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Increased mitochondrial content in remyelinated axons: implications for multiple sclerosis

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Mitochondrial content within axons increases following demyelination in the central nervous system, presumably as a response to the changes in energy needs of axons imposed by redistribution of sodium channels. Myelin sheaths can be restored in demyelinated axons and remyelination in some multiple sclerosis lesions is extensive, while in others it is incomplete or absent. The effects of remyelination on axonal mitochondrial content in multiple sclerosis, particularly whether remyelination completely reverses the mitochondrial changes that follow demyelination, are currently unknown. In this study, we analysed axonal mitochondria within demyelinated, remyelinated and myelinated axons in post-mortem tissue from patients with multiple sclerosis and controls, as well as in experimental models of demyelination and remyelination, *in vivo* and *in vitro*. Immunofluorescent labelling of mitochondria (porin, a voltage-dependent anion channel expressed on all mitochondria) and axons (neurofilament), and ultrastructural imaging showed that in both multiple sclerosis and experimental demyelination, mitochondrial content within remyelinated axons was significantly less than in acutely and chronically demyelinated axons but more numerous than in myelinated axons. The greater mitochondrial content within remyelinated, compared with myelinated, axons was due to an increase in density of porin elements whereas increase in size accounted for the change observed in demyelinated axons. The increase in mitochondrial content in remyelinated axons was associated with an increase in mitochondrial respiratory chain complex IV activity. *In vitro* studies showed a significant increase in the number of stationary mitochondria in remyelinated compared with myelinated and demyelinated axons. The number of mobile mitochondria in remyelinated axons did not significantly differ from myelinated axons, although significantly greater than in demyelinated axons. Our neuropathological data and findings in experimental demyelination and remyelination *in vivo* and *in vitro* are consistent with a partial amelioration of the supposed increase in energy demand of demyelinated axons by remyelination.

Keywords: multiple sclerosis; axon; demyelination; mitochondria; remyelination

Introduction

Mitochondria are important for cellular function and reflect local metabolic needs (Morris and Hollenbeck, 1993; Waxman and Ritchie, 1993). Energy demand of axons is met by a network of mitochondria, over 70% of which are stationary, while the remainder move at speeds ∼1 µm/s (Kiryu-Seo et al., 2010). The content of mitochondria within axons is determined by transport to and from neuronal soma as well as by fission and fusion (Hollenbeck and Saxton, 2005; Chen and Chan, 2009). Mitochondria are needed in axons to maintain ionic balance and structural integrity, which is compromised in chronic demyelinating diseases such as multiple sclerosis as well as in anoxic injury of the white matter (Caldwell et al., 1960; Waxman et al., 1992; Waxman, 2006; Trapp and Stys, 2009).

Multiple sclerosis is the most prevalent inflammatory demyelinating disease of the CNS (Compston and Coles, 2008). Following demyelination, nerve impulse conduction velocity decreases and axons become more vulnerable, particularly in inflammatory environments. Adaptive changes occur within demyelinated axons in an attempt to restore function (Felts et al., 1997; Trapp and Stys, 2009). An increase in mitochondrial content within axons following demyelination has been described as a potential adaptive process in multiple sclerosis, animal models of multiple sclerosis and in vitro (Mutsaers and Carroll, 1998; Sathornsumetee et al., 2000; Mahad et al., 2009; Witte et al., 2009; Kiryu-Seo et al., 2010). Energy demand of operating Na^+/K^+ ATPase, increased by diffusely expressed sodium channels with persistent leakage of sodium through Na_v 1.6 along the previously myelinated segments, is regarded as the most likely explanation for the increase in mitochondria within demyelinated axons (Craner et al., 2004; Waxman, 2006; Smith, 2007).

Remyelination is extensive in some multiple sclerosis lesions but incomplete or absent in others (Prineas and Connell, 1979; Raine and Wu, 1993; Patrikios et al., 2006; Patani et al., 2007). The beneficial effects of remyelination include restoration of saltatory conduction and protection of the majority of axons from further inflammatory insults (Smith et al., 1981; Kornek et al., 2000; Sathornsumetee et al., 2000; Irvine and Blakemore, 2008). The energy needs of axons would be predicted to change following remyelination as sodium channels recluster at newly formed nodes of Ranvier (Kuhlmann et al., 2002; Coman et al., 2006; Smith, 2006). Indeed, remyelination has been shown to decrease the demyelination-associated mitochondrial increase within axons in vitro and in acute and chronic animal models of multiple sclerosis (Mutsaers and Carroll, 1998; Sathornsumetee et al., 2000; Kiryu-Seo et al., 2010). Whether remyelination in multiple sclerosis changes axonal mitochondrial content and the mitochondrial characteristics within remyelinated axons are similar to myelinated axons are not known.

In this study, we examined axonal mitochondria in chronic multiple sclerosis lesions and, following ethidium bromide and lysolecithin-induced acute experimental demyelination, in vivo and in vitro (Shields et al., 1999; Penderis et al., 2003). Mitochondrial content within remyelinated axons remained elevated compared with myelinated axons in both multiple sclerosis

and following experimental demyelination. Mitochondrial respiratory chain complex IV activity was also increased within remyelinated compared with myelinated axons, suggesting that remyelinated axons demand more energy than myelinated axons. Findings in vitro suggest that the increased mitochondrial content in remyelinated axons is mostly due to an increase in stationary mitochondria. The lack of complete reversal of axonal mitochondrial parameters following remyelination may contribute to the generation of respiratory deficient neurons in the long term, through expansion of mitochondrial DNA deletions in neuronal soma (Campbell et al., 2011), and increase the potential to reach detrimental levels of axoplasmic calcium following exposure to a hypoxia-like insult, through release of calcium stored in mitochondria (Nikolaeva et al., 2005).

Materials and methods

Autopsy tissue and lesion classification

Brain tissue embedded in paraffin containing chronic multiple sclerosis lesions and control tissue were provided by the Medical University of Vienna and the Multiple Sclerosis Society Tissue Resource, London (Table 1). Cause of death for controls was either cardiorespiratory arrest or pneumonia. North Tyneside Local Research Ethics Committee (205/Q0906/182) and the Ethics Committee of the Medical University of Vienna (EK Nr. 535/2004) granted ethical approval. The cerebral white matter multiple sclerosis lesions were classified based on the sections stained with Luxol fast blue as well as by immunohistochemistry for myelin basic protein, HLA-D and leucocyte common antigen (Table 1) as described previously (Frischer et al., 2009). Chronic inactive lesions showed a lesion

Table 1 Details of autopsy cases

Case	Age/sex	Diagnosis	Disease duration (years)	Number of blocks	Number of lesions (D/R)
MS1	34/M	RPMS	4	2	2/3
MS2	41/M	SPMS	11.4	2	1/1
MS3	56/M	SPMS	30.5	2	1/0
MS4	62/F	SPMS	12	2	1/1
MS5	64/F	SPMS	28	2	2/3
MS6	75/F	PPMS	28	1	1/0
MS7	76/M	SPMS	31	2	1/2
MS8	77/F	PPMS	14	1	1/0
MS9	78/F	SPMS	31	3	3/2
MS10	84/F	SPMS	22	1	1/0
CON1	57/M	NA	NA	2	NA
CON2	64/M	NA	NA	1	NA
CON3	85/M	NA	NA	1	NA
CON4	89/M	NA	NA	2	NA
CON5	95/F	NA	NA	1	NA

CON = control; D = demyelinated lesions; F = female; M = male; MS = multiple sclerosis; NA = not applicable; PPMS = primary progressive multiple sclerosis; R = remyelinated lesions or shadow plaques and remyelinated regions or shadow plaque areas, as previously described (Patrikios et al., 2006); RPMS = relapsing progressive multiple sclerosis; SPMS = secondary progressive multiple sclerosis.

