Non-invasive diffusion tensor imaging detects white matter degeneration in the spinal cord of a mouse model of amyotrophic lateral sclerosis.

Clare K. Underwood[a], Nyoman D. Kurniawan[b], Tim J. Butler[a], Gary J. Cowin[b], Robyn H. Wallace[a,c].

[a]Queensland Brain Institute, [b]Centre for Advanced Imaging, [c]School of Chemistry and Molecular Biosciences, The University of Queensland, Brisbane, QLD, Australia.

Corresponding author: Robyn Wallace, Queensland Brain Institute, The University of Queensland, Brisbane, QLD, 4072, Australia. email: r.wallace2@uq.edu.au, telephone: +617 3346 6393, fax: +617 3346 6301

Abstract

Amyotrophic lateral sclerosis (ALS) is characterized by selective degeneration of motor neurons. Here we examine the ability of magnetic resonance imaging (MRI) to measure axonal degeneration in the lumbar spinal cord of the SOD1 mouse model of ALS. Diffusion tensor imaging (DTI) was successful in detecting axonal spinal cord damage *in vivo*. Fractional anisotropy (FA) values were reduced exclusively in the ventral white matter tracts of the lumbar spinal cord of ALS-affected SOD1 mice compared to wildtype littermates, with this effect becoming more pronounced with disease progression. The reduced FA values were therefore limited to white matter tracts arising from the motor neurons, whereas sensory white matter fibers were preserved. Significant decreases in water diffusion parallel to the white matter fibers, or axial diffusivity, were observed in the SOD1 mice, which correlated with the axonal degeneration observed by electron microscopy. At the same time, radial diffusivity perpendicular to the spinal column increased in the SOD1 mice, reflecting reduced myelination. These results demonstrate the usefulness of MRI in tracking disease progression in live animals and will aid in the assessment of treatment efficacy. This method could also potentially be adapted to aid the diagnosis and assessment of ALS progression in humans.

Keywords: amyotrophic lateral sclerosis, SOD1, fractional anisotropy, diffusion tensor imaging

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a devastating disease, in which affected individuals become progressively paralyzed and die within 3 to 5 years (Armon, 1994). ALS is characterized by selective degeneration of motor neurons in the motor cortex, brainstem and spinal cord. The only way to directly assess degeneration of these neurons in patients is by using magnetic resonance imaging (MRI). There have also been numerous studies of transcranial magnetic stimulation in ALS, many of which have been contradictory (Floyd et al., 2009); however, the diagnosis of ALS currently relies primarily on clinical assessment (Ferguson and Elman, 2007). Recognition of classical ALS is not difficult but, during the early stages of the disease, both false positive and false negative diagnoses are common. Careful examination, frequent follow-up and ancillary tests are therefore necessary to avoid erroneous diagnoses.

The SOD1 transgenic mouse model of ALS (Gurney et al., 1994) exhibits similar motor neuron degeneration to that observed in patients and is a useful model for assessing new ALS therapies. To date, *in vivo* MRI in the SOD1 mouse has been limited to the analysis of the brain, brain stem (Angenstein et al., 2004; Bucher et al., 2007; Niessen et al., 2006; Zang et al., 2004) and muscle (Brooks et al., 2004; Zhang et al., 2008). Only one of these studies evaluated diffusivity, by measuring the apparent diffusion coefficient (ADC). Increased ADC values were limited to two brain stem motor nuclei (facial and hypoglossal), while no significant differences in ADC were found in the spinal cord segment of the brain stem (Niessen et al., 2006). One MRI technique, diffusion tensor imaging (DTI), has the greatest diagnostic potential for ALS. DTI can differentiate between normal and ALS patients in group comparisons (Turner et al., 2009) and has the potential to be diagnostic in individual patients. DTI can provide sensitive quantitative information on axonal organization in the spinal cord, by measuring diffusion anisotropy. DTI has previously been used to detect both axonal and myelin spinal cord damage in mice following spinal cord injury (Kim et al., 2007) and in a mouse model of multiple sclerosis (Budde et al., 2008; Kim et al., 2006). Fractional anisotropy (FA), which reflects the directionality of water movement within tissues, is one such measure of the degree of diffusion anisotropy and provides an estimation of fiber tract atrophy (Bilgen et al., 2005;

Bonny et al., 2004). Using the DTI method, reduced FA values have been detected in the corticospinal tracts of the brain stem in human ALS sufferers (Sage et al., 2007). Post-mortem tissue has shown that within the white matter of the spinal cord of ALS patients there is a loss of large myelinated fibers in the corticospinal tracts and ventral roots. However all ALS patient and mouse model MRI studies to date have focused on corticospinal tracts in the brain and brain stem. In this study, we examine the utility of *in vivo* DTI to measure degeneration in the lumbar spinal cord of the SOD1 transgenic mouse model of ALS.

