Size-selective neuronal changes in the anterior optic pathways suggest a differential susceptibility to injury in multiple sclerosis

N. Evangelou,1,2 D. Konz, 2 M. M. Esiri, 2 S. Smith,1,2 J. Palace2 and P. M. Matthews1,2

1Centre for Functional Magnetic Resonance Imaging of the Brain and 2Department of Clinical Neurology, University of Oxford, UK

Correspondence to: Professor P. M. Matthews, Centre for Functional Magnetic Resonance Imaging of the Brain, John Radcliffe Hospital, Headley Way, Headington, Oxford OX3 9DU, UK
E-mail: paul@fmrib.ox.ac.uk

Summary
Axonal damage is found in both acute and chronic lesions of multiple sclerosis. Direct axon counting in post-mortem tissue has suggested that smaller axons might have a greater susceptibility to damage, but methodological limitations have precluded unequivocal interpretation. However, as neuronal and axonal sizes are linked and neuronal changes would be expected with retrograde or transsynaptic degeneration following axon injury, we hypothesized that an alternative strategy for studying this phenomenon would be to define multiple sclerosis-associated changes in neurones. To test this hypothesis, we measured both axonal loss and neuronal size changes in the anterior optic pathway [including the optic nerve (ON), optic tract (OT) and lateral geniculate nucleus] of the brains of eight patients who died with multiple sclerosis and in eight control brains. The ONs and OTs in brains from the multiple sclerosis patients showed a trend to smaller mean cross-sectional areas (ON, multiple sclerosis = 6.84 mm², controls = 9.25 mm²; and OT, multiple sclerosis = 6.45 mm², controls = 7.94 mm², P = 0.08) and had reduced axonal densities (ON, multiple sclerosis = 1.1 × 10⁵/mm², controls = 1.7 × 10⁵/mm²; and OT, multiple sclerosis = 1.4 × 10⁵/mm², controls = 1.8 × 10⁵/mm², P = 0.006). Estimated total axonal counts were reduced by 32 (OT)-45% (ON) in the patients relative to controls (ON, multiple sclerosis = 8.1 × 10⁵ axons, controls = 14.8 × 10⁵, P = 0.05; and OT, multiple sclerosis = 9.1 × 10⁵, controls = 13.3 × 10⁵, P = 0.02). The size distributions of the magnocellular cells in the lateral geniculate nucleus were similar for the two groups, but in multiple sclerosis brains the parvocellular cells were significantly smaller (mean sizes: multiple sclerosis = 226 µm², controls = 230 µm², P < 0.001) and had a larger variation in size, suggesting a greater proportion of atrophic neurones. Axon loss in the optic nerves of multiple sclerosis patients correlated strongly with measures of increased dispersion of cell sizes in the parvocellular layer (r = 0.8, P < 0.04). These data demonstrate that both atrophy and decreased density contribute to the substantial axonal loss in the anterior visual pathway of these patients. This appears related to a relatively selective atrophy of the smaller neurones of the parvocellular layer in the lateral geniculate nucleus, supporting the hypothesis that smaller axons may be preferentially susceptible to injury in multiple sclerosis.

Keywords: axonal loss; lateral geniculate nucleus; multiple sclerosis; neuronal changes; parvocellular; size

Abbreviations: LGN = lateral geniculate nucleus; ON = optic nerves; OT = optic tracts

Introduction
The clinical course of multiple sclerosis is extremely variable. Equally, there are a diversity of pathological mechanisms responsible for the clinical manifestations (Lucchinetti et al., 1996). Over the last few years in particular, attention has been focused on axonal loss in multiple sclerosis, as this appears to be a major cause of chronic disability (Matthews et al., 1998; Trapp et al., 1999).

Recently a study of axonal loss in the normal appearing white matter of the cervical spinal cord demonstrated relatively greater loss of smaller diameter axons, suggesting that smaller axons may be more susceptible to damage (Ganter et al., 1999). However, a concern about interpretation of this study is that axonal staining methods identify only a proportion of axons and preferentially stain larger diameter fibres. Smaller diameter axons may thus be more difficult to identify after even non-specific axonal dystrophy (Perry et al., 1998).
Grey matter pathology offers an alternative strategy for studying this problem. Neuronal atrophy or loss may be expected as a consequence of axonal transection in lesions, either as a consequence of Wallerian degeneration (Rawes et al., 1997) or from anterograde, transynaptic changes (Madigan et al., 1996). As demonstrated clearly in the anterior visual system (Sadun, 1986), in general there is a close relationship between the sizes of individual neurones and of their axons. Thus, size-selective axonal injury should be reflected in relatively size-selective atrophy or degeneration of the associated neurones.