border without macrophage infiltration or activated microglia. The multiple sclerosis tissue was selected from archival material used in the study by Frischer *et al*. (2009), where residual inflammation and axonal degeneration was minimal and similar to levels in age-matched controls. Normal appearing white matter bearing non-demyelinated or myelinated axons in multiple sclerosis was identified as white matter at least 1 cm distant from a lesion. Remyelinated lesions included those that were fully (shadow plaques) or partially (remyelinated regions) repaired, as previously reported (Prineas and Connell, 1979; Patrikios *et al*., 2006; Patani *et al*., 2007). In brief, remyelinated lesions were sharply demarcated from the normal appearing white matter and contained pale myelin staining due to reduction in myelin sheath thickness as well as shortened internodes and widened nodes of Ranvier. Myelin degradation products were absent within macrophages in remyelinated regions of chronic multiple sclerosis lesions and residual macrophages were minimal in shadow plaques, in which nearly all axons were remyelinated (Patrikios *et al*., 2006). Thus, remyelination was considered to be well established in shadow plaques and relatively early in remyelinated regions of chronic active multiple sclerosis lesions.

Animals and histological analysis

Focal demyelination was induced in caudal cerebellar peduncles of young female Sprague-Dawley rats (8–10 weeks of age, ~200 g), as previously described (Shields *et al*., 1999; Penderis *et al*., 2003), in compliance with UK Home Office regulations and Cambridge University guidelines. Anaesthesia was induced using 4% isofluorane in oxygen and warmed sterile saline was injected intraperitoneally to maintain hydration during surgical procedure. Stereotactic injections of 4 µl of 0.001% ethidium bromide were performed.

Animals were analysed for axonal mitochondrial changes following demyelination at four time points: when no remyelination was evident (Day 7), when remyelination was just complete (28 days) and when it had been established for several weeks (42 and 84 days). Three animals were used for each time point. Brain with brainstem were snap frozen in isopentane and stored at −80°C until mounting for cryostat sectioning in the sagittal plane. For large diameter axons (>2.5 µm) remyelination was complete at 28 days and still evident at 84 days (Shields *et al*., 1999; Penderis *et al*., 2003).

Experimental demyelination and remyelination *in vitro*

We used lysolecithin to demyelinate and removal of the toxin from culture media to investigate intra-axonal mitochondrial changes following remyelination, *in vitro*, in dorsal root ganglion and Schwann cell mixed cultures, as previously utilized to describe changes in size and speed of stationary and mobile mitochondria in axons (Kiryu-Seo *et al*., 2010). In this study, we determined the number of stationary

and mobile mitochondrial in remyelinated axons in the same experimental demyelination system. In brief, myelination was induced using ascorbic acid. Once myelination was complete, after ~6 weeks, lysolecithin (0.05%) was added for 15 h and demyelinated axons analysed 48 h following the addition of the toxin. Remyelination was assessed 2 weeks after the removal of the toxin. Mitochondria within neurons were labelled using Mito-DsRed2 with a mitochondrial targeting sequence subcloned to lentiviral vector. Presence or absence of myelin was established by immunocytochemistry and antibody against myelin basic protein.

Immunohistochemistry

For paraffin embedded multiple sclerosis tissue, following rehydration of fixed and paraffin embedded sections (5 µm thickness) antigen retrieval was performed using pressure cooker (15 psi for 5 min) in 1 mmol EDTA buffer pH 8.0. Non-specific antibody binding was prevented by blocking sections in 1% normal goat serum (Sigma) for 30 min prior to incubation with the mixture of two or three primary antibodies (Table 2) for 90 min. Two primary antibodies (SMI31 and porin, a voltage-dependent anion channels expressed in all mitochondria) were used for myelinated and demyelinated axons in control tissue and chronic inactive multiple sclerosis lesions, as all axons or none were demyelinated when serial sections of chronic multiple sclerosis lesions and control tissue were stained for myelin basic protein and SMI31. Three primary antibodies (SMI31, porin and myelin basic protein) were used for remyelinated axons in remyelinated regions and shadow plaques, to ensure that axons remaining demyelinated in these regions were not included in the analysis. After three washes in Tris-buffered saline, sections were incubated for 30 min with two or three secondary antibodies [Rhodamine Red-X (RRX)-conjugated goat anti-rabbit (Stratech Scientific Ltd) 1:200, Alexa Fluor® 633 goat anti-mouse IgG$_1$ 1:200 and Alexa Fluor® 488 goat anti-mouse IgG$_{2b}$ 1:200]. The sections were mounted using Vectashield with DAPI (Vector). Lack of cross reactivity and non-specific binding of secondary antibodies was determined using appropriate controls.

For snap frozen rat tissue, 8 µm thick cryostat sections were fixed in 4% paraformaldehyde prior to gentle antigen retrieval using boiling 1 mmol EDTA buffer pH 8.0 for 15 s in a microwave. Sections were stained as described above. As lesions at Day 7 were completely demyelinated and Day 28 were completely remyelinated, two primary antibodies (SMI31 and porin) were used for myelinated, demyelinated and remyelinated axons in the ethidium bromide-induced model.

Confocal microscopy

Immunofluorescently labelled axons (SMI31-positive) and mitochondria identified using porin and myelin (myelin basic protein for

Table 2 Details of antibodies

Antibody	Antibody type	Target	Manufacturer
Myelin basic protein	Rabbit polyclonal 1:100	Myelin basic protein	Abcam
HLA-DP, DQ, DR	Mouse IgG$_1$ 1:50	Human leukocyte antigen	Dako cytomation
LCA	Mouse IgG$_1$ 1:20	Leukocyte common antigen	Invitrogen
ED1	Mouse IgG$_1$ 1:1000	CD68	Serotec
Porin	Mouse IgG$_{2b}$ 1:400	Voltage-dependent anion channel	Mitosciences
SMI31	Mouse IgG$_1$ 1:1000	Phosphorylated neurofilament	Covance

LCA = leukocyte common antigen.

remyelinated regions in multiple sclerosis tissue) were imaged on a Leica scanning fluorescent microscope with an oil immersion lens at $\times 63$, as previously described. Sections (0.5-μm thick) for a total of 10-μm depth were imaged using a sequential system with the three fluorescent channels. Images were initially taken through sections in an x–y plane. To avoid inclusion of extra-axonal mitochondria located above and below the axon, the minimum number of x–y confocal images that contained an axon was selected separately for each axon at least 50 μm in length and without morphological features of injury (terminal ovoid's, constrictions and beading). The axonal location of mitochondria in the selected stack of confocal x–y images was confirmed in x–z images, as previously reported (Mahad *et al.*, 2009). Extra-axonal mitochondria in the selected stack of confocal x–y images, a rare occurrence, were not included in the analysis. For *in vitro* studies of stationary and mobile mitochondria, time-lapse images of DsRed2-positive elements were captured every 6.8 s for 20 min at 37°C with an inverted laser-scanning Leica confocal microscope.

Electron microscopy

Lesioned rats were deeply anaesthetized and perfused transcardially with phosphate-buffered saline followed by 4% glutaraldehyde, 0.008% CaCl$_2$ in phosphate-buffered saline. Tissue containing caudal cerebellar peduncles was post-fixed in the same fixative solution, then cut transversely into 1.0-mm thick blocks. Blocks were further fixed in osmium tetroxide at 4°C overnight, dehydrated through ascending ethanol washes and embedded in epoxy resin (TAAB Laboratories). One-micrometre sections were cut, stained with toluidine blue and examined by light microscopy, from which remyelination was identified using standard morphological criteria. Selected areas were examined by electron microscopy (Hitachi H600). Images of transverse sections in lesioned white matter of the unlesioned caudal cerebellar peduncles were acquired at $\times 6000$ magnification, and the digitized images were analysed with ImageJ (National Institutes of Health). The mitochondrial density of individual normal or remyelinated axons was obtained with measured areas and corresponding numbers of mitochondria.