2. Material and Methods

2.1 Mice

C57BL6 mice overexpressing the human SOD1 transgene carrying the G93A mutation (SOD1 mice) were obtained from The Jackson Laboratory (stock number: 004435). These mice develop symptoms of ALS at approximately 90 days of age and die prematurely at around 155 days. Control mice were wildtype (WT) C57BL6 littermates of the SOD1 mice, which lacked the SOD1 transgene. The University of Queensland Animal Ethics Committee approved all experiments.

2.2 Grip strength testing

Before MR imaging, the progression of ALS was evaluated by grip strength testing of the hind limbs (Meyer et al., 1979). All animals underwent brief training before imaging commenced. Mice held by the tail in front of the apparatus instinctively reached for the T-bar attached to the force meter (Muromachi Kikai Co., Tokyo, Japan). The experimenter then gently pulled the mouse away from the meter until its grip was released, with the maximum force (in grams) being recorded. Each mouse was tested ten times and the maximum grip strength used for statistical comparison. Differences between SOD1 and WT mice were assessed for statistical significance using a paired t-test. Correlation with FA values was calculated using Pearson's correlation coefficient.

2.3 *In vivo* MRI

The spinal cords of SOD1 and WT mice (n = 6 per group for the end-stage study and 3 per group for the longitudinal study) were imaged using a 16.4T Bruker (Karlsruhe,

Germany) NMR scanner with an 89 mm vertical bore magnet, 25 mT/m/A gradient coil set and Paravision 4.0 software. The mice were anesthetized using an isoflurane/oxygen mixture (maintained at 0.5-1.5 %) and the MRI data acquired using a 1.5 x 3 cm transmit/receive linear surface coil (M2M Imaging, Cleveland, OH). The mouse body temperature was maintained close to normal temperature by setting the gradient set within the magnet at 30°C and the respiration rate was monitored using Biotrig (SpinSystems, Brisbane, Australia). In order to minimize movement artefacts, the data were acquired with respiratory gating.

2.4 Diffusion tensor imaging and calculation of FA values

DTI data were acquired using a DTI spin-echo sequence, in an interleaved fashion using TR/TE = 1920/27.2 ms, acquisition matrix = 214 x 128 over the field of view 15 x 9 mm^2 (final resolution 70 µm x 70 µm). Slice thickness was 0.75 mm and the number of excitations was 2. The read, phase and slice planes were positioned at the left-right, dorsal-ventral, and rostral-caudal axes of the mouse, respectively. Diffusion sensitizing gradients (read, phase and slice) were applied in six orientations (1,1,0), (1,0,1), (0,1,1), (-1,1,0), (0,-1,1) and (1,0,-1) with the gradient strength b =1500 s/mm^2, 2 ms diffusion encoding and 20 ms diffusion separation. The DTI acquisition time was 2 hours, with a zero fill acceleration factor of 2. Signal-to-noise ratio was enhanced using a post-processing trapezoid window function. Ten axial images covering the lumbar spinal enlargement were acquired. Methods were based on previous reports (Bilgen et al., 2007; Bilgen et al., 2005; Kim et al., 2006; Kim et al., 2007; Niessen et al., 2006). The regions of interest (ROI) were manually outlined, as shown in Fig. 1B. The eigenvalues tensor parameters (λ_1, λ_2, λ_3) were computed using the Paravision 4.0 diffusion tensor calculation module. The axial diffusivity ($\lambda_\parallel$) is a measure of the diffusivity along the principal axis of the diffusion tensor ($\lambda_\parallel = \lambda_1$). The radial diffusivity ($\lambda_\perp$) is an average of the two minor diffusion axes ($\lambda_\perp = (\lambda_2+\lambda_3)/2$). The fractional anisotropy (FA) was calculated using the following equation: $FA = \sqrt{\tfrac{1}{2}} (\sqrt{((\lambda_1 - \lambda_2)^2 + (\lambda_1 - \lambda_3)^2 + (\lambda_2 - \lambda_3)^2)} / \sqrt{(\lambda_1^2 + \lambda_2^2 + \lambda_3^2)})$. Differences between SOD1 and WT mice were assessed for statistical significance using either repeated measures ANOVA or t-tests.

