Axonal loss in the multiple sclerosis spinal cord revisited

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Abstract

Preventing chronic disease deterioration is an unmet need in people with multiple sclerosis, where axonal loss is considered the key substrate of chronic disability. Clinically, chronic multiple sclerosis often presents as progressive myelopathy. Spinal cord cross-sectional area assessed using MRI is associated with increasing disability and has, by inference, been proposed as an indirect index of axonal degeneration. However, the association between cross-sectional area and axonal loss and their correlation with demyelination have never been addressed using comprehensive sampling of *post mortem* tissue. We extensively sampled whole *post mortem* spinal cords of people with multiple sclerosis and healthy controls to quantify axonal density and its association with demyelination and cross-sectional area.

Whole fixed *post mortem* spinal cords of thirteen people with multiple sclerosis (seven women, six men; mean disease duration= 29.2 years) and five healthy controls were dissected. Tissue blocks were embedded in paraffin and immuno-stained for myelin basic protein and phosphorylated neurofilaments. Measurements included total cross-sectional area, the areas of (i) lateral cortico-spinal tracts, (ii) grey matter, (iii) white matter, (iv) demyelination and the number of axons within the lateral cortico-spinal tracts. Axons above and below a 3μm diameter threshold were counted separately. Linear mixed models were used to analyse relationships.

A total of 396 tissue blocks were used. In multiple sclerosis spinal cord the reduction of cross-sectional area at cervical, thoracic and lumbar levels ranged between 19 and 24%, with borderline significance (p=0.055) for the largest reduction in the cervical cord. Atrophy of white (19-24%) and grey (17-21%) matter contributed equally to this overall reduction with no significant difference across levels. Axonal density in multiple sclerosis cortico-spinal tracts was lower by 57-62%, with no evidence of variation across levels (p=0.145). Axonal loss equally affected all fibres regardless of diameter. Demyelination affected 24-48% of the grey matter, most extensively at the thoracic level, whilst it affected only 11-13% of the white matter, with no significant differences across spinal levels. Disease duration was associated with reduced axonal density, however not with any area index. Significant association was detected between focal demyelination and axonal density whilst the latter did not correlate with the total area of demyelination.
Within nearly 30 years multiple sclerosis reduces axonal density by about 60% throughout the spinal cord. Spinal cord cross sectional area, reduced by about 20%, appears to be a poor predictor of axonal density as no association emerged between these two indices.

Keywords: multiple sclerosis; spinal cord; axons; demyelination; atrophy

Abbreviations: CST: Lateral cortico-spinal tract, aCST: cortico-spinal tract area, CDD: chronic disease deterioration, CSA: cross sectional area, CoV: coefficient of variation, EDSS: expanded disability status scale, GM: grey matter, MBP: myelin basic protein, WM: white matter.

Introduction

Multiple sclerosis (MS) is a common inflammatory demyelinating disease of the central nervous system leading to chronic disability [32]. The most evident pathologic findings in the CNS of people with multiple sclerosis (pwMS) include focal white matter (WM) demyelination and remyelination, inflammation, a variable degree of axonal preservation and loss, and gliosis [29] as well as extensive grey matter (GM) tissue damage [27].

The spinal cord is the most frequent location of clinically-apparent pathology at first presentation in pwMS [34]. It is also a common manifestation site of chronic disease deterioration (CDD) leading to loss of urinary and faecal sphincter control as well as progressive motor and sensory dysfunction of the limbs and trunk resembling the clinical syndrome of progressive myelopathy [33].

Although inflammation and demyelination are important features throughout the disease course, degeneration of chronically demyelinated axons is considered the major pathological correlate of CDD [3]. CDD may, at least in part, be mediated by mechanisms independent of the inflammatory penumbra associated with relapsing MS [2,40,14,1]. The reduction of spinal cord cross-sectional area (CSA), measured using magnetic resonance imaging (MRI), is widely considered an indirect measure of axonal degeneration [41,25,29].

