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Pathology of callosal damage in ALS: An *ex-vivo*, 7 T diffusion tensor MRI study

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ABSTRACT

Objectives: The goal of this study was to better understand the changes in tissue microstructure that underlie white matter diffusion changes in ALS patients.
Methods: Diffusion tensor imaging was carried out in postmortem brains of 4 ALS patients and two subjects without neurological disease on a 7 T MRI scanner using steady-state free precession sequences. Fractional anisotropy (FA) was measured in the genu, body, and splenium of the corpus callosum in formalin-fixed hemispheres. FA of the body and genu was expressed as ratio to FA of the splenium, a region unaffected in ALS. After imaging, tissue sections of the same segments of the callosum were stained for markers of different tissue components. Coded image fields were rated for pathological changes by blinded raters.
Results: The FA body/FA splenium ratio was reduced in ALS patients compared to controls. Patchy areas of myelin pallor and cells immunostained for CD68, a microglial-macrophage marker, were only observed in the body of the callosum of ALS patients. Blinded ratings showed increased CD68 + microglial cells in the body of the corpus callosum in ALS patients, especially those with *C9orf72* mutations, and increased reactive astrocytes throughout the callosum.
Conclusion: Reduced FA of the corpus callosum in ALS results from complex changes in tissue microstructure. Callosal segments with reduced FA had large numbers of microglia-macrophages in addition to loss of myelinated axons and astrogliosis. Microglial inflammation contributed to reduced FA in ALS, and may contribute to a pro-inflammatory state, but further work is needed to determine their role.

1. Introduction

Diffusion tensor imaging is a tool to evaluate diffusion properties in white matter (Basser, 1995; Pierpaoli et al., 1996) in living subjects, both qualitatively and quantitatively (Pierpaoli and Basser, 1996). Many studies have described changes of white matter diffusion parameters in patients with amyotrophic lateral sclerosis (ALS) (Ellis et al., 1999) which are thought to be caused by loss of integrity of axons undergoing degeneration (Song et al., 2003). A decline in the fractional anisotropy (FA) of the corticospinal tract is the most consistent finding in ALS (Agosta et al., 2010; Ciccarelli et al., 2009; Foerster et al., 2013) although decreased FA also occurs in the body of the corpus callosum (Filippini et al., 2010; Iwata et al., 2011). ALS patients with low FA of the corticospinal tract have shorter survival and more rapid progression (Agosta et al., 2010; Menke et al., 2012). Tissue changes thought to account for changes in diffusion measures in ALS patients are based on animal models that caused reduction in FA values by experimental manipulations that cause axonal degeneration or demyelination (Song et al., 2003; Thiessen et al., 2013). However, other tissue changes might also produce changes in diffusion measures. To date, there are few

Abbreviations: AD, axial diffusivity; ALS, Amyotrophic lateral sclerosis; DTI, diffusion tensor imaging; DWI, diffusion weighted imaging; DW-SSFP, Diffusion Weighted Steady State Free Precession; FA, fractional anisotropy; GFAP, glial fibrillary acidic protein; MD, mean diffusivity; MRI, magnetic resonance imaging; PMI, *post mortem* interval; PSI, scan interval (death to scan); RD, radial diffusivity; SNR, signal to noise ratio; VOI, volume of interest

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Table 1
Summary of demographic data.

Subject	Age	Gender	Diagnosis	C9orf72	Disease duration (months)	PMI (hours)	PSI (days)	Histology
1	43	M	Control	−	−	12	46	+
2	53	M	Control	−	−	24	9 years	−
3	79	F	ALS	−	11	14	49	+
4	57	M	ALS	−	−	31	30	+
5	70	M	ALS	+	24	29	71	+
6	69	M	ALS	+	48	6	34	+

PMI: Postmortem interval (*i.e.* time from death to fixation). PSI – interval from death to scan.

studies correlating changes in diffusion measures with tissue histology in neurodegenerative diseases.

Over the past ten years, techniques to obtain diffusion imaging in postmortem brains have greatly improved: higher magnetic fields, stronger gradients, signal-to-noise (SNR) optimization and better shimming techniques, among other factors, have allowed imaging of *ex-vivo* human brain tissue at high resolution. New MRI steady-state free precession (SSFP) pulse sequences provide superior diffusion weighted imaging (DWI) of postmortem brain tissue (Buxton, 1993; Foxley et al., 2014; McNab et al., 2009; Miller et al., 2012), compared to classical, spin echo DWI methods (Stejskal and Tanner, 1965; D'Arceuil and de Crespigny, 2007; Pfefferbaum et al., 2004). DW-SSFP methods allow a detailed view of the white matter architecture, as well as quantitative analysis of diffusivity parameters. Although tissue fixation decreases the mean diffusivity (MD) of tissue, FA values are thought overall to remain unchanged over a range of fixation times (Guilfoyle et al., 2003; Sun et al., 2005). *Post mortem* interval (PMI; interval from death to fixation) significantly affects diffusivity measures (Foxley et al., 2014; D'Arceuil et al., 2007). In an animal study comparing 1-, 4-, and 14-day PMIs to immediate fixation, all diffusivity measures in white matter declined with increasing delay of fixation: axial diffusivity (AD) declined most rapidly by 1 day PMI, FA was relatively unchanged at 1-day PMI, but exhibited decline between the 1- and 4- day PMIs (D'Arceuil and de Crespigny, 2007). Consequently, the absolute FA values of postmortem human brains are not directly comparable to *in vivo* imaging.

