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Regional axonal loss in the corpus callosum correlates with cerebral white matter lesion volume and distribution in multiple sclerosis

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Summary

Previous imaging studies have suggested that there is substantial axonal loss in the normal-appearing white matter (NAWM) of brains from multiple sclerosis patients and that this axonal loss may be an important determinant of disability. Recently, substantial axonal loss in the NAWM has been confirmed directly in post-mortem tissue. Whether the NAWM changes occur as a consequence of damage to axons traversing lesions or to a more diffuse injury process is uncertain. Using formalin-fixed brains of eight multiple sclerosis patients and eight age-matched controls, we examined the relationship between demyelinating lesion load in three volumes of the cerebral white matter and the loss of axons in NAWM of the corresponding three projection regions (anterior, middle, posterior) in the corpus callosum (CC). There was a significant loss of calculated total number of axons crossing the CC in each of the three regions relative to the non-multiple sclerosis controls. Strong correlations were found between the regional lesion load and both the axonal density (r = –0.673, P = 0.001) and the total estimated number of axons crossing the corresponding projection area in the CC (r = –0.656, P = 0.001) for the patients. This suggests that Wallerian degeneration of axons transected in the demyelinating lesions makes a major contribution to the substantial, diffuse loss of axons in the NAWM in multiple sclerosis. These findings emphasize the need to consider the consequences of multiple sclerosis lesions in terms of both local and distant effects in functionally connected regions of the brain.

Keywords: multiple sclerosis; neuropathology; axon; corpus callosum; Wallerian degeneration

Abbreviations: CC = corpus callosum; NAWM = normal-appearing white matter

Introduction

While less prominent than demyelination, loss of axons in multiple sclerosis lesions is well described in the neuropathological literature (Greenfield and King, 1936). The importance of axonal loss in the pathogenesis of the symptoms of multiple sclerosis has recently become the focus of attention (Matthews *et al.*, 1998). Using magnetic resonance spectroscopy to measure levels of *N*-acetylaspartate, more specific evidence for the role of axonal dysfunction in axon loss has been found both in multiple sclerosis lesions and in the normal-appearing white matter (NAWM) of living multiple sclerosis patients (Arnold *et al.*, 1990; Fu *et al.*, 1998).

Studies of post-mortem brains first confirmed the early loss of axons in acute multiple sclerosis lesions (Ferguson *et al.*, 1997; Trapp *et al.*, 1998). We reported recently a quantitative pathological study of axonal loss in the NAWM, which demonstrated a reduction of >50% in the total number of axons passing through the corpus callosum (CC) in multiple sclerosis patients compared with controls who died of unrelated diseases (Evangelou *et al.*, 2000). The cause of the axonal loss was hypothesized to be Wallerian degeneration of the axons transected in multiple sclerosis lesions, but the possibility of a more generalized axonopathy could not be discounted.

To investigate this hypothesis, we extended our pathological studies in order to examine the relationship between the regional multiple sclerosis lesion load in the cerebral hemispheres and distant axonal loss in the corresponding projection areas of NAWM in the CC. We reasoned that, if Wallerian degeneration of the axons that are

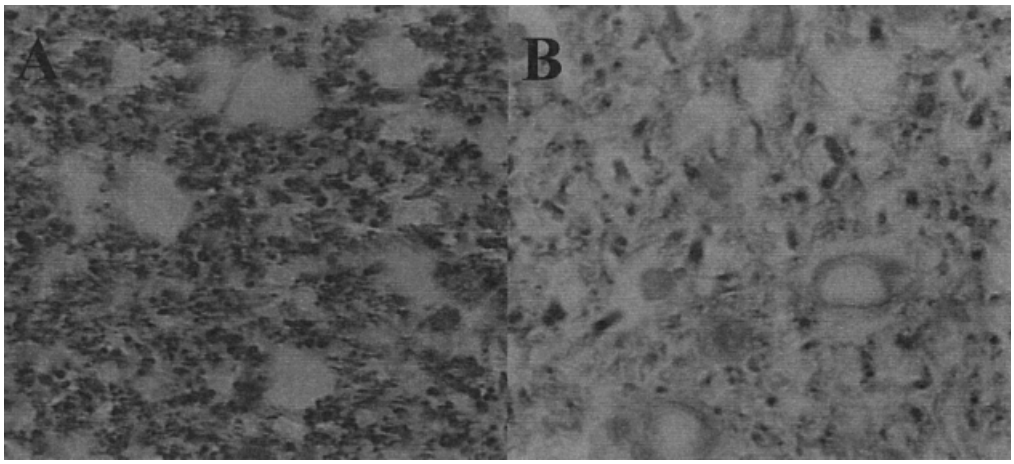

Fig. 1 Representative examples of Palmgren-stained corpus callosum sectioned in axial cross-section from a normal control (**A**) and a patient with multiple sclerosis (**B**). Note the substantially reduced axon density in **B**.

transected in demyelinating lesions is a dominant mechanism of axon loss in NAWM, then a strong relationship should be found between these measures.