Though axonal loss has been described in MS for over 160 years [5,6,31], a number of fundamental issues regarding axonal pathology in the chronic MS spinal cord have remained unresolved. Firstly, the reported degree of axonal loss after a life with MS has been highly variable ranging from 19-60% [30,10,15]. Secondly, axonal loss in the MS cortico-spinal tracts (CSTs) was found to be size-selective, with small diameter fibres more affected than large ones [10]. However, examination of the proportional rather than the absolute loss has, to our knowledge, never been undertaken [15,10,38]. Thirdly, the distribution of lesions, degree of axonal loss, and reduction of CST [10] and of cross-sectional area (CSA) [19] has been described as uneven across the cord length, with most pronounced changes detected in the upper cervical portion of the MS spinal cord . However, these findings were based on relatively selective sampling strategies [10,11,20,19 ,18], sometimes leading to surprising results, such as the complete lack of GM atrophy after a mean disease duration of 17 years [19] a finding challenged by MRI evidence suggesting MS spinal cord GM atrophy as a strong predictor of disability [36]. Finally, whilst the role of inflammatory demyelination for axonal damage and loss in acute multiple sclerosis is well established [13,39], the association of axonal loss with demyelination in chronic MS has remained unclear, with some groups detecting no difference in axonal counts between lesions and non-lesional spinal cord [30].

In order to provide more definitive pathology indices and to explore the potential of spinal cord CSA as a predictor of axonal loss, we comprehensively sampled whole *post mortem* spinal cords from pwMS and controls focussing our analysis on: (i) axonal loss in the CSTs (large and small diameter fibres), (ii) presence and degree of GM and WM demyelination and atrophy, and (iii) their association with CSA.

Materials and Methods

Whole *post mortem* spinal cords of pwMS and control subjects who died of non-neurological conditions were used. Tissues had been donated through the donor scheme of the UK Multiple Sclerosis Tissue Bank (Research Ethics Committee reference number 08/MRE09/31). A single case (MS0) was provided by Professor Joanne Martin through the tissue donor programme of The Royal London Hospital (Barts Health NHS Trust, London, UK). All tissues were fixed in 4% formaldehyde solution. Clinical notes were reviewed to obtain demographics, disease onset and course and to estimate the degree of disability. The quality and the preservation of each spinal cord was macroscopically assessed at the beginning of the study by a neuropathologist (FS) to exclude any obvious compression or artefactual damage due to tissue handling.

Tissue sampling and immunohistochemistry

Spinal cord levels were identified based on the presence of the thinnest nerve root at thoracic level 1. The remaining nerve roots were subsequently identified and marked. Each cord was then dissected axially to result in at least one tissue block per available nerve root level (Fig. 1a). Each tissue block was then marked with tissue dye to retain information on its spatial orientation and processed for embedding in paraffin wax. Of each tissue block 10µm –thick sections were cut using a Shandon Finesse ME+ microtome (ThermoScientific, UK) Care was taken to cut them in a plane perpendicular to the anterior spinal artery. The sections were mounted on Superfrost+ slides (VWR, UK) and left in a 60^{o}C oven overnight. The distance between the exit of the second thoracic (T2) and fifth lumbar (L5) pair or nerve roots was measured to be used as a ‘proxy’ of total cord length, for the statistical analysis of confounding by cord length (see below).

Serial sections of each block were stained for H&E, phosphorylated neurofilaments (SMI-31, mouse monoclonal, 1:1000, Abcam, UK) and myelin basic protein (MBP, SMI-94, mouse monoclonal, 1:100, Covance, Princeton, NJ, USA) by following a modified protocol [17,38]. To assess microglial activation and macrophages a select number of sections was also stained for CD68 (mouse monoclonal, 1:100, Abcam, UK). After cutting, sections were dewaxed in xylene and rehydrated in industrial methylated spirit (IMS) and water. After antigen retrieval was performed for 10 minutes in citrate buffer at pH6, the sections were left to cool before undergoing blocking with 2.5% normal horse serum (Vector Labs, Burlingame, CA) and avidin/biotin block (Thermo Fisher Scientific, Waltham, USA). Primary antibodies were incubated for 60 min at room temperature followed by appropriate biotinylated secondary antibody incubation for 30 min, which were detected using the ABC method (Vector Labs, Peterborough, UK). 3, 3'-diaminobenzidine (DAB) was applied for 5 min followed by counterstaining with haematoxylin. Sections were mounted in Di-N-Butyle Phthalate in Xylene (DPX, VWR, UK) and left to dry. To enable direct comparison of our results with previous work by others [10]we repeated the analysis of axonal density between control and MS using only two randomly selected blocks per anatomical region.

