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Axonal damage in the making: Neurofilament phosphorylation, proton mobility and magnetisation transfer in multiple sclerosis normal appearing white matter☆

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ABSTRACT

Aims: Multiple sclerosis (MS) leaves a signature on the phosphorylation and thus proton binding capacity of axonal neurofilament (Nf) proteins. The proton binding capacity in a tissue is the major determinant for exchange between bound and free protons and thus the magnetisation transfer ratio (MTR). This study investigated whether the MTR of non-lesional white matter (NLWM) was related to the brain tissue concentration of neurofilament phosphoforms.
Methods: Unfixed post-mortem brain slices of 12 MS patients were analysed using MTR, T1 at 1.5 T. Blocks containing NLWM were processed for embedding in paraffin and inspected microscopically. Adjacent tissue was microdissected, homogenised and specific protein levels were quantified by ELISA for the Nf heavy chain (NfH) phosphoforms, glial fibrillary acidic protein (GFAP), S100B and ferritin.
Results: Averaged hyperphosphorylated NfH (SMI34) but not phosphorylated NfH (SMI35) levels were different between individual patients NLWM. The concentration of hyperphosphorylated NfH-SMI34 correlated with T1 ($R = 0.70$, $p = 0.0114$) and — inversely — with MTR ($R = -0.73$, $p = 0.0065$). NfH-SMI35 was not correlated to any of the MR indices.
Conclusions: Post-translational modifications of axonal proteins such as phosphorylation of neurofilaments occur in NLWM and may precede demyelination. The resulting change of proton mobility influences MTR and T1. This permits the in vivo detection of these subtle tissue changes on a proteomic level in patients with MS.

Introduction

Multiple sclerosis (MS) is an inflammatory, demyelinating disease of the central nervous system (CNS) with associated neurodegeneration (Lassmann et al., 2007; Trapp and Nave, 2008). The most obvious pathological finding in MS brain tissue is focal demyelination, which may affect any part of the CNS (Chard and Miller, 2009). New MS lesions may result in neurological symptoms and/or signs, a clinical situation called "relapse", though patients with non-relapsing (primary or secondary progressive) MS also develop new lesions, albeit less frequently (Petzold, 2008).

It is well known that areas of focal demyelination (MS lesions) represent only a fraction of MS pathology, with additional contribution of non-lesional changes of brain parenchyma (Bjartmar et al., 2001; Howell et al., 2010; Kutzelnigg et al., 2005; Zeis et al., 2008) and meninges (Magliozzi et al., 2007). Both lesional and non-lesional MS brain parenchyma is affected by axonal damage (Albert et al., 2007; Kutzelnigg et al., 2005; Peterson et al., 2001; Petzold, 2005; Trapp and Nave, 2008). Neurodegeneration is the major cause of irreversible neurological disability in patients with MS (Trapp and Nave, 2008). The evolution of axonal loss, however remains enigmatic (Moore et al., 2000). This is partly because axonal damage in people with MS is difficult to visualise *in vivo* at a resolution conducive to the investigation of underlying mechanisms.

Magnetisation transfer (MT) is a quantitative magnetic resonance imaging (MRI) technique, based on the exchange of magnetisation

Abbreviations: AL, acute lesion; BSP, brain-specific proteins; CDP, chronic disease progression; CNS, central nervous system; CTRL, control group; EDSS, Expanded Disability Status Scale; ELISA, enzyme linked immunoabsorbent assay; GM, grey matter; IQR, interquartile range; MS, multiple sclerosis; MRI, magnetic resonance imaging; MTR, magnetisation transfer ratio; NLWM, normal lesional white matter; Nf, neurofilament; NfH, neurofilament heavy chain; PP, primary progressive; RR, relapsing remitting; SAPK, stress-activated protein kinase; SP, secondary progressive; WM, white matter.

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between protons in at least two pools: mobile protons and protons bound to macromolecules (Tofts et al., 2003). Changes in MT derived measures including the MT ratio (MTR) in the CNS of patients with MS have been considered to reflect changes in the amount of myelin (Barkhof et al., 2003; Schmierer et al., 2007). This has clearly been shown in studies combining lesional and non-lesional brain tissue (NLBT). The extent to which tissue changes may be detectable with MTR applied to NLBT alone is less well known. In this study we investigated the association between MTR and proteomic alterations of axons in MS non-lesional white matter (NLWM).

Based on histological studies it is well accepted that damage already occurs to axons in the NLWM (Lovas et al., 2000; Trapp et al., 1998). The typical picture is that of irregular changes of the axonal diameter and axonal end bulbs (Trapp et al., 1998). Immunohistochemical studies suggest that protein phosphorylation increases, at least for the neurofilament heavy chain (NfH) and tau (Anderson et al., 2008; Petzold et al., 2008; Schneider et al., 2004). These histological and immunohistochemical data are consistent with changes on a proteomic level (Dutta et al., 2006; Herrero-Herranz et al., 2008; Petzold et al., 2008). Protein phosphorylation appears to be an important biochemical mechanism by which axonal injury is mediated (Petzold et al., 2008). Many of the well established mechanisms driving neurodegeneration (glutamate excitotoxicity, mitochondrial failure) are related to Ca^{2+} influx, activation of kinases and phosphorylation of NfH (Akassoglou et al., 2002; Brownlees et al., 2000; Grant and Pant, 2000; Petzold, 2005; Schwarzschild et al., 1999; Xia et al., 1996).