Material and methods

Using the archival material of the Department of Neuropathology at the Radcliffe Infirmary, we examined the brains of the most recent eight patients to have died with multiple sclerosis and to have had an autopsy examination, and eight brains from subjects as closely matched as possible for age and sex who died of non-neurological conditions. All brains were fixed in formalin.

We bisected each brain in the mid-sagittal plane and measured the cross-sectional area of the CC as described previously (Evangelou *et al*., 2000). The CC was divided into three sections for analysis. The first section included the rostrum, the inferior genu and the superior genu, the second section included the posterior genu and the mid-body, and the third section included the isthmus and the splenium (Aboitiz *et al.*, 1992). Sections of the CC (10 μm) were stained either for myelin with luxol fast blue and cresyl violet or for axons with the Palmgren silver stain (Fig. 1). After regions of the corpus callosum with demyelinating lesions had been excluded (fewer than ~5% of the total CC studied), we measured the axonal density in each of the three regions of the CC using a previously validated automated method (Evangelou *et al.*, 2000). The total estimated number of axons crossing the CC was calculated for each region by multiplying the density of the fibres by the cross-sectional area.

We sectioned the cerebral hemispheres coronally every 1 cm. We identified and then measured the cross-sectional area of all of the macroscopically identified multiple sclerosis lesions using the stereological technique of point counting (Howard and Reed, 1998). The volume of the lesions was calculated by multiplying the surface area by the depth of the section (1 cm). We then calculated the separate lesion loads in each of three cerebral white matter volumes (anterior, middle and posterior) projecting into the corresponding regions of the CC.

The results are reported as mean ±1 SD unless otherwise noted. Comparisons between groups were performed using the Wilcoxon signed rank test, and one-tailed Pearson correlations were calculated (assuming that volume or axon number increases were not possible in the patient group). The relationship between relative changes in lesion volumes and regional NAWM axon counts was tested by multiple regression analysis. All statistical calculations were performed with SPSS for Windows version 8.

Results

Individual results for patients and mean values for controls are given in Table 1. The ages of the normal controls (median 63 years, range 40–77 years, $n = 8$) and the multiple sclerosis patients (median 58 years, range 30–71 years, $n = 8$) at death were similar. Two patients had relapsing–remitting multiple sclerosis and the other six had secondary progressive disease. The median duration of disease was 21 years (range 5–34 years).

The mean cross-sectional areas were smaller in the multiple sclerosis brains than in control brains in each of the three anatomical regions of the CC [in mm^2, median (range): anterior, multiple sclerosis patients 152 (96–304), controls 232 (176–352); middle, multiple sclerosis patients 230 (154–267), controls 315 (240–465); posterior, multiple sclerosis patients 182 (111–338), controls 297 (260–349)]. The mean axonal densities in the multiple sclerosis brains were also reduced in all three regions compared with controls [in axons/mm^2: anterior, multiple sclerosis patients 9.2×10^4 ($2.0–15 \times 10^4$), controls 1.4×10^5 ($1.1–1.9 \times 10^5$); middle, multiple sclerosis patients 7.8×10^4 ($2.1–18 \times 10^4$), controls 1.3×10^5 ($0.8–1.6 \times 10^5$); posterior, multiple sclerosis

Table 1 *Multiple sclerosis plaque volumes, cross-sectional area of the corpus callosum, measured axon density in the corpus callosum, and calculated total axon number in the corpus callosum*