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Area measurements

Images of cross-sectional whole tissue blocks were acquired using a x4 objective (Fig. 1b-d) using a standard microscope (Nikon eclipse 80i) equipped with a motorized stage (mbf bioscience, Williston, USA) and saved as TIFF files. Images of sections stained for SMI-31 were opened with ImageJ (version 1.47; http://imagej.nih.gov/ij/) to manually outline and measure the following: (i) the CSA, (ii) the areas of GM and WM and (iii) the area of the lateral cortico-spinal tract (aCST; Fig. 2a). The latter was outlined following anatomic criteria: a horizontal line extended laterally from the most posterior part of the grey matter commissure out to the lateral border of the cord, which was used to define the anterior border. The outer margin of the spinal cord and the dorsal horn were used as the lateral and medial borders, respectively [10,38,37].

Measurement of demyelination

Where present, GM and WM demyelination were manually outlined on MBP images at x4 magnification and their area measured. The total areas of GM and WM demyelination in each block containing lesions was calculated as the sum of all lesion areas of the respective tissue type in the section (Fig. 1d). GM and WM demyelination were then expressed as percentages. Only tissue blocks affected by WM demyelination were used for analysis of the relationship between demyelination and axonal loss.

Assessment of axonal count and density

Using SMI-31 immuno-stained sections, four images at x40 magnification were randomly acquired from both the left and the right cortico-spinal tracts of each tissue block and saved as TIFF files. Care was taken that these images, which were subsequently used for quantifying the number of axons, were placed well within the acknowledged boundaries of the CST (Fig. 2a). Each image was then opened and a counting frame (sized: 120 μm x 120 μm) applied (Fig. 2b and c). After adjusting the white balance and transforming the area outlined by the counting frame to 8-bit black and white, axons were detected using ImageJ applying an object detection threshold to quantify the number of large (sized >3μm) and small (sized ≤3μm) diameter axons separately [10,30] (Fig. 2d and e).

Axonal density in each CST was calculated as the sum of large and small diameter axons divided by the total area of the four counting fields expressed as the number of axons/mm^2. Given no significant difference was detected between left and right CST axonal densities, the density of axons per tissue block was then expressed as a mean of left and right for all the subsequent statistical analysis.

Reproducibility of our histological measurements was verified by repeating three times the quantification of axonal density in one tissue block at each cord level (cervical, thoracic and lumbar) in six cases (three MS and three controls) by N.P. The coefficient of variation (CoV) was calculated as the square root of the mean variance, divided by the mean of the measurements.

Analysis of focal demyelination and axonal loss

Blocks containing 'isolated' WM lesions were obtained irrespective of the spinal cord proportion (cervical, thoracic, lumbar), and the following criteria applied: lesions were considered isolated if the CSTs appeared normally myelinated in at least 5 adjacent blocks (each approximately ~0.5cm thick) above and below the lesion. Regions of interest were then placed in the lesion, and (i) corresponding ipsilateral areas of non-lesional tissue that included the pathway of the CST in the block immediately above and below the lesion, as well as (ii) homotopic contralateral areas of non-lesional tissue at each corresponding level (lesion block, above lesion block, below lesion block). Axonal density was established as described above, and compared amongst groups (Fig. 3).