For the present study, we chose to characterize morphological changes in grey matter neurones of the lateral geniculate nucleus (LGN) and in the anterior visual pathway. Anterograde changes in the LGN after optic nerve lesions are well described (Goldby, 1957; Madigan et al., 1996). The anatomy of the LGN also lends itself well to a study of possible effects of size-selective neuronal changes, as cell types are well separated into six laminae. Laminae 1 and 2 (the magnocellular layers) are comprised of larger cells, whereas laminae 4–6 (the parvocellular layers) have smaller neurones. The parvocellular layers receive their retinal input predominantly from small diameter, slowly conducting axons, whereas the faster conducting Y-type large retinal ganglion cells with large axons project mainly to the magnocellular layers in the LGN.

Here we report measurements of axon loss in the anterior visual pathway of patients with multiple sclerosis. We then quantitatively compare size distributions of magnocellular and parvocellular neurones of the LGN to test for the relatively selective small neurone injury pattern expected if there was a particular susceptibility of smaller axons to damage from multiple sclerosis.

Methods
Selection of brains for study
We reviewed the pathology notes of 11 consecutively received multiple sclerosis brains in the archival material of the Department of Neuropathology at the Radcliffe Infirmary. Three cases were excluded because of concomitant neurological diseases (intractable epilepsy since childhood, episode of disseminated intravascular coagulopathy and cerebral ischaemic episode). Brains from eight multiple sclerosis patients (six females and two males) and eight controls (four males and four females) without prior history of neurological symptoms and a normal neuropathological examination were studied. The mean age at death of the multiple sclerosis patients was 56 years old (range 30–71) and of the controls 49 years old (range 21–71). The duration of the disease in the multiple sclerosis group ranged from 5 to 34 years with a mean of 21 years. One of the patients had relapsing–remitting disease and the other seven had secondary progressive multiple sclerosis. All brains examined were weighed and formalin-fixed. In all cases, permission for use of autopsy tissue for research had been obtained from a close relative.

Section preparation
We examined the right-sided anterior optic pathways [including the optic nerve (ON), the optic tract (OT) and the LGN] from all patients and controls. The brains were dissected previously for standard pathological examination, and in two cases (one multiple sclerosis, one control) parts of the optic pathways were damaged during brain removal thus reducing the sample size. The final sample included 15 right ONs, 14 right OTs and 16 right LGNs. Both the ON and OT were sectioned in orthogonal direction to the optic pathway. The optic nerves were sectioned 5 mm anterior to the chiasm to avoid the von Willebrand’s knee. The optic tracts were cut at least 5 mm posterior to the chiasm. Following the dissection of the optic pathways, the study sections were coded by a member of the department not involved in the study to blind the observer (N.E.) to the sources of the specimens.

Tissue blocks were embedded in paraffin and 10 µm thick sections were cut on a microtome. All sections were stained with Palmgren for axons and Luxol Fast Blue with Cresyl Violet stain for myelin and neuronal Nissl substance. Multiple sections (on average 15) of each optic nerve and tract were stained and results were averaged for each mean value reported. Sections chosen for study did not include demyelinating lesions.

Image analysis
Stained sections of the LGN neurones were examined and images of individual neurones were captured with an Olympus BX40 microscope and an ×100 oil immersion objective lens. Only sections with six clearly visualized distinct LGN layers were examined. The maximum cross-sectional areas of neurones was measured only when the nucleolus was clearly visible. Neurones from each of the six layers were captured in an orderly fashion and images saved for later measurements of cell sizes. Using the Vernier scale on the microscope stage, images were captured in a raster search pattern so that regions counted were distributed evenly throughout the individual layer. This was done in the following way. The slide was placed on the stage and the x, y coordinates of the section were recorded. The microscope stage then was moved along the x dimension at regular intervals until the objective lens had passed over the region of interest (i.e. a parvocellular or magnocellular layer). The stage then was moved along one interval in the y dimension and the objective moved back over the region of interest, capturing images at the same regular intervals. Measurements were taken from cells in all layers of the LGN in the same way. The cross-sectional areas of the optic nerves and tracts were measured from sections captured with a JVC TK-1070E camera on a Silicon Graphics computer and imported to the Scion Image B3b image analysis program as described previously (Evangelou et al., ...
Measures were taken from five sections of each optic nerve or tract.