	Patients								Non-MS controls
	1	2	3	4	5	6	7	8	
Anterior									
Lesion volume (cm^3)	8.38	2.18	1.98	9.64	0.12	0.20	0.56	2.36	0
CC area (mm^2)	143	304	147	96	198	157	172	103	232
CC density ($10^{-1} \times$ axons/mm^2)	2940	8245	10134	2042	2870	14083	15306	15333	14000
Total calculated number of axons	4.2×10^6	2.5×10^7	1.5×10^7	1.9×10^6	5.6×10^6	2.2×10^7	2.6×10^7	1.5×10^7	3.3×10^7
Middle									
Lesion volume (cm^3)	13.86	7.18	6.30	11.26	0.12	0.16	0.00	2.08	0
CC area (mm^2)	193	267	232	154	254	227	254	193	315
CC density ($10^{-1} \times$ axons/mm^2)	2736	6236	9431	2083	3795	14769	17505	12021	13000
Total calculated number of axons	5.3×10^6	1.7×10^7	2.2×10^7	3.2×10^6	9.7×10^6	3.3×10^7	4.6×10^7	2.3×10^7	3.5×10^7
Posterior									
Lesion volume (cm^3)	9.00	3.68	4.44	9.86	0.00	1.04	0.00	3.32	0
CC area (mm^2)	189	339	175	111	260	167	206	137	297
CC density ($10^{-1} \times$ axons/mm^2)	2729	6708	9979	3144	5146	13884	18243	8736	12000
Total calculated number of axons	5.1×10^6	2.3×10^7	1.8×10^7	3.5×10^6	1.3×10^7	2.3×10^7	3.6×10^7	1.2×10^7	3.8×10^7

Values are given for three anatomically based regions of the corpus callosum. The control data are reported as mean values for the group (see Results for ranges).

patients 7.7×10^4 ($2.7–18 \times 10^4$), controls 1.2×10^5 ($1.0–1.8 \times 10^5$)]. The calculated total numbers of axons crossing the CC in the multiple sclerosis brains were therefore reduced significantly in each region relative to normal controls (anterior: patients, median 1.7×10^7, controls 3.3×10^7, $P < 0.01$; middle: patients, median 2.0×10^7, controls 3.5×10^7, $P < 0.04$; posterior: patients, median 1.6×10^7, controls 3.8×10^7, $P < 0.01$). There were no significant differences between patients and controls in the relative regional reductions in CC area or axon density, or in calculated numbers of axons crossing the CC.

The patients had a median lesion load of 10.2 cm^3 (range 0.2–31.2 cm^3). Similar lesion loads were found in each of the three volumes of cerebral white matter defined on the basis of the region of projection through the CC [median (range): anterior 2.1 (0.1–9.6) cm^3; middle 4.3 (0–13.8) cm^3; posterior 3.5 (0–9.8) cm^3]. The multiple sclerosis lesion loads in each cerebral white matter volume (anterior, middle and posterior) correlated with both the axonal density ($r = –0.673$, $P = 0.001$) (Fig. 2A) and the total number of axons crossing the CC in the corresponding region ($r = –0.656$, $P = 0.001$) (Fig. 2B). We found that when the relationship between lesion loads in the different cerebral white matter volumes and regional CC axon changes was tested for data from individual patients by multiple regression analysis, this correlation remained strong ($r = 0.620$, $P = 0.001$), excluding the possibility that the patients just with higher lesion loads might be biasing the group results.

Discussion

It has long been known that there can be extensive axonal loss in gliotic lesions with chronic multiple sclerosis (Greenfield and King, 1936). More modern post-mortem studies have demonstrated injury and loss of axons in acute lesions (Ferguson *et al.*, 1997; Trapp *et al.*, 1998) also, confirming the earlier magnetic resonance spectroscopic studies (Arnold *et al.*, 1990). Evidence has emerged recently for a substantial loss of axons in areas of the brain that do not appear to be affected by the disease, this loss being associated with signal intensity changes on conventional MRI of the brain (Narayanan *et al.*, 1997; Fu *et al.*, 1998; De Stefano *et al.*, 1999) or on direct neuropathological examination (Evangelou *et al.*, 2000). There is evidence that the extent of axonal loss in the NAWM plays an important role in determining the clinical disability (De Stefano *et al.*, 1998; Fu *et al.*, 1998; Matthews *et al.*, 1998).

Two possible pathogenetic mechanisms have been proposed for the decrease of axonal numbers in the NAWM. One possibility is that there is a diffuse axonopathy in multiple sclerosis. Although such a process might be particularly important in primary progressive disease (in which the inflammatory lesion load is low), it may also contribute to axon loss in more typical disease. Secondly (and not mutually exclusively), loss of axons in the NAWM might simply be the result of Wallerian degeneration of axons that are transected in multiple sclerosis lesions.