Sequential complex IV histochemistry and immunofluorescent labelling of axons

Mitochondrial respiratory chain complex IV active elements were detected in unfixed cryostat sections from snap frozen tissue blocks, as previously described (Mahad *et al.*, 2009). In brief, cytochrome c oxidase (COX or complex IV) medium (100 mM cytochrome c, 4 mM diaminobenzidine tetrahydrochloride and 20 mg/ml catalase in 0.2 M phosphate buffer, pH 7.0) was applied for 55 min at 37°C followed by three washing steps in Tris-buffered saline. Following blocking of non-specific binding, using 1% normal goat serum, the sections were incubated with primary antibody against phosphorylated neuro-filament, SMI31. Combined bright field and confocal images were obtained using a Zeiss Axio Imager Z2 (Apotome) system, as recently reported (Zambonin *et al.*, 2010). To determine possible biochemical consequences of possible damage to or depletion of mitochondrial DNA by ethidium bromide, we used sequential complex IV (COX)/complex II [SDH (succinate dehydrogenase)] histochemistry (Mahad *et al.*, 2009).

Quantitation of axonal mitochondrial content in tissue and number of stationary and mobile mitochondria *in vitro*

The axonal mitochondrial content was determined as the area occupied by axonal mitochondria, identified by porin labelling and located to axons using x–y and x–z confocal images, as a percentage of the area of axon in x–y images, as previously described (Mahad *et al.*, 2009). Control sections were stained and imaged with multiple sclerosis tissue in each batch of experiments and values for axonal mitochondrial content were normalized using the same control tissue sections in order to correct for batch-to-batch variability in staining and imaging parameters. In addition, the size and number of porin elements within each axon were determined by manually outlining axonal porin elements and axons. Axons with continuous segments of at least 50-μm length were included in the analysis to ensure morphological integrity and to take into account the variability of inter-mitochondrial distance within axons (Mahad *et al.*, 2009). To avoid inclusion of axons that may not have been remyelinated in remyelinated multiple sclerosis tissue, we identified remyelinated axons in shadow plaques and remyelinated regions of chronic multiple sclerosis lesions by triple immunofluorescent labelling of SMI31, porin and myelin basic protein (Table 2). To identify stationary and mobile mitochondria *in vitro*, kymographs were generated, as previously described (Kiryu-Seo *et al.*, 2010). The number of mobile mitochondria that moved past two fixed points 40 mm apart, three times the average distance between adjacent stationary sites in myelinated and demyelinated axons, during the 20 min study period was calculated in kymographs.

Statistics

Parametric tests (two-way ANOVA) were used to assess differences between more than two groups. To compare differences between two selected groups of interest Bonferroni test was used. Statistical Package for the Social Sciences version 14 was used and $P < 0.05$ were considered statistically significant.

Results

Mitochondrial content within remyelinated axons is greater than in myelinated axons in multiple sclerosis

In order to compare remyelinated axons with myelinated and demyelinated axons, we determined the mitochondrial content within remyelinated and chronically demyelinated axons as well as myelinated axons in normal appearing white matter from multiple sclerosis cases and in normal white matter of control cases. In chronic inactive multiple sclerosis lesions, where inflammation and axonal degeneration had subsided to levels in age-matched controls (Frischer *et al.*, 2009), the chronically demyelinated axons contained significantly increased mitochondrial content compared with myelinated axons (Figs. 1A, B and 2). In remyelinated regions, closely adjacent to demyelinated areas in chronic multiple sclerosis lesions, we did not detect a
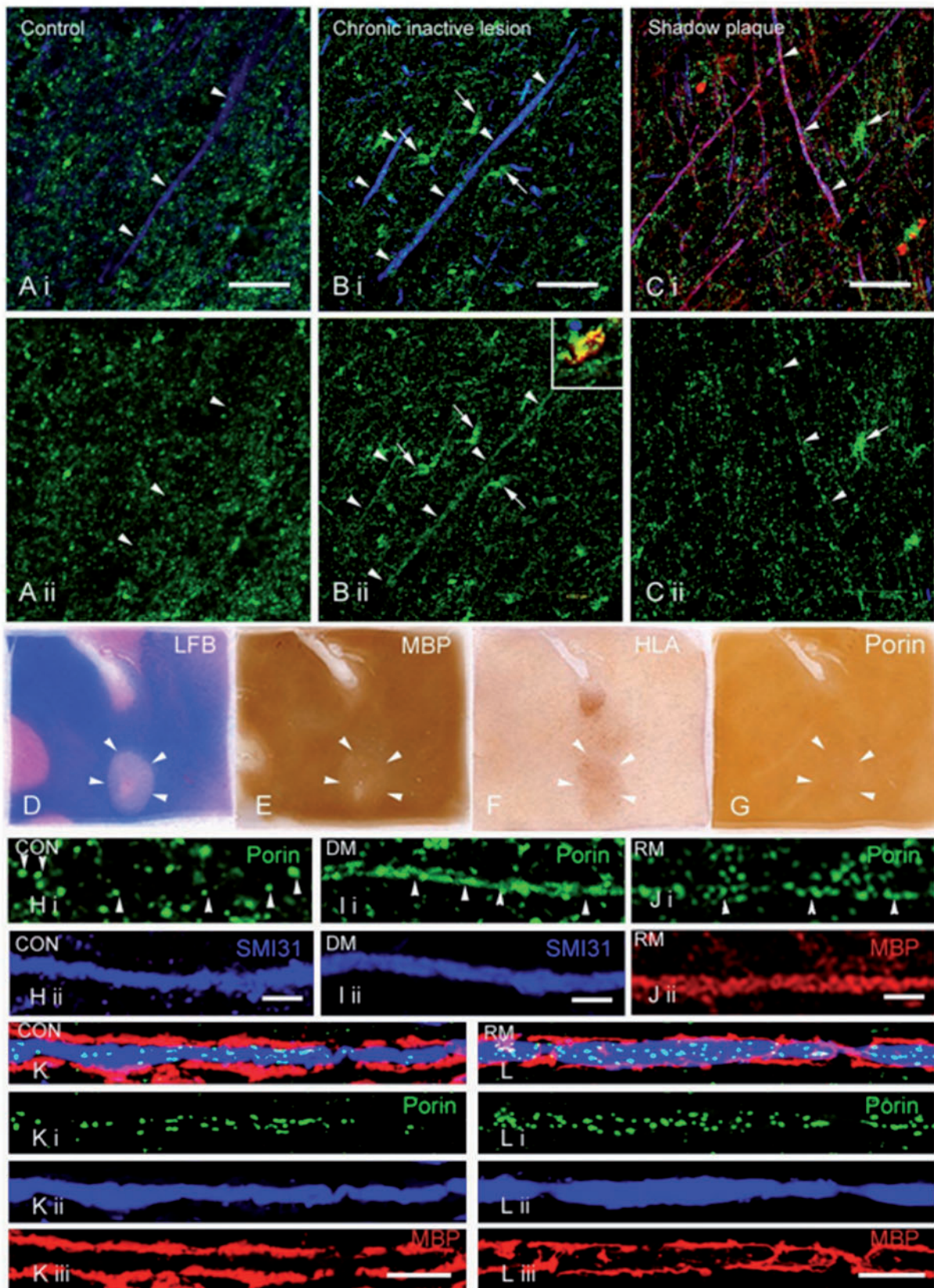
Figure 1 Axonal mitochondrial content in multiple sclerosis and control brain tissue. (**A–C**) Confocal images of phosphorylated neurofilament (SMI31) staining axons (blue, arrowheads) and porin staining mitochondria (green) with (**C**, **J**, **K** and **L**) or without (**A**, **B**, **H** and **I**) myelin basic protein (MBP, in red) staining showed numerous axonal and non-axonal mitochondria in control white matter (**A**), chronic inactive multiple sclerosis lesions (**B**) and shadow plaques (**C**) [**B**(ii) and **C**(ii), arrows showing glial cell bodies and insert in **B**(ii)
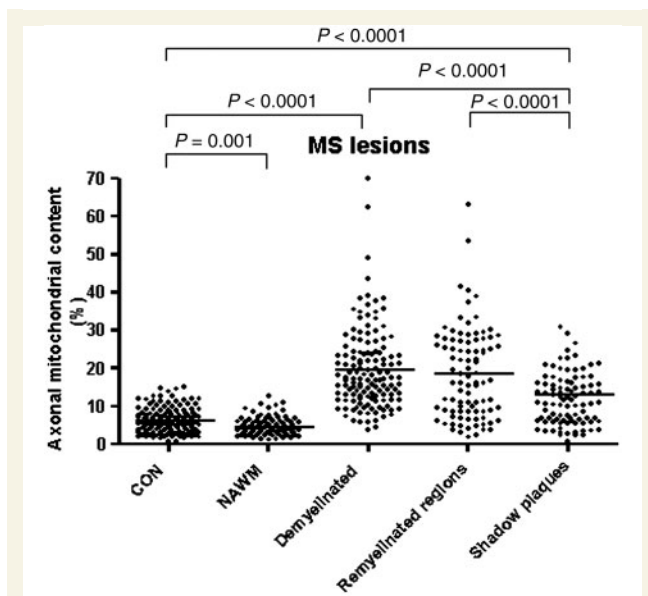
Continued

