significant challenge for imaging. In this study, we achieved a spatial resolution of 0.08 mm $\times$ 0.08 mm (transverse) $\times$ 0.1 mm (longitudinal) by employing a 3D imaging sequence,

which has higher signal-to-noise ratio than the 2D imaging sequences used in previous studies at comparable resolution but it required significantly longer imaging time. To enhance the imaging efficiency and to keep the imaging time in a reasonable range we excised the sciatic nerves for simultaneous *ex vivo* imaging. With the current spatial resolution, our results show that DTI technology can measure axon regeneration *ex vivo*. The techniques for *in vivo* imaging of the mouse sciatic nerve require further optimization to reach the same resolution as achieved in our *ex vivo* studies. Optimization would include magnet strength and parallel imaging capability, which will help to reduce the imaging time and to enhance the spatial resolution to apply DTI as outcome measure in high throughput animal studies.

Several recent studies emphasize the growing interest in high resolution DTI technology to assess the longitudinal evolution of axonal injury and regeneration in animals and humans (DeBoy, et al., 2007, Song, et al., 2003, Sun, et al., 2008, Takagi, et al., 2009, Zhang, et al., 2009). Our findings are compatible with the previous studies, which indicate that FA is a sensitive marker for axonal degeneration. Reduced FA correlates with the degree of axon degeneration in different models of CNS injury (Kim, et al., 2007, Song, et al., 2003, Sun, et al., 2008, Sun, et al., 2007). Two separate studies suggest that Wallerian degeneration reduces anisotropy in rat and frog peripheral nerve segments (Beaulieu, et al., 1996, Stanisz, et al., 2001). Our findings are in line with a study published earlier this year showing that DTI and tractography can detect axonal degeneration and regeneration in rat nerves (Takagi, et al., 2009). This study also found that changes in $\lambda_{\parallel}$ corresponded to alterations in FA. Absence of significant changes in $\lambda_{\parallel}$ values with nerve degeneration after transection in our studies likely reflects imaging of fixed nerves. This is supported by Sun et al. study that compared various diffusion indices acquired *in vivo* and *ex vivo* (from fixed tissue) and reported that the sensitivity of parallel and perpendicular diffusivities to axon and myelin injuries are reduced in postmortem fixed tissue, while FA remains relatively unchanged (Sun, et al., 2006). These findings may explain why the FA values showed significant differences between completely transected, regenerating, and normal nerves, while differences between parallel and perpendicular diffusivities were masked in these groups.

Our approach of using FA obtained from completely denervated nerves was critical to successfully assessing regeneration in the crush model at a single time point. However, an important observation emerging out of our studies is the fact that completely denervated segments of the nerve retain significant proportion of FA signal compared to uninjured nerves. This suggests that the decrease in FA values with complete disruption of axons and associated changes in myelin only partly contribute to DTI parameters/FA and that certain microstructural organizations in the nerve are preserved even after complete nerve transection. An important implication of these findings is that there is a workable dynamic range of change in FA values that correlate with axonal degeneration and regeneration. It is anticipated that for broad application of this technology to assess axonal degeneration and regeneration, both determination of DTI parameters and tractography will be useful. Tractography allows the opportunity to visualize the longitudinal extent of axon regeneration and this would be most relevant to clinical situations in which regeneration is incomplete and/or not measurable by standard electrophysiology (i.e., in proximal nerve trunks). To perform tractography in individual subjects it will be important to obtain DTI parameters in completely denervated nerves to set thresholds because in most preclinical and clinical situations of interest nerve degeneration and regeneration are typically incomplete/partial. For clinical studies patients with complete traumatic nerve injuries present a special opportunity to determine DTI parameters in completely denervated segments of human nerves for subsequent applications in other clinical situations with partial axon degeneration and regeneration.

Taken together our study shows that DTI technology can clearly delineate peripheral nerves in mice and provide parameters that are suitable for measurement of axonal regeneration in *ex vivo* studies on small rodent nerves. Given the recent progress in imaging human peripheral nerves with DTI (Hiltunen, et al., 2005, Khalil, et al., 2008, Stein, et al., 2009, Viallon, et al., 2008) we anticipate further development of this technology for application to living animals and humans to measure axon degeneration and regeneration.

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Figure 1. Axonal regeneration in four different mouse inbred strains

Compound muscle action potential (CMAP) amplitudes recorded in the hindpaw on day 9, 15 and 17 after nerve crush were similar in PWK/PHj (■), WSB/EiJ (▲), DBA/2J (◆) and C57BL/6 (●) mice.

Figure 2. Changes in fractional anisotropy (FA) after transection and 17 days after nerve crush Representative light micrographs of an uninjured sciatic nerve (A), 10 days after nerve transection (B), and 17 days after nerve crush (C) (bar = 75 μm). (D) Average FA values of sciatic nerves after transection and 17 days after crush injury. In comparison to control (black bars) nerves, FA is reduced in transected and in regenerated sciatic nerve segments after crush injury.

Figure 3. Correlation between morphology and diffusion tensor imaging (DTI) parameters
Correlations between DTI parameters and numbers of myelinated axons in PWK/PHj (■), WSB/EiJ (▲), DBA/2J (◆) and C57BL/6 (●) mice. **(A)** The fractional anisotropy (FA) and numbers of regenerating axons are significantly correlated. **(B)** The parallel diffusivity ($\lambda_{||}$) correlates with axon counts. **(C)** The perpendicular diffusivity ($\lambda_{\perp}$) is not correlated with the number of regenerating axons.

Figure 4. Diffusion tensor imaging with 3D reconstruction and tractography of ex vivo mouse peripheral nerves

Fiber tracking can visualize axon degeneration and regeneration in the mouse sciatic nerve injury. Fiber tracking with a FA threshold of 0.5 was performed on diffusion tensor data collected from uninjured nerves, transected nerves and in regenerating nerves 17 days after crush injury. Seed points were selected at the proximal end of the sciatic nerves. For each case, the entire nerve tissue specimen (left, gray, reconstructed from T2-weighted images) and fiber tracking result (right, red) are displayed. Arrows mark the transection (yellow) and crush site (white), respectively.

Figure 5. Comparison of conventional T2 (T2W) and DTI-based contrast (anisotropy map [FA] and direction encoded color maps [DEC])

Axial and horizontal slices with different angles were extracted from 3D images of a fixed mouse hind limb. White arrows indicate location of sciatic nerves. It is difficult to distinguish nerve in T2-weighted images. Anisotropy and color maps provide a much better contrast to identify peripheral nerves. The color map visualizes the orientation of axonal fibers with RGB color. This further distinguishes nerve from surrounding tissues due to unique and coherent color along its trajectory that is dictated by the orientation of axons/ nerve fibers (bar = 1mm).

Figure 6. 3D reconstruction of ex vivo mouse peripheral nerves

In the left panel, muscle is indicated by transparent gray structures, which is removed in the middle panel for better visualization of the nerves. The light gray structure is the bone. The 3D trajectories of the sciatic nerves are reconstructed from 3D DTI data. The three major branches of the sciatic nerves are displayed with different colors: yellow = tibial, blue = superficial peroneal, red = deep peroneal nerves, green = saphenous nerve. The branching can be clearly appreciated in the right panel.