For pilot power calculation estimation, we measured the size of 200 neurones. We calculated that 200 cells from both the parvo- and the magnocellular layers were needed to reduce the standard error of the means below 0.05. We therefore initially aimed to measure at least 100 cell bodies from each of the six layers, using two sections from each LGN.

Manually counting axons is an extremely time-consuming method with suboptimal reproducibility, predominantly due to observer fatigue. Under optimal conditions, a trial of repeated axon counting of sections by trained observers gave a correlation coefficient of only 0.85 (Nagy et al., 1999). To allow more efficient and reproducible measurements of axonal loss, we therefore developed an automated image analysis method for counting axons based on pixel intensity cluster counting. The histological images were captured by a camera mounted to an Olympus microscope with a ×100 oil immersion objective and transferred to a workstation (Silicon Graphics) for analysis. Images then were intensity thresholded to establish outlines of the (stained) axons. In preliminary work, we used receiver–operator characteristic curve analysis with different segmentation thresholds to optimize the accuracy and reproducibility in measurements of axons. The images were analysed after application of a spatial filter (as implemented in MedX, Sensor Systems, Inc.).

With the automated method, axons that were very close together were unable to be counted separately, consistently reducing the total number of axons measured by the automated method relative to manual counting. The extent of this bias was determined by the size of the spatial filter. Using a local $3 \times 3$ mm$^2$ filter with a segmentation threshold set for $22.5\%$ of the maximum pixel intensity range on an individual image, an optimal correlation between the automated method and manual counting was achieved ($r = 0.96, P < 0.001$). To account for the consistent bias towards smaller numbers of axons, the regression equation relating the automated and manual counts on test data was used to calculate a correction term, which then was applied to each measurement.

**Statistics**

SPSS v.7 (www.spss.com) was used for statistical testing. For most comparisons, the Mann–Whitney test was employed. Spearman’s correlations were performed to test the relationships between two continuous variables. The Kruskal–Wallis H test was used to test whether more than two

---

**Fig. 1** (A) Images ($\times14.8$) of representative cross-sections of the optic tracts (control, 1; multiple sclerosis, 3) and optic nerves (control, 2; multiple sclerosis, 4) from brains of a control and a multiple sclerosis patient. (B) Images ($\times740$) of representative Palmgren-stained cross-sections of optic tracts (control, 1; multiple sclerosis, 3) and optic nerves (control, 2; multiple sclerosis, 4). Each discrete punctate area of dark staining marks an axon. Relatively reduced axonal density in both the OT and the ON is appreciated in the images from the multiple sclerosis patients.
independent samples were likely to have come from the same population.

Results
Optic nerve
The cross-sectional area of the right ON was measured in seven controls (post-mortem damage precluded measurements on one subject) and eight multiple sclerosis cases (Fig. 1). The ON showed a trend to be smaller in multiple sclerosis patients than in controls (median multiple sclerosis = 6.84 mm², range 4.59–7.90 mm²; median controls = 9.25 mm², range 6.39–12.6 mm², $P = 0.08$). Mean axonal density in the right ON from the multiple sclerosis group (median = $1.1 \times 10^5$ axons/mm², range 1.0–1.2 $\times 10^5$ axons/mm²) was smaller than in the controls (median = $1.7 \times 10^5$ axons/mm², range 1.2–2.1 $\times 10^5$ axons/mm², $P = 0.05$) (Fig. 1). The calculated total number of axons of the right ON therefore also was 45% smaller in the multiple sclerosis group (median multiple sclerosis = 8.1 $\times 10^5$, range 6.7–9.5 $\times 10^5$; median controls = $14.8 \times 10^5$, range 10.4–18.8 $\times 10^5$, $P = 0.05$).