2.5 Electron microscopy

Two mice from each cohort were used for electron microscopy. Following the final MRI scan, mice were administered an overdose of sodium pentobarbital (Lethabarb, Virbac, Milperra, Australia), after which the lumbar spinal cords were removed and placed in gluteraldehyde. Survey sections were cut at 500nm thickness and stained with 1% toluidine blue 1% sodium tetraborate to identify the lateral ventral motor neuron populations. Ultrathin sections including this area and the adjacent white matter were cut at 70nm thickness, mounted on copper grids, stained with uranyl acetate followed by lead citrate, then viewed and photographed with a JEOL 1010 transmission electron microscope (Tokyo, Japan). To quantify axonal degeneration in the white matter, the total numbers of axons in 5 random 20 x 20 micron fields were averaged.

3. Results

Lumbar cord segments were identified in reference to the intersection point of the lowest rib at vertebra T13 on coronal scout images (Fig. 1A). Ten axial images covering the lumbar spinal enlargement were used for the data analysis, with slice 1 located at the top of T12 and slice 10 at the bottom of L1. A representative FA map calculated from an axial section within the lumbar region of a WT mouse is shown in Fig. 1B. The dorsal, dorsolateral, ventral and ventrolateral regions of interest within the white matter were manually outlined (Fig. 1B) and FA values were obtained. The ventrolateral regions contain the motor tracts of interest, whereas the central ventral region contains tracts associated with vestibular control. The dorsal region largely comprises ascending sensory fibers and the corticospinal tract. The dorsolateral regions contain ascending spinothalamic and spinocerebellar tracts (Watson et al., 2009).

3.1 FA values are reduced in ventral white matter regions of SOD1 mice

FA values were calculated for each slice across the lumbar spinal enlargement within the dorsal, right dorsolateral, left dorsolateral, ventral, right ventrolateral and left ventrolateral regions of interest of SOD1 and WT animals aged 145 days. The dorsal region showed no difference in FA values between the two cohorts of mice (Fig. 2A), consistent with the fact that the ascending sensory axons in this region are preserved in the SOD1 mice. The left and right dorsolateral regions also showed no difference in FA values between SOD1 and WT mice (data not shown). However, the left and

right ventrolateral regions (Fig. 2C & 2D) showed a significant decrease, from 0.84±0.007 at slice 5 in WT mice to 0.75±0.01 at slice 5 in SOD1 mice ($p<0.0001$), reflecting degeneration of the motor neuron axons. The FA values in the ventral (Fig. 2B) region also showed a significant decrease, from 0.85±0.01 at slice 5 in WT mice to 0.77±0.01 at slice 5 in SOD1 mice ($p<0.0001$). In all regions, the FA values varied between slices along the length of the spinal cord. However, as repeated measures ANOVA showed no interaction between slice and genotype, the FA mean of all slices was subsequently used to test the correlation of FA with age (section 3.3) and disease severity (section 3.4) in the SOD1 mice.

3.2 Changes in axial and radial diffusivity are associated with axonal degeneration

The left and right ventrolateral regions of interest were combined to calculate axial and radial diffusivity in the motor tracts of SOD1 and WT mice. Calculation of diffusivity values in the axial (longitudinal) plane of the ventrolateral regions of the lumbar spinal cord showed that diffusivity decreased in SOD1 mice (Fig. 3A), whereas in the radial (transverse) plane, diffusivity increased (Fig. 3D). Following the final MRI scan, the lumbar spinal cords were assessed for axonal degeneration and loss of myelin using transmission electron microscopy (Fig. 3 B,C,E,F), SOD1 41.2 (±12.5) WT 120.1 (±6.8) $p=0.0009$ allowing ~~correlation~~ of the MRI findings with the degree of tissue damage within the spinal cord. In SOD1 mice, decreases in axial diffusivity reflect axonal degeneration (Fig. 3 A-C) while increases in radial diffusivity were associated with loss of myelin (Fig. 3 D-F).