Figure 2 Quantitation of axonal mitochondria in multiple sclerosis (MS) and control (CON) tissue. Quantitation of axonal mitochondria, as a percentage of the area of axon, showed a significant increase in axonal mitochondrial content in chronically demyelinated axons (19.61 ± 5.67, n = 136) compared with myelinated axons in multiple sclerosis tissue (4.67 ± 1.28, n = 102) and controls (6.26 ± 1.73, n = 126). The mitochondrial content within remyelinated axons in remyelinated regions of chronic multiple sclerosis lesions (18.45 ± 5.89, n = 92) was not significantly different compared to demyelinated axons. A significant reduction in mitochondrial content within remyelinated axons was noted in shadow plaques (11.79 ± 4.79, n = 93), where remyelination was established throughout the lesions, compared with demyelinated axons in chronic multiple sclerosis lesions. Interestingly, remyelinated axons in shadow plaques still showed a significantly greater mitochondrial content than myelinated axons. Each data point indicates the area of axonal mitochondria as a percentage of the area of a single axon. Myelinated axons in normal appearing white matter (NAWM) of multiple sclerosis cases contained significantly less mitochondria than in corresponding axons of controls.

significant difference in axonal mitochondrial content between remyelinated and demyelinated axons (Fig. 2). In shadow plaques, where remyelination was established throughout the lesion, a significant reduction in mitochondrial content was noted in remyelinated axons compared with chronically demyelinated axons in multiple sclerosis lesions (Figs 1C and 2). At low magnification, the increase in porin expression, previously reported in chronic active multiple sclerosis lesions (Mahad *et al.*, 2009), was no longer apparent in shadow plaques (Fig. 1D–G and Supplementary Fig. 1).

Although remyelination is thought to decrease the energy needs of axons, based upon reclustering of sodium channels, the content of mitochondria within remyelinated axons did not return to levels apparent within myelinated axons of the normal appearing white matter in multiple sclerosis and in myelinated axons of control white matter (Fig. 1H and J–L). Interestingly, we detected a difference in size and density of porin elements within demyelinated and remyelinated axons compared with myelinated axons. The increased mitochondrial content within remyelinated axons in multiple sclerosis compared with myelinated axons was mainly due to an increase in number rather than the size of porin elements (Fig. 1H–L and Table 3). In contrast, an increase in size of porin elements accounted for the greater mitochondrial content within chronically demyelinated axons.

Changes in axonal mitochondria in ethidium bromide-induced experimental demyelination and remyelination are consistent with findings in multiple sclerosis

As multiple sclerosis is a chronic disorder, changes in axonal content following remyelination may be influenced by the chronicity of the disease as well as defects of mitochondria within neurons (Dutta *et al.*, 2006). We next asked whether axonal mitochondrial changes differed between recently remyelinated fibres and those where remyelination had been established for some time, following focal demyelination and where demyelination was acute and neurons were not directly involved. To do this, we used an acute

Figure 1 Continued

showing double labelling of HLA in red and porin in green]. Myelin basic protein was labelled in multiple sclerosis lesions with remyelinated regions and shadow plaques. As all and none of the axons were demyelinated in chronic multiple sclerosis lesions and control tissue, respectively, when determined in serial sections (data not shown), we did not label myelin in chronic inactive multiple sclerosis lesions and control tissue. Mitochondrial elements were abundant in demyelinated [**B**(ii), arrowheads] and remyelinated axons [**C**(ii), arrowheads] and less apparent in myelinated axons in control tissue [**A**(ii), arrowheads]. Scale bar = 20 μm. (**D–G**) Low magnification images of serial sections from a case with multiple sclerosis stained for Luxol fast blue (LFB), myelin basic protein, human leukocyte antigen (HLA) and porin show a shadow plaque (arrowheads). The intensity of porin immunoreactivity in shadow plaque with established remyelination (arrowheads) was similar to normal appearing white matter at low magnification, unlike the increased intensity of porin reported previously in centre of chronic active multiple sclerosis lesions (Mahad *et al.*, 2009; Witte *et al.*, 2009). (**H–J**) The elongated and larger mitochondrial elements were apparent in demyelinated (DM, **I**) compared with myelinated (CON, **H**) and remyelinated axons (RM, **J**) at ×100 magnification. Scale bar = 8 mm. (**K** and **L**) Differences in axonal mitochondria in remyelinated compared with myelinated axons were observed in all shadow plaques from different cases with multiple sclerosis. Remyelinated axons [**L** and **L**(ii), SMI31 in blue] with thinner myelin sheaths [**L**(iii), myelin basic protein in red] contained more mitochondria [**L**(i), porin in green] than myelinated axons [**K** and **K**(i–iii)], shown in a separate multiple sclerosis case to that in **J**. Scale bar = 20 μm.

Table 3 Size and density of porin immunoreactive elements within axons in multiple sclerosis and experimental demyelination

Controls	Normal appearing white matter	Demyelinated	Partially remyelinated	Fully remyelinated
Multiple sclerosis				
5.22 ± 0.31 (58)	4.32 ± 0.63 (52)	15.67 ± 1.80# (64)	4.49 ± 0.23 (45)	4.95 ± 0.25 (42)
3.36 ± 0.07	3.54 ± 0.11	4.92 ± 0.12	6.54 ± 0.06#	6.72 ± 1.05#
Controls	**Normal appearing white matter**	**Demyelinated**	**Early remyelinated[a]**	**Late remyelinated[b]**
Experimental demyelination				
2.48 ± 0.17 (38)	2.14 ± 0.14 (52)	7.18 ± 1.41# (149)	5.50 ± 0.65# (97)	2.27 ± 0.17 (106)
5.01 ± 0.17	4.97 ± 0.16	4.93 ± 0.17	6.06 ± 0.20†	8.09 ± 0.19#

[a] Early and [b]Late remyelinated axons were analysed at 28 and 42 days post ethidium bromide injection in animal models whereas in multiple sclerosis remyelinated axons in partially or fully repaired lesions were analysed separately.
#$P < 0.001$; †$P = 0.013$.
Unshaded = mean size or area of axonal porin immunoreactive elements in μm^2. Shaded = mean density or number of axonal mitochondria (porin immunoreactive elements) per $100\,\mu m^2$ of axons. The number of axons analysed is shown in parentheses.

model of CNS demyelination induced by direct injection of ethidium bromide into adult rodent white matter (Fig. 3A). At Day 7, axons were demyelinated and myelin basic protein immunoreactivity was localized to macrophages in lesions. At Day 28 and beyond, remyelination was complete, as previously reported (Shields *et al.*, 1999).