Optic tract
The cross-sectional area and axonal density of the right OT were measured in seven multiple sclerosis cases and seven controls (Fig. 2). There was a trend for the OT to have a smaller cross-sectional area in the multiple sclerosis than in control brains (median multiple sclerosis = 6.45 mm², range = 3.60–8.92; median controls = 7.94 mm², range = 5.42–10.30; $P = 0.08$). The axonal density of the right OT in the multiple sclerosis group (median = $1.4 \times 10^5$ axons/mm², range 1.1–1.7 $\times 10^5$ axons/mm²) was smaller than in controls (median = $1.8 \times 10^5$ axons/mm², range 1.7–2.1 $\times 10^5$ axons/mm², $P = 0.006$). From these data, we estimated that the OTs in the multiple sclerosis group had >30% fewer axons than in the controls (median multiple sclerosis = 9.1 $\times 10^5$ axons, median controls = $13.3 \times 10^5$, $P = 0.02$).

There were no significant differences between the ratios of the cross-sectional areas of the ON or the OT to the total brain weight for either the multiple sclerosis or the control groups (data not shown, $P > 0.5$), suggesting that the ON and OT were not relatively more atrophic in the multiple sclerosis patients than the rest of the brain.

Lateral geniculate nucleus
The maximum cross-sectional areas of LGN cells in the parvocellular and magnocellular layers were measured in brains from all 16 subjects. In total, the cross-sectional area of 7812 cells was measured in the LGNs from normal controls: 2791 derived from the magnocellular layers and 6371 from the parvocellular layers. In the normal controls, the average cell size was not significantly different across the four parvocellular layers ($P = 0.5$) or between the two magnocellular layers ($P = 0.5$).

For each brain, the cross-sectional areas measured for all of the parvocellular and magnocellular neurone cell bodies were averaged. The mean of these averaged cross-sectional areas was not significantly different between the multiple sclerosis and the control groups ($P > 0.5$), but this comparison does not take into account differences in variation of the cell sizes in the two groups (Table 1). The multiple sclerosis group showed a trend for larger dispersion of the neuronal cross-sectional areas than the controls in the parvocellular layers ($P = 0.07$) but not in the magnocellular layers.

We therefore considered the distributions of cross-sectional neuronal areas for individual parvocellular and magnocellular cells in the multiple sclerosis and control LGNs (Fig. 2). The distributions of cross-sectional areas of the magnocellular cells were almost identical in the multiple sclerosis and control groups (median multiple sclerosis = 366 μm², median control = 365 μm², $P = 0.58$). However, the parvocellular cells were significantly smaller in the multiple sclerosis group than in the controls (median multiple sclerosis = 226 μm², median controls = 230 μm², $P < 0.001$).

Histograms of cell size distributions were not significantly different for the magnocellular layers between the multiple sclerosis and control groups, but the parvocellular histogram in the multiple sclerosis group was significantly skewed to the right (mean multiple sclerosis skew = 0.89, mean control skew = 0.65) with the mode considerably smaller than the control group (multiple sclerosis mode = 160 μm², control mode = 210 μm²) (Fig. 2).

Relationship between changes in the optic nerve, optic tract and the lateral geniculate nucleus
For the multiple sclerosis patients, the axonal density in the OT correlated strongly with multiple measures of the dispersion of neuronal sizes in the parvocellular layer (standard deviation of cell body areas, $r = 0.82$, $P = 0.02$; skewness, $r = 0.85$, $P = 0.01$; kurtosis, $r = 0.78$, $P = 0.04$) (Fig. 3). No significant correlation between these variables was found for the control group. Consistent with the absence of changes described above, the dispersion of the neuronal sizes of the magnocellular layers did not correlate with axonal density of the OT in either the multiple sclerosis or the control group.

Discussion
Although multiple sclerosis is a primary demyelinating disease affecting the CNS, axonal loss was noted even in early pathological descriptions (Greenfield and King, 1936). It has become appreciated more recently that axonal injury and transection are characteristic of both acute and chronic multiple sclerosis lesions (Ferguson et al., 1997; Trapp et al., 1997).
Neuronal changes in optic pathways in multiple sclerosis

**Fig. 2** Histograms of individual cell size ($\mu$m$^2$) in parvocellular and magnocellular layers. Values for the distribution modes and skews are shown. Note the similar distributions of the magnocellular neurones and the larger skew of the parvocellular neurone sizes in the multiple sclerosis group relative to controls.