Statistical analysis

Differences between patients and controls were examined using linear mixed models with the measure being compared as the response variable and a fixed effect group indicator; fixed effect cord region indicators were included in all models, and other potential confounders (age at death dichotomised at <66, 66+ years to give roughly equal numbers, gender and cord length, dichotomised at <29, 29+ cm) were included singly as fixed effect covariates. These models used the cord slice as the unit of analysis, with a random subject intercept to account for the ownership of slices by subjects. An advantage of the mixed model is that it enables all available cord slices to be used in the analyses. Possible variations in patient vs control differences by region were examined by adding a group × region interaction term to the models. Linear mixed models were also used to investigate associations between cord measures and potential confounders. Residuals were examined to check model assumptions, as a result of which axonal density was log transformed to improve residual normality and homoscedasticity; this has two consequences: i) the calculated estimates and their confidence intervals represent proportional, not absolute loss; ii) it suggests that proportional loss in axonal density may be a more reliable description of patient vs control difference than absolute loss. Restricted maximum likelihood estimation (REML) was used except where there was evidence of residual heteroscedasticity, when maximum likelihood was used with robust standard errors. Analyses were performed in Stata 13 (Stata Corporation, College Station, Texas, USA), all measures were reported as average ± standard deviation (SD), except where noted. Significance is reported at 5%, unless otherwise indicated.

Results

Subjects

Post mortem tissue of eighteen subjects, thirteen (seven women, six men) with MS and five people (three men and two women) with no evidence of neurological disease, who donated their CNS to the UK MS Tissue Bank, was used (Table 1). The mean age at death of pwMS was 65.3 ± 11 years and for healthy controls 82 ± 7 years. Disease duration in pwMS was 29.2 ± 11.2 years. None of the patients had been treated with disease modifying drugs.

Spinal cord preservation and microscopic tissue features

The wet tissue was of good quality with no macroscopically evident signs of compression and/or other artefactual damage. The samples had been fixed in formalin for 30 ± 24 months before processing. The time between death and tissue fixation was 35.5 ± 16.5 hours. The mean cord length of all spines was 27.2 ± 4.8 cm, with controls being 28.5 ± 4.4 cm and MS being 26.8 ± 5.0 cm. The number of tissue blocks analysed ranged from 17 to 35 in controls and from 11 to 32 in MS. In total 396 tissue blocks were used for in this study. A shrinkage factor was not established in the current experiment, however a recent study pursued in our lab investigating cortical MS pathology revealed a shrinkage factor of 30% years [4], which is virtually identical to a previously reported factor in the spinal cord [10].

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Assessment of cellularity

H&E and CD68 (immuno-) stained sections were used for inspection of MS and control tissue. Characteristics of the cellular composition were assessed in areas that included the CST (Fig. 4). Whilst within MS lesions cellularity was decreased compared to the surrounding non-lesional tissue, the latter was indistinguishable from control tissue with no change in cellularity suggestive of inflammation. CD68 immuno-staining did not reveal any difference between lesional and non-lesional MS tissue. Against this backdrop, no systematic assessment of inflammatory cells was undertaken.

Reproducibility of histological indices

The calculation of the CoV for the aCST measurement, plotted as an average per slide between the left and right CSTs and combining all spinal levels for both patient and control tissue, was 10%. When separating the same measurement by level and testing for significant differences the CoV was 10% at cervical, 11% at thoracic and 4% at lumbar level with no evidence of patient versus control difference in measurement error (p=0.35). The CoV for the axonal density, when plotted as an average combining all regions and both patient and control cases, was also 10%. A separate analysis by level showed a significantly lower variability at the cervical level (6%, p=0.0008) when compared to thoracic (13%) and lumbar (10%). There was no evidence of patient versus control difference in measurement error (p=0.829).

Spatial distribution of tissue loss along the multiple sclerosis spinal cord

Table 2 shows unadjusted patient vs control differences by spinal cord levels. The multiple sclerosis spinal cord CSA was lower than control by between 19% and 24%, quite small proportional variation between cord levels; however, there was borderline significant evidence, p=0.055, of variation in absolute differences by region, with the largest absolute loss in the cervical region (Fig. 5a).

aCST was decreased in the multiple sclerosis spinal cord by between 25% and 37% (Table 2). There was significant evidence of variation in absolute differences across spinal cord levels, p= 0.017. The greatest absolute reduction was observed at thoracic, followed by cervical level (Fig. 5b).