The results of our study are most consistent with the

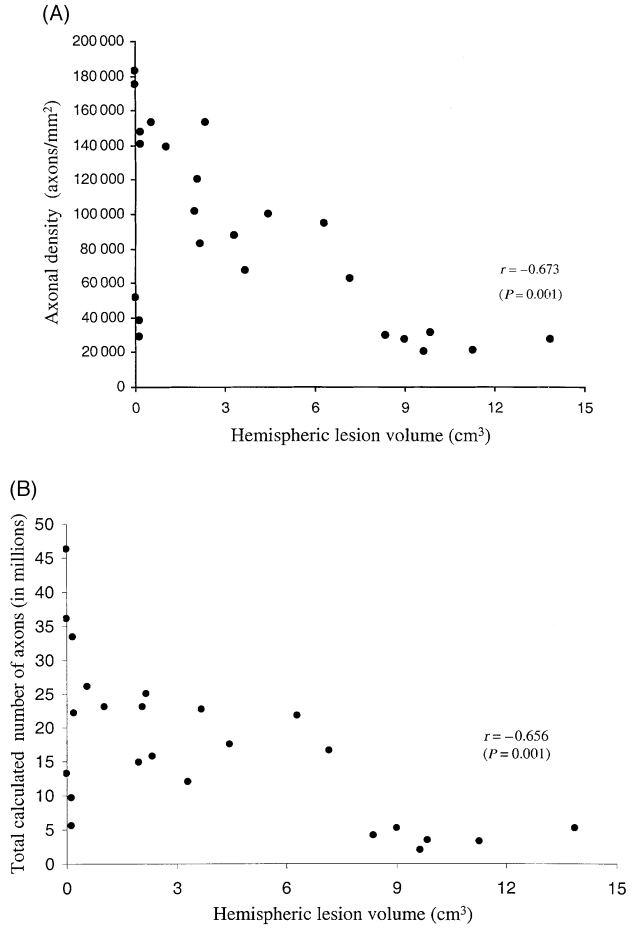

Fig. 2 Correlations of hemispheric demyelinating lesions with axonal density (**A**) and the total calculated number of axons (**B**) crossing the NAWM of the corresponding area of the corpus callosum.

second hypothesis. Despite the variation in the number of axons across the CC of neurologically normal controls, we found a significant and strong correlation between regional hemispheric lesion volume and axonal density in the corresponding projection region of the CC in multiple sclerosis patients. Supportive results have been presented recently in a study of the cervical cord of multiple sclerosis patients (Ganter *et al.*, 1999), but the CC offers advantages for the quantitative assessment of axon loss. Although many of the fibres are unmyelinated (and the smallest cannot be visualized in paraffin-embedded material, potentially contributing to underestimation of axon loss) (Aboitiz *et al.*, 1992), the high degree of order in axon orientation within the CC allows them to be counted accurately in transverse sections.

Quantitative relationships between more distant white matter lesions and axon loss in the CC is also possible. Most of the axons of the CC appear to connect homologous areas of the two cerebral hemispheres (de Lacoste *et al.*, 1985; Pandya and Seltzer, 1986). In the adult corpus callosum the genu connects the prefrontal cortices, the midbody connects the motor, sensory and auditory cortices, and the splenium carries temporal, parietal and occipital fibres (de Lacoste *et al.*, 1985; Pandya and Seltzer, 1986). Therefore, estimates of regional axonal densities and total fibres can be related to the volume of multiple sclerosis lesions within the regions from which these fibres arise.

Wallerian degeneration follows quickly after axonal transection. Imaging studies after cerebral infarction have demonstrated that the earliest changes occur within 72 h to 14 days (Igarashi *et al.*, 1998; Castello *et al.*, 2000), with subsequent evolution over up to 4 months after injury (Orita *et al.*, 1994).

If disability in multiple sclerosis is caused predominantly by injury to axons traversing inflammatory lesions, then efforts to minimize damage to and loss of axons should work in cooperation with current efforts directed at reducing the frequency and extent of inflammation. Pharmacological targeting of potentially toxic inflammatory factors (Rothwell and Hopkins, 1995) or the use of neurotrophic agents may be helpful (Skaper and Walsh, 1998). Potential genetic markers of increased susceptibility to more severe disease may provide further new targets for treatment, as may the identification of genes specifically determining Wallerian degeneration.

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