We detected a significant increase in mitochondrial content within acutely demyelinated axons 7 days after ethidium bromide injection, compared with myelinated axons in corresponding non-lesioned tissue (Fig. 3B–D). At 28 days following ethidium bromide injection, when remyelination has recently been completed, the axonal mitochondrial content was not significantly different compared with acutely demyelinated axons (Fig. 3B–E). At 42 days following ethidium bromide injection, however, there was a significant decrease in the axonal mitochondrial content compared with both demyelinated and early remyelinated axons (Fig. 3B–F). Mitochondrial content within remyelinated axons remained greater than in myelinated axons, similar to the findings in multiple sclerosis tissue. The changes in size and density of porin elements in relation to demyelination and remyelination were also similar to the findings in multiple sclerosis (Table 3). The increased mitochondrial content within remyelinated axons in ethidium bromide-induced model at 42 days was due to the increase in density of porin elements whereas changes in size of porin elements accounted for the increase in acutely demyelinated axons.

In order to further investigate mitochondrial content following remyelination, we performed electron microscopy at 84 days post ethidium bromide injection. At this stage remyelination was well-established (Fig. 4A and B). Mitochondrial density, defined as the number of discrete mitochondria per square micrometre of axons in cross section, remained more numerous in late remyelinated than in myelinated axons of similar diameter ($>2.5\,\mu m$, Fig. 4A–C). The greater mitochondrial content within remyelinated axons was associated with a significant increase in area of complex IV active mitochondrial elements as a percentage of axonal area (Fig. 4D–F), when determined using previously reported methods (Mahad *et al.*, 2009; Zambonin *et al.*, 2010). Mitochondrial

elements lacking complex IV but with intact complex II activity, a marker of biochemically relevant mitochondrial DNA depletion or mutations, were not observed within lesions in the *in vivo* model (Supplementary Fig. 2).

Greater mitochondrial content within remyelinated compared with myelinated axons is due to an increase in number of stationary mitochondria

An *in vitro* experimental demyelination system using lysolecithin was used to determine whether the increased content of mitochondria within remyelinated compared with myelinated axons was due to changes in mobile and/or stationary mitochondria (Kiryu-Seo *et al.*, 2010). The number of mobile mitochondria that moved at a steady speed in remyelinated axons was not significantly different from myelinated axons (Fig. 5A–G and movie clips in Supplementary material). Interestingly, there was a trend towards a decrease in the number of mobile mitochondria that moved at a steady speed following demyelination, which recovered following remyelination (Fig. 5G). There was a significant increase in the number of stationary mitochondria in remyelinated compared with myelinated and demyelinated axons (Fig. 5A–F and H).

Discussion

The increase in mitochondrial content in axons following demyelination is well-established (Mahad *et al.*, 2009; Witte *et al.*, 2009; Kiryu-Seo *et al.*, 2010). In this study, we report changes in mitochondrial content within remyelinated axons in multiple sclerosis and following experimental demyelination, *in vivo* and *in vitro*. Remyelination appeared to remodel mitochondria within axons and was associated with a significant decrease in axonal mitochondrial content compared with demyelinated axons. However, the mitochondrial content in remyelinated axons remained significantly
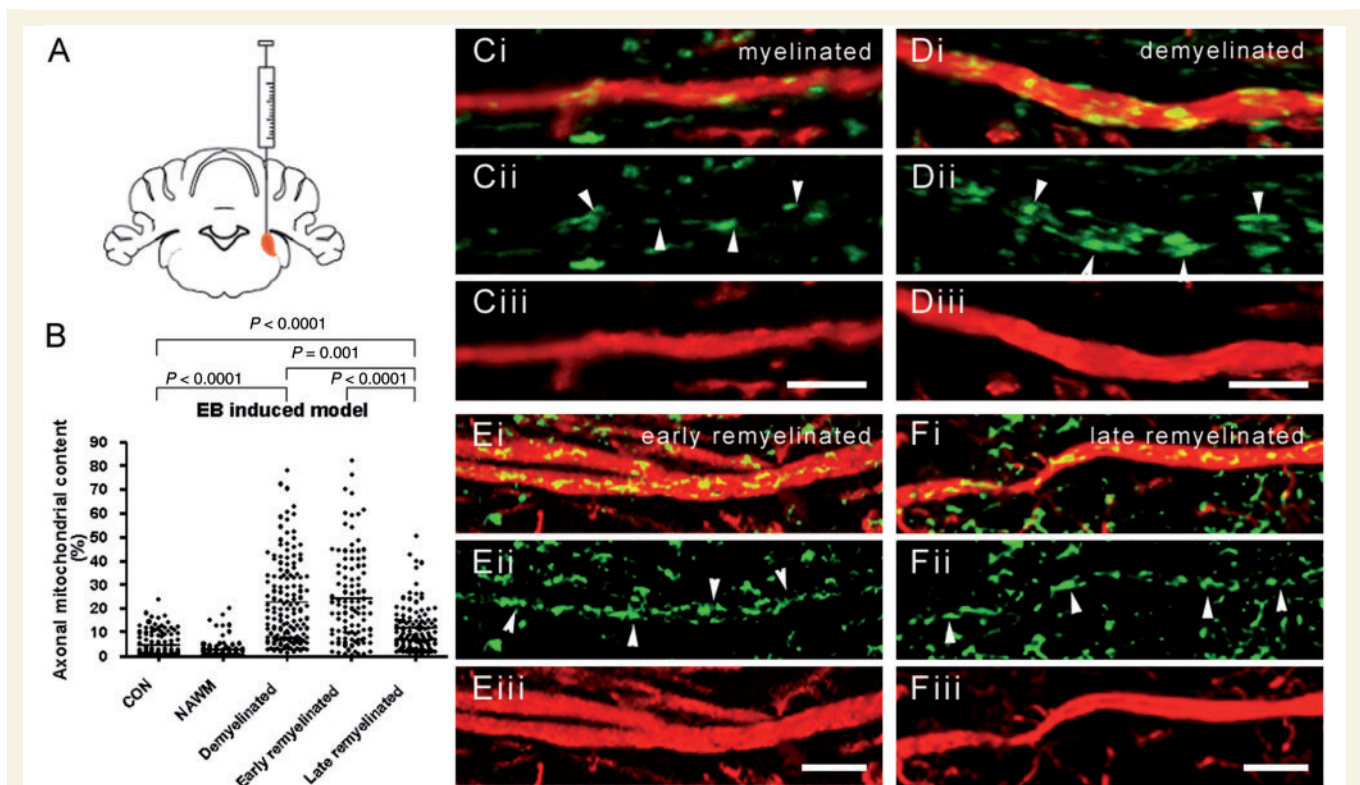
Figure 3 Axonal mitochondrial content in ethidium bromide-induced model of multiple sclerosis. (**A**) Experimental demyelination was induced by stereotactic injection of ethidium bromide (EB) to caudal cerebellar peduncle. At Day 7, axons were demyelinated (not shown) and at Day 28 remyelination was complete (early remyelinated) as previously reported (Shields et al., 1999). (**B**) Axonal mitochondrial content was determined as for multiple sclerosis tissue in Fig. 1. There was a significant increase in mitochondrial content within acutely demyelinated axons at Day 7 (demyelinated, 22.70 ± 8.42, $n = 143$) following ethidium bromide injection to caudal cerebellar peduncles. Remyelinated axons at Day 28 (early remyelinated stage, 24.15 ± 8.96, $n = 104$) contained similar content of mitochondria compared with acutely demyelinated axons. At 42 days, following ethidium bromide injection (late remyelination stage, 11.85 ± 5.46, $n = 109$), there was a significant reduction in axonal mitochondrial content compared with demyelinated and early remyelinated axons. As observed in multiple sclerosis tissue, remyelinated axons in ethidium bromide-induced model contained significantly greater mitochondrial content than myelinated axons within normal appearing white matter (NAWM) in ethidium bromide model (2.91 ± 2.12, $n = 78$) as well as control (CON) tissue (4.68 ± 3.10, $n = 120$). Each data point indicates the area of axonal mitochondria as a percentage of the area of a single axon. (**C–F**) Axonal mitochondria were identified by the location of porin elements (green) within phosphorylated neurofilament (SMI31) staining axons (red) in xyz and xzy confocal images. At $\times 100$ magnification, the mitochondrial elements were elongated and larger in demyelinated (**D**) compared with myelinated (**C**) and early (28 days) and late (42 days) remyelinated axons (**E** and **F**, respectively). There were more porin elements in remyelinated (**E** and **F**) than myelinated axons (**C**). Scale bar = $8 \mu m$. [**C–F**(i)] Overlap confocal images of mitochondria (porin) and phosphorylated neurofilament (SMI31). [**C–F**(ii)] Porin elements (green). [**C–F**(iii)] SMI31 labelling in red.