3.3 FA values decrease with age in SOD1 mice

To determine whether DTI could detect progressive axonal degeneration, a second cohort of SOD1 and WT mice were imaged at 45, 75, 110 and 145 days of age. FA values were calculated within the same regions of interest as described in section 3.1, and were summed across all slices of the lumbar spinal enlargement (Fig. 4). At 45 and 75 days of age, no difference in FA value between SOD1 and WT mice was detected in any region of the lumbar spinal cord. Hind limb grip strength was also not significantly different between the two groups at 45 and 75 days of age (Fig. 4E). At 110 days, FA values were significantly lower in the ventrolateral regions of the SOD1 mice (0.73±0.01) compared to the WT controls (0.79±0.02). At 145 days, FA values

showed a further decrease in the SOD1 mice (0.68±0.02) compared to the WT controls (0.74±0.01). By 145 days a decrease was also detected in the ventral axonal tracts of SOD1 mice (0.69±0.02) compared to WT mice (0.73±0.01). The progressive decrease in FA values highlight a diminishing integrity of the overall white matter structure in SOD1 mice.

3.4 Grip strength decreases with age in SOD1 mice

3.4 FA values correlate with disease severity in SOD1 mice

The decrease in FA values in SOD1 mice was not detected until after the onset of ALS symptoms, as measured by grip strength (Fig. 4E). At 110 days, the grip strength decreased from 147±10 grams in WT mice to 82±25 grams in SOD1 mice ($p=0.007$, Fig. 4E). At 145 days, the grip strength decreased further in SOD1 mice, to 67±21 grams ($p=0.03$). The correlation between hind limb grip strength and FA values (averaged across all slices) of SOD1 and WT animals aged between 110 and 145 days (i.e. from disease onset) is shown in Fig. 5. As expected, WT mice all had high FA and grip strength values. In SOD1 mice, FA values decreased with decreasing grip strength exclusively in the ventral ($r = 0.75$, $p=0.02$) and ventrolateral regions (right: $r = 0.66$ ($p=0.02$); left: $r = 0.78$ ($p=0.03$)) of the spinal cord.

4. Discussion

ALS is characterized by selective loss of motor neurons in the brain and spinal cord. With the loss of motor neurons, there is concomitant formation of cytoplasmic neurofilament bodies and axonal spheroids, as well as vacuolization and reactive gliosis. There is also loss of large myelinated fibers in the corticospinal tracts and ventral roots, and axonal degeneration (Bruijn et al., 2004).

At present, there is no useful objective measure of the effectiveness of ALS therapy. In mice, using histological methods to directly count neurons in affected areas requires sacrifice of the animal. In this respect, novel techniques need to be developed to monitor therapies that might slow or prevent the death of motor neurons in live experimental animals. Previous work using MRI techniques have detected differences in T_2 values within several brain stem motor nuclei in SOD1 transgenic mice compared to WT animals (Angenstein et al., 2004; Bucher et al., 2007; Niessen

et al., 2006; Zang et al., 2004). Although it has been observed that there is reduced FA in the corticospinal tracts and other brain regions of human ALS sufferers (Blain et al., 2007), the change in FA in the lumbar spinal cord with the progression of ALS has not been assessed.

In the present study we have demonstrated that DTI can detect axonal degeneration in live SOD1 mice. The accumulation of neurofilaments and corresponding decreased axonal transport may also contribute to the observed decrease in FA values. Furthermore, the decrease in FA was restricted to the motor tracts of the spinal cord (Fig. 2). When axial diffusivity was examined, we observed a decrease across all slices in the motor tract of the lumbar enlargement in the SOD1 mice (Fig. 3A). This decrease suggests that water is diffusing more slowly along the length of the spinal cord in the SOD1 mice and TEM images support that this is probably due to a decrease in the overall number of axons. In addition, diffusivity values in the radial plane increased in the SOD1 mice (Fig. 3B). Tightly wrapped myelin usually forces water movement in the longitudinal plane. In SOD1 mice the loss of myelin (Fig. 3F) would allow more abnormal water movement in the radial plane in the spinal cord white matter.