An increase of NfH phosphorylation changes the charge depended proton binding capacity of the protein. Non-phosphorylated NfH only binds two protons (Chang et al., 2009). Phosphorylated NfH in contrast has the ability to bind up to 82 protons, a remarkable difference (Chang et al., 2009). The biological relevance of NfH phosphorylation in the MS brain is not known. On a proteomic level phosphorylation of NfH causes radial extension of sidearms out of the dense polymer structure. This is explained by charge repulsion and locally altered entropic and electrostatic interactions of the neurofilament network (Kim et al., 2011; Petzold, 2005). Here we report preliminary evidence that changes in the phosphorylation status of NfH, and hence the proton binding capacity of the neurofilament network, is detectable using MT imaging in MS NLWM.

Material and methods

This study was approved by the Joint Ethics Committee of the UCL Institute of Neurology and The National Hospital for Neurology & Neurosurgery, London, UK. The MRI data sets and paraffin embedded tissue blocks were used alongside snap frozen samples of NLWM obtained from the brain tissue in unfixed condition. The brain slices had been donated by 10 women and 2 men with MS to the UK Multiple Sclerosis Tissue Bank (MSTB) based at Imperial College London. Brain slices used were collected from the MSTB within a mean of 17 (SD = 6) hours post mortem. Demographic data including age, disease duration, course and brain weight were obtained from the case records collected at the MSTB and based on information from the same source. Disability was estimated using the expanded disability status score (EDSS) scale (Kurtzke, 1983). Some of the MRI data sets used in this study had been used previously to investigate changes of MRI data in the white matter following fixation (Moore et al., 2000; Schmierer et al., 2008).

Tissue handling

In each case a coronal brain slice (≈1 cm thick) from one hemisphere had been dissected at the level of the mamillary bodies, sealed in a plastic bag and stored in a refrigerator at 2–8 ?C. Three hours before MRI brain slices were taken out of the fridge, carefully wrapped in polyethylene film and left to warm up to scanner room temperature (≈20? C). Immediately after MRI, samples of NLWM (size = 0.5–1 cm^3) were obtained from the brain slices and snap frozen in liquid nitrogen. Digital images of the brain slices were taken showing the location where snap frozen samples had been obtained. Thereafter, brain slices were immersed in 10% buffered formalin.

MRI scanning and parameter map calculation

Scans were acquired on a GE Signa Horizon Echospeed 1.5 T system (General Electric, Milwaukee, WI, USA) using a birdcage head coil. The MRI plane was positioned parallel to the coronal surface and in the centre of each brain slice. The following data sets were acquired using an imaging slice thickness of 5 mm, a field of view (FOV) of 24×24 cm^2 and a matrix size of 256×256 (giving a pixel size of 0.94×0.94 mm^2):

- 2D dual spin-echo (SE) proton density (PD)- and T_2-weighted (T_2w) images with parameters TR = 2000 ms and TE = 30/120 ms, respectively.
- 2D PD and T_1w gradient echo images TR/TE/flip angle = 1500 ms/11 ms/45° and 36 ms/11 ms/45 °, respectively), from which T_1 maps were generated as previously described (Moore, 2003).
- 2D dual SE images (TR/TE1/TE2 = 1720 ms/30 ms/80 ms) obtained with (M_{sat}) and without (M_0) a sinc shaped saturation prepulse applied 1 kHz off water resonance, from which MTR maps were calculated according to $MTR = 100 \times (M_0 - M_{sat})/M_0$ (Schmierer et al., 2007).

Definition of ROI

All scans and maps were displayed on a Sun workstation (Sun Microsystems, Mountain View, CA, USA) using DispImage (Grimaud et al., 1996; Plummer, 1992). Areas of NLWM were defined on T2w SE images as areas of white matter that was free from hyperintense signal suggesting MS lesions. The absence of lesions in areas where snap frozen samples had been obtained was confirmed by visual inspection of the T2w scans using the digital images of brain slices obtained in the dissection theatre as a reference. Accuracy of the correspondence between areas of NLWM detected on MRI and their histological substrates in brain slices were further enhanced through the use of a previously described stereotactic procedure in all cases (Schmierer et al., 2003).

Pathological procedures

Tissue blocks sized approximately 1.5×1.5×1 cm and containing the areas of NLWM detected on MRI were dissected. The blocks were cut in half using a 5 mm deep iron angle resulting in two blocks of approximately 5 mm thickness each with the cutting plane corresponding to the centre of the MRI plane. Blocks were processed for embedding in paraffin and sections stained for haematoxylin & eosin (H&E), Luxol-Fast blue (LFB) and Bielschowsky's silver impregnation. Sections were inspected to confirm no areas of demyelination or remyelination (Brück et al., 2003; Schmierer et al., 2004) had been sampled for protein extraction (see below).

Protein extraction

Snap-frozen blocks of brain tissue from MS cases were cut and resuspended at 1:5 g/mL in Tris-HCl buffer (100 mM Tris, pH 8.1 with 1% Triton X-100). A protease inhibitor cocktail (Sigma, P 8340) was added in a dilution 1:100. Samples were homogenised on ice by sonication, triturated 3 times through 19 and 21 gauge needles and spun at 20,000 *g*. In order to de-lipidise the sample diisopropyl ether was added. After extensive mixing, the sample was spun at 20,000 *g*. The supernatant was covered by a myelin layer. A needle was put through