**Table 1** Neurone cell body areas in the magnocellular and parvocellular layers of multiple sclerosis patients and controls

<table>
<thead>
<tr>
<th>Location of cell body</th>
<th>Neurone cell body area</th>
<th>Magnocellular</th>
<th>Parvocellular</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Multiple sclerosis</td>
<td>Controls</td>
</tr>
<tr>
<td>Magnocellular layer</td>
<td>Mean</td>
<td>377</td>
<td>384</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>135</td>
<td>132</td>
</tr>
<tr>
<td>Parvocellular layer</td>
<td>Mean</td>
<td>226</td>
<td>238</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>85</td>
<td>69</td>
</tr>
</tbody>
</table>

Mean cell body areas were determined for each brain and their values were averaged for the two groups. Although the parvocellular layer cells are slightly smaller and the standard deviation of mean values larger than for the multiple sclerosis patients, the difference relative to controls is not significant.

This axonal injury and loss appear to be the major cause of chronic disability in multiple sclerosis. Both cross-sectional and longitudinal studies have demonstrated a correlation between decreases in the concentration of $N$-acetyl aspartate (an acetylated amino acid localized to neurones in the adult that can be measured using magnetic resonance spectroscopy to allow non-invasive estimation of axonal density) and disability (Davie et al., 1997; Fu et al., 1998; Lee et al., 2000). MRI studies of the brain and cervical spinal cord in multiple sclerosis patients have shown a correlation between atrophy (representing axon and supporting tissue volume...
loss) and clinical disability (Losseff et al., 1996; Bitsch et al., 2000). T1-hypointense lesions on conventional MRI, which are associated with axon loss and matrix destruction, also show a strong correlation with progression of disability (Bitsch et al., 2000).

Multiple mechanisms could contribute to neuronal damage in grey matter. It has long been recognized that inflammatory changes are prominent in grey matter in multiple sclerosis, even though macroscopic lesions are not common (Lumsden, 1970). Diffusible, neurotoxic inflammatory factors (e.g. tumour necrosis factor-α) could give rise to neurone damage even if the primary inflammatory event is in the white matter (Gimsa et al., 2000). As axon damage becomes more substantial in the white matter, additional mechanisms could be important. Retrograde injury responses characteristically develop with Wallerian degeneration from axonal transection. In addition, transynaptic dystrophy follows loss of afferent axons in some nuclei (Sadun, 1986; Madigan et al., 1996). To the extent that either of these mechanisms play major roles, there should be a correlation between neuronal changes and axonal loss. A recent PET study supported the above notion reporting significant correlations between the local volume of multiple sclerosis plaques and regional cortical cerebral metabolism (Truyen et al., 1996).

Recent studies have suggested that smaller axons may be injured preferentially in multiple sclerosis (Ganter et al., 1999; Bitsch et al., 2000). However, unequivocal interpretation of relative counts of small axons is difficult, particularly when there is potentially substantial axonal dystrophy. Smaller axons do not stain as well as larger axons with either silver stains or immunohistochemistry (particularly when shrunken), making their identification more difficult. Additionally, there are reports of increased axonal diameter due to oedema in demyelinating lesions which could contribute to an apparent relative loss of small axons, with normal diameter outside the lesions (Porciatti and Sartucci, 1996). However, to the extent that antero- or retrograde changes occur with axonal injury and loss, neuronal cell body morphology can be used as a marker for axonal pathology. As quantitative measurement of neuronal cell diameters is less likely to be subject to the artefacts that limit the interpretation of axonal size distribution changes, determination of whether there are changes in specific neuronal groups can provide an important test of whether axon loss is size-selective or not.