The goal of this study was to better understand the changes in tissue microstructure that underlie white matter diffusion changes in ALS patients. To accomplish this, we carried out DW-SSFP imaging of postmortem brains of ALS patients and subjects with no known history of neurological disease in a 7 T scanner. The corpus callosum was examined histopathologically. The corpus callosum was chosen for analysis because anatomical segments are differentially affected in ALS, and can be easily identified in different subjects. DTI changes occur in ALS in the body of the corpus callosum and occasionally in the genu, but the splenium is unaffected (Filippini et al., 2010; Iwata et al., 2011). To control for potential differences in PMI across different brains, the FA of the genu and the body of the corpus callosum were expressed as ratios to the FA of the splenium within each subject. Histologic changes that might explain the abnormal diffusion parameters, such as gliosis, inflammation or axonal degeneration, were analyzed qualitatively and semi-quantitatively by blinded ratings of the histological material.

2. Methods

2.1. Subjects

Six cerebral hemispheres (five males, one female; aged 43–79 years) were obtained from the National Institutes of Health (Bethesda, MD) and from the University of Maryland Brain and Tissue Bank (Baltimore, Maryland) for imaging studies. Informed consent for brain donation was obtained prior to death or from the next of kin. Brains were extracted en-bloc from the skull, hemisected and immersed in 10% formalin (mean postmortem interval, 19.3 ± 10.1 h; range 6–31 h). Brains were stored in formalin at room temperature during the fixation period. Histological studies were carried out on five of the hemispheres, comprised of 4 ALS patients (subjects #3 to #6) and 1 control with no known neurological disease (subject #1). Histology was not carried out for one control hemisphere (#2) because of concerns that the markedly longer fixation time would affect the immunostaining. The mean age of the five subjects with imaging and histology was 63.6 ± 13.9 years. The interval from death to scanning (PSI) ranged from 4 to 10 weeks for these subjects. All ALS patients met revised El Escorial Criteria (Brooks et al., 2000) for definite ALS. Two ALS patients (subjects #5 and #6) carried the C9orf72 hexanucleotide expansion mutation (Renton et al., 2011). A premortem DTI scan had been done on one ALS patient (#6) on a 3 T scanner. Clinical information is summarized on Table 1.

2.2. Imaging methods

2.2.1. Specimen preparation for imaging

The surface of the hemispheres was briefly rinsed with a few hundred mls of phosphate buffered saline before the hemisphere was placed in a Plexiglas container filled with Fomblin (Solvay Solexis, NJ), a low proton- fluid which has no MRI signal (D'Arceuil et al., 2007). Air bubbles were removed with vacuum suction, facilitated with periodical gentle shaking of the container for 24 h before scanning.

2.2.2. MRI acquisition

Hemispheres were imaged using a 7 T MRI scanner (Magnetom Siemens, Erlangen, Germany), which has a gradient strength of 70 mT/m and a slew rate of 200 T/m/s with a 32 channel receiver coil. All acquisitions for each hemisphere were obtained in the same scan session. Scanning was performed at room temperature for all specimens. B1 maps based on the Bloch-Siegert approach (Duan et al., 2013) were acquired at the beginning of the sequence protocol to help obtain accurate FA and mean diffusivity (MD) measurements. A 3D balanced SSFP pulse sequence (TE 3.8 ms, TR 7.58 ms, flip angle 35°), which has previously successfully been applied to *ex-vivo* human brain tissue, was used to achieve gray-white matter differentiation (Buxton, 1993; Foxley et al., 2014; McNab et al., 2009; Miller et al., 2011). Four structural 3D balanced SSFP pulse sequences were acquired, divided in two pairs, each with two radiofrequency phase cycling increments of 0° and 180° (Miller et al., 2012). Pairs of balanced SSFP images were acquired before and after the DW-SSFP sequences. The two radiofrequency phases in each pair were averaged to reduce susceptibility artifacts. The second pair was used for evaluating tissue motion and scanner drift. T1 maps were derived from inversion recovery 3DFSE data at eight different inversion times. T2 maps were derived from 3D FSE data at eight different echo times.

Diffusion weighted images were acquired with a DW-SSFP pulse sequence (Buxton, 1993) (resolution 1.0 × 1.0 × 1.0 mm). Diffusion weighting was applied in 49 non-collinear directions, with an applied b effective (Foxley et al., 2014) value of 4000 s/mm^2, gradient amplitude of 56 mT/m and a gradient duration of 15 ms. Matrix size was 180 × 176 × 176, with a TE/TR of 25/34 ms and a flip angle of 30°.