GM area differences across the spinal cord showed a reduction in multiple sclerosis spinal cord ranging from 17% to 21%. There was only borderline significant evidence that the absolute differences varied across the three levels of the neuraxis, p= 0.0515. All values are shown in Table 2 and plotted in Fig. 5c.

The pattern of WM area across the spinal cord showed a reduction in multiple sclerosis varying from 19% to 24%. There was no significant evidence of variation in absolute differences by region (p= 0.165). All values are shown in Table 2 and Fig. 5d.

Adjustment for age, gender or cord length did not materially alter estimated differences.

Axonal loss affects all spinal cord levels equally and is independent of axonal diameter

Axonal density in the multiple sclerosis cortico-spinal tract was significantly reduced by between 57% and 62% (Table 3, Fig. 6a), with no evidence of variation in this loss across spinal cord levels (p=0.145). These estimates were obtained using the log transformed density, which better satisfied normality assumptions than the raw density. There were similar reductions in both large and small diameter axons in multiple sclerosis spinal cords of 60.6% (large) and 60.2% (small), with no evidence that the proportional reduction in small diameter axons is greater than in those with a large diameter, p=0.973 (Fig. 6b and c). These estimates were not materially affected by adjustment for age, gender or cord length.

Assessing the extent of axonal loss using only two blocks per anatomical region showed that the axonal density was reduced by 64% at the cervical, 66% at the thoracic and 64% at the lumbar level. Only marginal, not statistically significant, difference in the percent change of axonal density between control and MS was detected compared to the analysis including all tissue blocks (see above).

Grey matter demyelination is more extensive than white matter demyelination

When analysing only tissue blocks that contained 'any' area of GM demyelination its percentage ranged from 24% to 48% (Fig. 6d, Table 3b), with evidence of a significant variation across the three spinal cord levels (p=0.001), with the most extensive GM demyelination affecting the thoracic level. Thoracic GM demyelination was significantly greater than at both cervical (p=0.001) and lumbar (p=0.018) levels.

Focusing on tissue blocks that contained 'any' area of WM demyelination only its percentage ranged from 11 % to 13%, with no evidence of variation across the three cord levels (p= 0.936). (Fig. 6e, Table 3b)

Across the multiple sclerosis spinal cord GM demyelination as a percentage of total GM was more extensive than WM demyelination; including all cord levels the mean percent difference between GM and WM demyelination was 26% (95% CI 15.4, 36.7, p<0.001). Region-specific percent differences between GM and WM demyelination are shown in Table 3b.

Axonal density correlates with disease duration

In multiple sclerosis, after adjusting for region, higher axonal density was borderline significantly associated with shorter disease duration (p=0.055). Disease duration did not correlate with cortico-spinal tract, GM or WM area. After adjusting for subject group and region, no significant association was detected between cord length as well as age at death and (i) axonal density, (ii) cortico-spinal tract, and (iii) GM or WM areas. No significant correlation emerged either between axonal density and (i) aCST and (ii) CSA (Fig. 7a and b).

Axonal loss is strongly associated with focal white matter demyelination, however overall lesion volume does not reflect this

In 10/13 cases included in this study, 11 'isolated' lesions were identified. Focusing on the analysis within CSTs affected by an isolated lesion revealed that axonal density within and below these lesions was lower by 49% and 40%, respectively, compared to the density measured above the lesions (p<0.001). Homotopic contralateral areas showed no difference between the two sides above the lesion level, however significant decrease at the lesion level (-47%) and below (-42%; p<0.001) (Table 4, Fig. 7c). Including all blocks in which WM demyelination was detected no association emerged between the total extent of WM demyelination and axonal loss (Fig. 7d).

Discussion

Although axonal loss in MS has been described as a pathological hallmark contributing to functional disability, results reporting the extent of axonal pathology have been inconsistent. Whilst this may be due to differences among tissue collections, other limitations including sampling bias may have contributed to variable results reported [10,30,3,37,15]. We therefore employed a comprehensive sampling strategy to investigate axonal loss and its relationship with volume changes and demyelination across the whole spinal cord in multiple sclerosis.