greater than myelinated axons in multiple sclerosis and following experimental demyelination and remyelination in vivo. A significant increase in mitochondrial number, rather than size, contributed to the differences between remyelinated and myelinated axons. In vitro studies of experimental demyelination and remyelination showed that the increased mitochondrial content in remyelinated compared with myelinated axons to be mostly due to an increase in the number of stationary mitochondria. The increase in mitochondrial number in remyelinated axons is unlikely to be because of mitochondrial transport disturbance or mitochondrial respiratory chain dysfunction as the number of mobile mitochondria was similar to myelinated axons and complex IV activity was increased in remyelinated axons. The supposed metabolic demand of myelinated, demyelinated and remyelinated

axons, based on the distribution of sodium channels, is consistent with the changes in mitochondrial content within axons (Waxman, 2006; Trapp and Stys, 2009).

Previous studies consistently showed changes in axonal mitochondria following remyelination (Mutsaers and Carroll, 1998; Sathornsumetee et al., 2000; Kiryu-Seo et al., 2010). However, whether remyelination completely reverses the mitochondrial changes in axons that follow demyelination has not been comprehensively addressed. In the study by Mutsaers et al. (1998), remyelination was thought to return axonal mitochondria to 'normal refractiveness', as the difference in mitochondrial density between myelinated and remyelinated optic nerve axons was not statistically significant, although on average, remyelinated axons contained twice as many mitochondria as myelinated axons
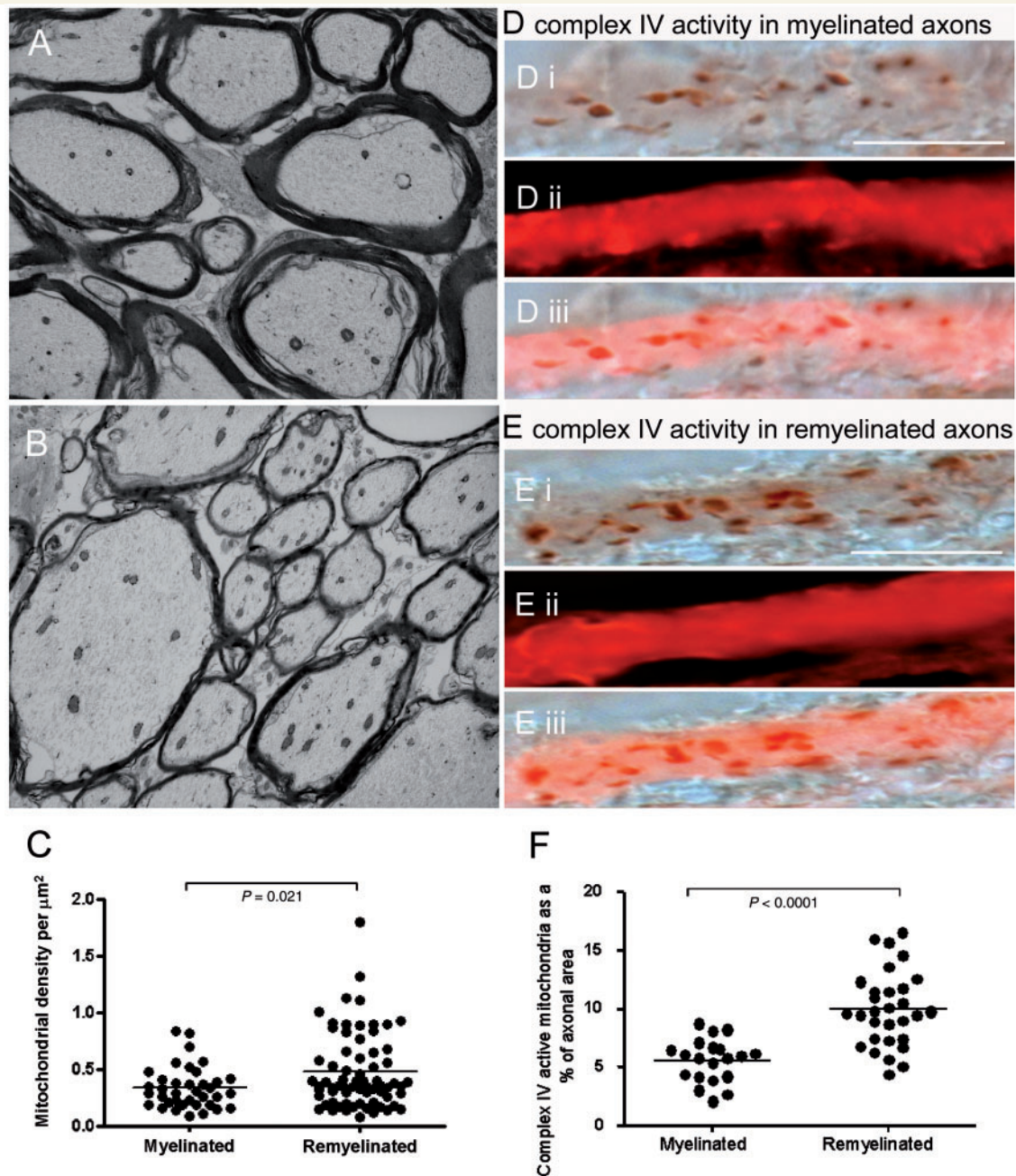
Figure 4 Electron microscopic analysis of mitochondrial density and histochemical analysis of mitochondrial respiratory chain complex IV activity within myelinated and remyelinated axons following ethidium bromide-induced experimental demyelination, *in vivo*. (**A** and **B**) Imaging of remyelinated axons at 84 days following ethidium bromide injection to caudal cerebellar peduncle by electron microscopy confirmed complete remyelination. Mitochondria were identifiable within cross sections of myelinated (**A**) and remyelinated axons (**B**). (**C**) The density of mitochondria in cross sectional images was significantly greater in remyelinated (0.47 ± 0.13, $n = 36$) than myelinated axons (0.33 ± 0.09, $n = 68$). (**D** and **E**) Sequential cytochrome c oxidase (complex IV or COX) histochemistry and immunofluorescent labelling of phosphorylated neurofilament (SMI31) was performed in the same cryostat section. Brightfield [**D** (iii) and **E** (iii)] and confocal images [**D** (ii) and **E** (ii)] captured using Zeiss Axio Imager Z2 (Apotome) system and superimposed to identify complex IV active mitochondrial elements within axons [**D**(i) and **E**(i)]. Scale bar = 20 μm. (**F**) The area occupied by complex IV active elements within axons as a percentage of the area of axons was calculated per myelinated and remyelinated axon. A significant increase in complex IV activity was detected within remyelinated (10.01 ± 0.73, $n = 30$) than myelinated axons (5.63 ± 0.41, $n = 20$).
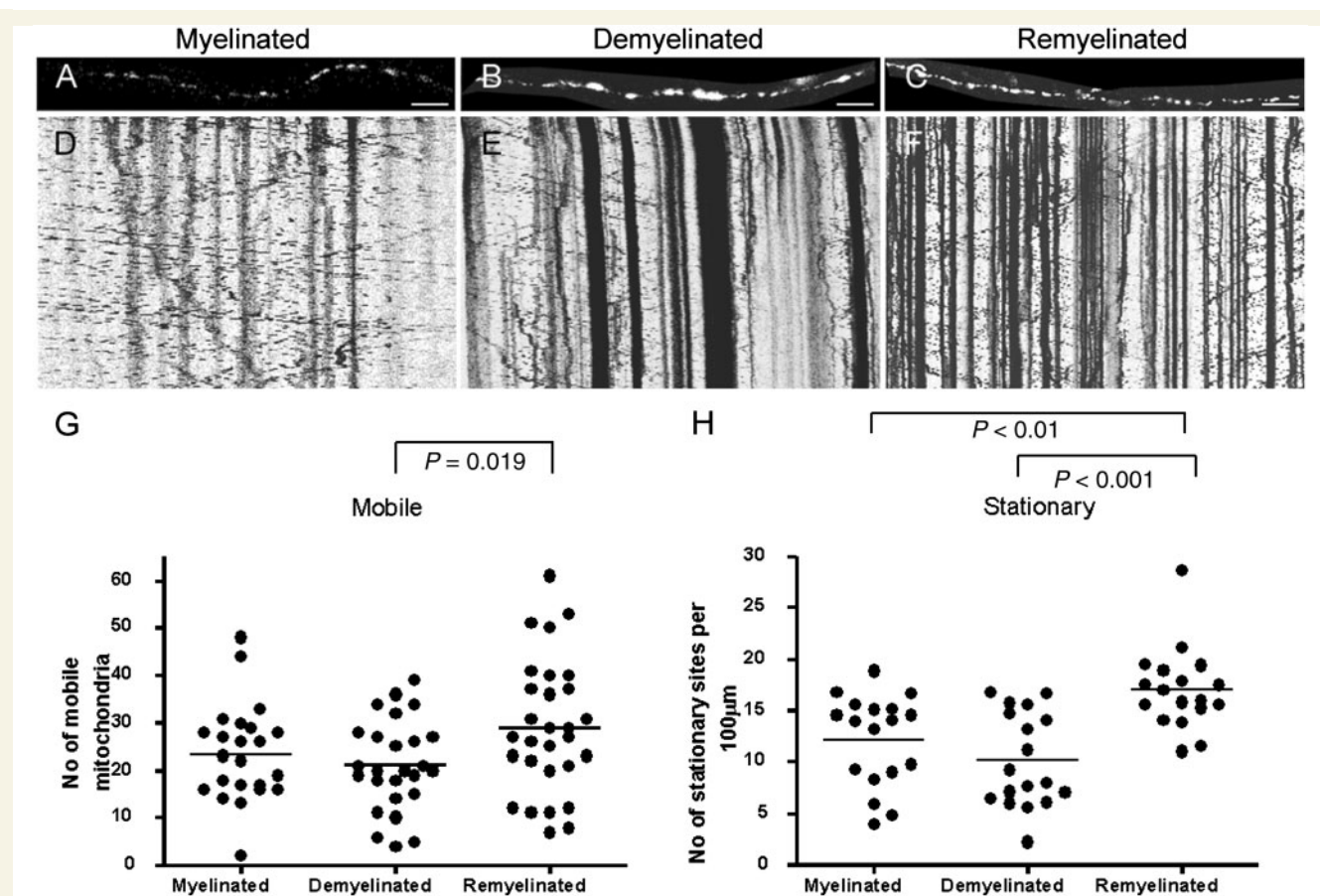