Here we have assessed the distributions of neuronal sizes in the well-defined magnocellular and parvocellular layers of the LGN and correlated these with axonal densities and cross-sectional fibre tract areas in the anterior visual pathway. As in our previous study of the corpus callosum (Evangelou et al., 2000b), we found substantial axonal loss in the normal appearing white matter of the anterior visual pathways. Decreases in axonal density and volume contribute approximately equally to the total axonal loss in the optic nerve and tract. Comparing specimens from patients and controls, we found evidence for pathological changes in neurones of the LGN as a consequence of multiple sclerosis. The observation that the extent of neuronal changes was correlated with the extent of axonal damage in the optic nerve and tract (which projects to the LGN) suggests that these changes could be a result of the remote inflammatory white matter damage of multiple sclerosis. In addition, a significant difference in the distribution of sizes of neurones between patients and controls was found only in the parvocellular layer (smaller neurones). This is as expected if the cells in the LGN had undergone either retrograde or transsynaptic atrophy as a consequence of axonal injury preferentially affecting smaller axons.

The observation of significant changes only in the parvocellular layer suggests that differences in the sizes of neurones and their axons are important determinants of susceptibility to injury. These size-dependent differences in susceptibility could be a consequence of differences in the surface area : volume ratio (e.g. if a non-specific, diffusible toxin such as nitric oxide is responsible) or other factors, such as the particular metabolic or antigenic characteristics of the different classes of cells. This confirms the earlier observation of a relative loss of smaller axons in the cervical cord (Ganter et al., 1999) and suggests that factors related directly to axonal size may be critical to the genesis of the axonal injury responsible for chronic disability in multiple sclerosis. An alternative interpretation is that larger axons may have a greater ability to recover from damage associated with multiple sclerosis. Experimental peripheral nerve grafting to optic nerve stump following axotomy in ferrets showed also that the larger cells exhibited more axonal regeneration potential than the small ones, for example (Quan et al., 1999).
This possible increased susceptibility to injury for small diameter axons (or greater resistance to injury by the larger fibres) is not simply a non-specific characteristic of nerve damage. In vasculitic peripheral neuropathies, for example, large myelinated fibres are affected earlier than are small fibres (Shintaku et al., 1988).

There remain limitations to interpretation of our study. First, the numbers of tissues studied was low and only limited pre-mortem clinical information was available. It is difficult to assess how representative the sample is for a disease as heterogeneous as multiple sclerosis. Because of loss of optic nerves and tracts during brain removal, the groups were not identical. This probably contributes to the modest differences (13%) in the mean numbers of axons measured in the two white matter regions, although we regard this degree of agreement between the figures to be rather good, given the difficulties of quantitative histology. While we found a correlation between injury in the optic nerve and changes in the LGN, it was not possible to distinguish between the effects of anterograde (from lesions in the optic nerve) and retrograde (from lesions in the optic radiations) changes. However, this distinction may not be essential to testing the primary question that there are size-selective changes. Finally, the observation of such correlations of course does not prove that axonal injury is the cause of the neuronal changes. A similar result might be found, for example, if grey matter inflammation was directly responsible for the neuronal changes, provided only that the extent of grey and white matter inflammatory changes were strongly correlated. The results would still be of significance, but would need to be interpreted as evidence for size-selective neuronal damage.

It is intriguing to consider the potential functional consequences of selective injury to axons and neurones of the parvocellular system. This network is involved particularly in colour vision, in contrast to the relative motion sensitivity of cells in the magnocellular system. Increased susceptibility to injury in the parvocellular system could contribute to the characteristically early impairment of colour vision of multiple sclerosis patients who have suffered from optic neuritis (Blinkenberg et al., 2000).

More generally, better defining the nature and extent of neuronal injury is likely to help in understanding the neuropsychological deficits associated with multiple sclerosis. Secondary neuronal damage and dysfunction enhance the diffuseness of cortical functional impairments and further limit direct correlations of specific white matter lesion and functional deficits. The results also offer possible hope for new strategies to limit progression of chronic disability by slowing axon damage and loss. Defining the specific factors that make larger axons less susceptible to injury could be important for neuroprotection in multiple sclerosis. Such an approach would be expected to work in synergy with efforts to control inflammation.

Acknowledgements
The Multiple Sclerosis Society of Great Britain and Northern Ireland (P.M.M. and J.P.) and the Medical Research Council (P.M.M.) supported the research reported in this paper.

References


Received February 21, 2001. Revised May 14, 2001. Accepted May 14, 2001