Our CSA measurements indicate that in chronic MS spinal cord atrophy of approximately 20% occurs and that this atrophy evenly affects all levels of the spinal cord. No material difference was detected between the spinal cord levels when analysing GM and WM atrophy separately. The WM loss detected was between 15 % and 21% whilst GM loss was approximately 17% at cervical and lumbar levels, with similar trend difference at the thoracic level. Area reduction was also detected specifically affecting aCST, though this was statistically significant only at the cervical and thoracic levels. The degree of aCST reduction at the thoracic level (36.5%) suggests that the loss of CST fibres may be disproportional to the overall spinal cord atrophy, which affected all areas equally.

Axonal density was reduced by 57-62% across all levels of the spinal cord. This degree of axonal loss across the entire spinal cord is remarkably similar to the loss reported by Tallantyre and co-workers [37] (based on a single cervical cord section), approximately twice the loss reported by de Luca and co-workers [10] (five sections in total at cervical, thoracic and lumbar levels), and about 1/3 higher compared to Ganter and co-workers [15] (two sections at levels C_3 and T_2). In addition, the cohorts of MS cases used in these three studies had an average disease duration of 25, 17 and 14 years, respectively, while it was 29 years in our cohort, suggesting that axonal loss accrual is indeed proportional to clinical progression duration. This interpretation is also supported by the fact that in our cohort, and after adjusting for anatomical and clinical confounders, the longer was the progression the lower was the axonal density within the CSTs.

MRI-derived spinal cord atrophy indices are increasingly being used in studies [26], including treatment trials (NCT00731692), of people with CDD due to multiple sclerosis. However, despite sufficient statistical power, no association emerged in our study between CSA and axonal density (Fig. 7) and the significant difference between CSA reduction (20%) and axonal loss (about 60%) suggests spinal cord atrophy significantly underestimates the degree of axonal loss, a putative key substrate of CDD. These findings corroborate observations in animal models of MS in which the time courses was monitored [21]. Whilst previous studies using human *post mortem* MS samples have investigated CSA changes [19] and axonal loss [15, 3, 30, 10, 11, 39], correlation between CSA and axonal density has, to our knowledge, never been systematically explored.

If spinal cord volume does not provide a straightforward correlate of axonal density, other factors need to be considered. The expected degree of atrophy based on axonal loss could be offset by 'space filling' tissue components such as oedema, inflammation and gliosis. For example, in a clinical study of the sodium channel blocker lamotrigine pseudo-atrophy (here: of the brain) due to anti-oedematous effects confounded the detection of a (putative) neuroprotective effect [24]. However, although in our study fluid was removed as a result of tissue processing and fixation, it is unlikely that oedema would account for the large difference detected between CSA and axonal density. Gliosis is a rather more likely candidate given it evidently counteracts the space reducing effect of nerve fibre loss [21]. Taken together, these observations suggest that spinal cord atrophy is only partially reflective of the pathological feature considered crucial for CDD, i.e. axonal loss. Translated into the clinical setting of pwMS, rather than relying on atrophy measures (alone), there appears to be an urgent need for better techniques reflecting axonal damage in the spinal cord [16].

Contrary to earlier studies no difference in the degree of axonal loss between large and small diameter axons ($p=0.973$) was detected, questioning the selective vulnerability of small diameter axons as previously suggested [38,10,30]. It was not possible for us to verify whether respective data in previous studies were normally distributed. However, analysing the entire spinal cord and applying log transformation to improve residual normality and homoscedasticity of our not-normally distributed data suggests that axons of all sizes are equally affected.

It should be kept in mind, however, that axonal diameter may decrease in the process of neurodegeneration [22], possibly due to a decrease in neurofilament gene and nerve growth factor receptor expression [35]. It is therefore possible that large diameter axons in chronic MS become atrophic over the course of the disease. As a result, the number of small diameter axons detected may consist of both axons that always were of small diameter as well as large diameter axons that have become atrophic.