Figure 5 Changes in the number of stationary and mobile mitochondria following remyelination *in vitro*. (**A–C**) Single frame obtained from the stack of time lapse confocal images of myelinated (**A**), demyelinated (**B**) and remyelinated (**C**) axons *in vitro,* showed stationary and some mobile mitochondria *in vitro,* judged according to the kymographs. The increase in size of stationary mitochondria in demyelinated axons and reversal following remyelination were evident. The presence or absence of myelin was determined using immunofluorescent cytochemistry with antibodies against myelin basic protein, as previously described (Kiryu-Seo *et al.*, 2010). Scale bar = 10 μm. (**D–F**) Kymographs of time lapse confocal images showed the increase in number of stationary mitochondria, which were smaller than in demyelinated axons, in remyelinated axons compared with myelinated and demyelinated axons. Mitochondria that moved at a steady speed were apparent in all three kymographs. There were also numerous mitochondria that moved at a variable speed and distances shorter than 40 μm in 20 min, which were not included in the quantitation of mobile mitochondria. (**G** and **H**) There was a trend towards a decrease in number of mitochondria that moved at a steady speed beyond 40 μm in 20 min within demyelinated (21.12 ± 1.87, $n = 26$) compared with myelinated axons (23.61 ± 2.10, $n = 23$). The difference in the number of such mobile mitochondria between remyelinated (29.01 ± 2.61, $n = 29$) and myelinated axons was not significant although there was a significant difference between remyelinated and demyelinated axons. Quantitation of number of stationary mitochondrial sites revealed a significant increase in remyelinated (17.05 ± 0.93, $n = 18$) compared with myelinated (12.21 ± 1.05, $n = 18$) and demyelinated (10.20 ± 1.08, $n = 18$) axons. The average length of axons included in the quantitation was 97.44 ± 11.25 (μm) for demyelinated, 110.40 ± 9.89 for demyelinated and 126.67 ± 16.41 for remyelinated axons. Dynamics of DsRed2 labelled axonal mitochondria are shown in the online video clips in the Supplementary material.

(Mutsaers and Carroll, 1998). Interestingly, mitochondrial density in remyelinated axons did not return to levels in myelinated axons in Theiler's murine encephalomyelitis virus (TMEV)-induced inflammatory demyelination, where some axons remained chronically demyelinated up to the latest time point (195 days), remyelination occurred late and large diameter axons were preferentially injured, unlike in multiple sclerosis (Sathornsumetee *et al.*, 2000; DeLuca *et al.*, 2004). Axonal mitochondrial changes were also observed following demyelination and remyelination in an *in vitro* system, which was relatively free of inflammation. We

previously reported a number of changes in axonal mitochondria that were induced by demyelination and reversed by remyelination, *in vitro* (Kiryu-Seo *et al.*, 2010). Mitochondrial transport velocity and size of stationary mitochondria were similar in remyelinated and myelinated axons, *in vitro,* which led to the conclusion that remyelination reversed demyelination-induced mitochondrial changes. However, the number of stationary mitochondrial sites in remyelinated axons and the number of mobile mitochondria in axons were not analysed in the previous study. Additional *in vitro* findings in this study showed that not all

changes in axonal mitochondria following demyelination are completely reversed by remyelination. In particular, there were more stationary mitochondrial sites in remyelinated axons compared with myelinated and demyelinated axons. The differences in mitochondrial dynamics *in vitro* provide additional information that is complementary to the *in vivo* observations. Furthermore, *in vitro* observations highlight the importance of studying mitochondrial dynamics following experimental demyelination and remyelination in the CNS, *in vivo*.