Ascending sensory axons are the major component of the dorsal region of interest. FA values were similar in SOD1 and WT mice at all ages in the dorsal fiber tract, as would be expected given that sensory neurons are unaffected in SOD1 mice. In mice, the dorsal region also contains the corticospinal tract (located laterally in humans). In human ALS, the corticospinal (or pyramidal) tract shows decreased FA (Sage et al., 2007). However, the SOD1 mouse displays no light microscopic evidence of degeneration in the corticospinal tract (Leichsenring et al., 2006). This is consistent with our DTI results, which also show no decrease in FA values, suggesting the corticospinal tract is spared in SOD1 mice. The importance of corticospinal connections in motor control in humans has led to the assumption that the tract is also of major importance to motor control in non-primate mammals. However, the evidence for this is generally poor, especially for projections below the cervical cord (Watson et al., 2009).

Based on grip strength data at 45 and 75 days of age, SOD1 mice are phenotypically normal (Fig 4E). The FA values at these ages show no difference between SOD1 and WT animals (Fig 4 A-D). At 75 days, FA values increased in both SOD1 and WT mice, probably due to increased myelination during this developmental period. At 110 days, the FA values in the SOD1 mice are significantly reduced exclusively in the motor tracts (Fig. 4 C&D). By 145 days, the vestibular tract in the ventral region also shows reduced FA in SOD1 mice (Fig. 4 B), suggesting the nerve fibers that control posture and balance are also affected in the late stages of the disease. FA values in the ventral and ventrolateral regions show a strong correlation with disease progression, as assessed by grip strength (Fig 5).

5. Conclusions

This is the first study to demonstrate *in vivo* that DTI can be used to measure disease progression in SOD1 mice. There is a significant reduction in the FA values within the motor tracts of the lumbar spinal cord in SOD1 transgenic mice compared to WT mice during late stage ALS. The decrease in FA values correlates with disease progression and reflects reduced axon number and demyelination (confirmed by electron microscopy). Thus, DTI may provide a useful non-invasive, quantitative *in vivo* method to monitor the progression of ALS, allowing earlier or more accurate diagnosis, as well as assessment of treatment efficacy.

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Fig. legends

Fig. 1. MRI animal alignment and definition of regions of interest (ROIs). **A**. The lumbar spinal enlargement was identified in reference to the lowest rib (vertebra T13), with slices numbered from T12 (* = slice 1 referred to in subsequent figures). **B**. Representative fractional anisotropy (FA) map calculated from an axial section within the lumbar region of a WT mouse. The dorsal (D), ventral (V), right and left ventrolateral (Lr, Ll) and right and left dorsolateral (DLr, DLl) ROI within the white matter were manually outlined on the FA maps.

Fig. 2. Diffusion tensor imaging identifies decreased FA values in ventral white matter regions of affected SOD1 mice. FA values calculated for each slice across the lumbar spinal enlargement (rostral-caudal) within A: dorsal, B: ventral, C: left ventrolateral and D: right ventrolateral regions of interest of SOD1 (n = 6) and WT (n = 6) animals aged 145 days. Error bars = standard error of the mean (SEM).

Fig. 3. Changes in axial and radial diffusivity reflect axonal degeneration in SOD1 mice. Following the final MRI scan and calculation of diffusivity values (μm^2/sec) in the axial (A) and radial (D) planes, the lumbar spinal cords were examined using transmission electron microscopy (TEM). Photos of the white matter were taken from areas close to the motor neurons. B and C: TEM images showing axonal degeneration (scale bar = 5µm). E and F: TEM images showing loss of myelin (scale bar = 2µm). Error bars = standard error of the mean (SEM). SOD1 (n = 6) and WT (n = 6) animals aged 145 days.

Fig. 4. FA values in ventral white matter regions decrease with disease progression. FA values were summed across all slices across the lumbar spinal enlargement within the dorsal (A), ventral (B), left ventrolateral (C) and right ventrolateral (D) regions of interest of SOD1 (n=3) and WT (n=3) animals imaged at 45, 75, 110 and 145 days of age. The hind limb grip strength of each mouse was measured prior to every MRI scan (E). Error bars = standard error of the mean (SEM). Actual p values: Ventral 145 days, p=0.02; Ll 110 days, p= 0.009; Ll 145 days, p=0.005; Lr 110 days, p=0.006; Lr 145 days, p=0.005; grip strength 110 days, p=0.007; grip strength 145 days, p=0.03.

Fig. 5. The correlation between the hind limb grip strength (grams) and FA values (averaged across all slices) in the dorsal (A), ventral (B), right ventrolateral (C) and left ventrolateral (D) regions of interest of SOD1 and WT animals aged between 110 and 145 days (r = Pearson's correlation coefficient, $p<0.05$).