Mitochondrial networks in axons are dynamic structures and change rapidly through transport, fission and fusion and possibly mitochondrial biogenesis within axons (Morris and Hollenbeck, 1993; Amiri and Hollenbeck, 2008; Kiryu-Seo *et al.*, 2010). *In vitro* findings elegantly showed how rapidly the changes in mitochondrial parameters take place within axons following demyelination (2 days) and remyelination (at 14 days) (Kiryu-Seo *et al.*, 2010). In our *in vivo* model, complete remyelination occurred early (28 days), allowing us to assess how axonal mitochondria adapt with remyelination over time. The lack of complete reversal of mitochondrial changes at 84 days following acute demyelination in our animal model cannot be explained by inadequate time for a complete reversal to occur. Furthermore, neurons in the *in vivo* model were not exposed to ethidium bromide and we did not see evidence of a biochemical defect in mitochondrial respiratory chain complex IV activity, which is frequently effected when mitochondrial DNA is depleted. Instead, complex IV activity was increased in remyelinated compared with myelinated axons. The increase in complex IV activity favours the mitochondrial changes as an active response to remyelination than a consequence of a toxin or inflammatory mediator-related mitochondrial damage. Furthermore, injured mitochondria within axons are likely to undergo retrograde movement and be degraded by mitophagy in neuronal soma (Miller and Sheetz, 2004; Chen and Chan, 2009). Similarities in mobile mitochondria between remyelinated and myelinated axons point against the disruption of mitochondrial transport machinery in remyelinated axons accounting for the changes in mitochondrial content. Hence, the lack of complete reversal of axonal mitochondria following remyelination in the models is likely to be because of remyelination *per se*. The shorter internodes, wider nodes and binary nodes in remyelinated axons, and the resulting reclustered sodium channels, are plausible explanations for supposed increase in energy demand and mitochondrial content in remyelinated axons (Coman *et al.*, 2006; Howell *et al.*, 2006). The apparent remodelling of mitochondrial networks within axons identifies a role for mitochondrial transport and fusion following demyelination while mitochondrial fission is likely to be important following remyelination (Tondera *et al.*, 2009). A disturbance in mitochondrial fusion and/or fission may play a role in axonal degeneration particularly in inflammatory demyelinating conditions of the CNS.

The balance between demand for and supply of energy in axons would depend on mitochondrial biogenesis in neuronal soma. We recently reported mitochondrial DNA deletions at low levels in multiple sclerosis cortical grey matter that had expanded to high levels in a proportion of neurons (Campbell *et al.*, 2011). In primary mitochondrial disorders, the expansion of mitochondrial DNA mutations and respiratory deficiency are most notable in structures that are metabolically highly active, such as muscle fibres, choroid plexus epithelium and neurons (McFarland *et al.*, 2010). An increase in mitochondrial DNA replication rate and expansion of mitochondrial DNA mutations (clonal expansion) has been proposed as a likely mechanism of tissue injury in these disorders. The supposed increase in energy demand of remyelinated and demyelinated axons is likely to influence the expansion of induced mitochondrial DNA deletions in multiple sclerosis. Such an intrinsic process is more likely to render neurons with remyelinated or demyelinated axons respiratory deficient over the course of multiple sclerosis than myelinated axons. The combination of genetically determined respiratory deficiency and inflammation in the CNS may be catastrophic, as illustrated by cases with Harding's disease (Kovacs *et al.*, 2005). Mitochondria store calcium and produce reactive oxygen species. When mitochondria are exposed to extrinsic factors that inhibit the mitochondrial respiratory chain, such as hypoxia or hypoxia-like insult, calcium stored in mitochondria is released into the axoplasm, which triggers the common pathway of axonal degeneration (Nikolaeva *et al.*, 2005). Mitochondrial changes in remyelinated axons observed in this study may have consequences for neuronal and axonal integrity when the mitochondrial respiratory chain dysfunctions due to intrinsic and/or extrinsic factors. Interestingly, we found the mitochondrial content in myelinated axons in multiple sclerosis to be lower than myelinated axons in controls. Magnetic resonance spectroscopy studies have identified a decrease in *N*-acetylaspartate levels in the normal appearing white matter in multiple sclerosis and suggested axonal loss as an explanation (Aboul-Enein *et al.*, 2010). Our findings suggest that depletion of mitochondria in myelinated axons may also contribute to the magnetic resonance spectroscopy changes in multiple sclerosis. The mechanisms by which the axonal mitochondrial changes occur in the normal appearing white matter in multiple sclerosis need further investigation. A limitation of this study is the exclusion of small diameter axons ($<2.5\,\mu$m), which was intended to minimize error in localization of axonal mitochondria using *xyz* and *xzy* confocal images. Hence, our findings may not be applicable to small diameter axons, which apparently are preferentially lost during demyelination in multiple sclerosis and as a result may be less abundant in remyelinated regions (DeLuca *et al.*, 2004).

The changes in axonal mitochondrial content following demyelination were at first thought to be potentially detrimental to axons but now are increasingly recognized as a compensatory mechanism (Andrews *et al.*, 2005; Mahad *et al.*, 2009; Witte *et al.*, 2009; Kiryu-Seo *et al.*, 2010). We previously reported an increase in mitochondrial content within approximately half of demyelinated axons in chronic active and inactive multiple sclerosis lesions (Mahad *et al.*, 2009). Changes in mitochondria within axons were also observed following acute demyelination *in vitro* in the absence of inflammatory cells (Kiryu-Seo *et al.*, 2010). We extended the previous observations in chronically demyelinated axons by using chronic inactive multiple sclerosis lesions where axonal degeneration and inflammation had subsided to age-matched control levels (Frischer *et al.*, 2009). The expectation was to find the changes in axonal mitochondrial content in these chronic multiple sclerosis lesions to be subtle or absent, if the

mitochondrial changes were directly pathogenic to axons. Finding approximately a 4-fold increase in mitochondrial content in these chronically demyelinated and non-degenerating axons provides further support to the concept that mitochondrial changes following demyelination at least in part are an adaptive process, occurring independent of but related to inflammation. Interestingly, a small subset of remyelinating and late remyelinated lesions has been reported to show extensive axonal injury in multiple sclerosis, at levels comparable to active demyelinating lesions. This finding in principle indicates the vulnerability of remyelinated axons particularly in inflammatory environments (Kuhlmann *et al.*, 2002; Smith, 2006). Inflammation is a potent suppressor of mitochondrial function (Smith and Lassmann, 2002; Qi *et al.*, 2006). If energy was in short supply due to mitochondrial defects, our findings suggest that demyelinated and remyelinated axons may have a greater tendency to degenerate than myelinated axons (Kuhlmann *et al.*, 2002; Smith, 2006).

In summary, based on the changes in axonal mitochondria following demyelination and remyelination, the potential impact of a mitochondrial defect or an energy deficient state is likely to be greatest for demyelinated axons and greater for remyelinated than myelinated axons in multiple sclerosis. Thus, protection of mitochondria within neurons and axons within the CNS may be beneficial in multiple sclerosis even when remyelination is achieved.

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Supplementary material

Supplementary material is available at *Brain* online.

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