In line with previous studies, demyelination was significantly more extensive in the GM than the WM, underlining the nature of MS as a condition that affects all CNS tissue compartments [20,18] and evidently rendering the previously held belief that MS is a merely 'white matter' disease outdated. Several groups reported a lack of association between demyelination and axonal loss in the MS spinal cord, a surprising result given the (multi-) focal nature of the disease [11,38,30,15]. Even in studies employing a highly focussed approach, differences in axonal density within and outside lesions did not always emerge [30].

Whilst the overall plaque load in our study did not correlate with the total extent of axonal loss, we detected reduced axonal density, by 49%, in focal CST lesions and the associated distal non-lesional CST (Wallerian degeneration). Evidence suggests acute axonal damage [14,39] and Wallerian degeneration [12] are characteristic features of newly emerging lesions. Our data indicate that even in the very late stage of MS spinal cord demyelination has significant impact on tract specific axonal loss distal to lesions, whereas 'dying back' neuropathy, as previously suggested, seems of lesser importance [7].

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The apparent discrepancy between analysing our data using (i) all blocks containing demyelination and their associated axonal density and (ii) only blocks containing lesions that affect the CST may have been due to at least two factors. Firstly, in the former analysis we included the total area of demyelination, i.e. including areas outside the CST, whilst axonal density was established in the CST only. This may have masked the effect of focal demyelination on axonal density. Secondly, although we comprehensively examined the entire length of the spinal cord, we were unable to account for the potential effects of supra-spinal (brain) tissue pathology on spinal cord axonal density.

The effect of focal demyelination on CST axonal loss in our and previous studies [37] are quite different from those reported by Lovas and co-workers who did not detect reduction in axonal density, but in axonal thickness [38,30]. Our data suggest differences in the methodology of quantification may explain these discrepancies. Whilst we are not aware whether Lovas, et al. excluded any lesion pathology above or below their lesion of interest we confirmed the absence of tract specific demyelination in five tissue blocks above and below the index lesion in our 'isolated lesion' experiment. We thereby significantly increased the likelihood that remote effects of demyelination did not bias our measurements. An additional, or alternative, explanation could be differences in lesion size with longer lesions hypothetically being more damaging, as has been described using MRI in people with optic neuritis [23]. However, lesion size was not assessed, neither in our, nor to the best of our knowledge in previous *post mortem* studies. There could be merit adding such measures in the future to provide further insights into the effect of demyelination on axonal density.

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The lack of a significant difference in the detected axonal loss when including only two tissue blocks per anatomical region compared to the analysis of all tissue blocks produced suggests that to investigate axonal loss *in isolation* two blocks per anatomical level appear to be sufficient. However, sampling the entire cord not only enables the total magnitude of other pathological features to be assessed, but also to rule out remote effects on specific areas of interest, such as in our 'isolated' lesion analysis. Such lesions were also quite rare underpinning the merit of dissecting the entire cord, though MRI guidance may be an alternative solution as has been shown before for sampling lesions in the *post mortem* brain [8].

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The association detected between axonal density and disease duration suggests time is a key determinant of clinically significant tissue damage in chronic MS. Although our data have been obtained using *post mortem* material, *in vivo* evidence of brain atrophy assessed using MRI suggests that a chronic "slow burning" axonopathy may be a feature of MS from the earliest stages of its evolution [10], over and above lesion-associated acute axonal damage [13,39,14]. This may be triggered by the inflammatory responses that drive relapsing attacks, as seen experimentally [21]. However, these smouldering lesions increase with disease duration and chronicity in MS and probably contribute to worsening disability [14].

In conclusion, our study provides robust evidence that after a life with MS for, on average, nearly 30 years about 60% of spinal cord CST axons are lost. It is likely this loss is an important driver of disease deterioration and permanent disability in pwMS and affects axons of any calibre throughout the length of the spinal cord. The

magnitude of spinal cord atrophy, about 20%, does not reflect well the degree of axonal loss, which in itself appears strongly associated with focal demyelination. MRI indices of spinal cord volume obtained *in vivo* are likely to underestimate the extent of axonal damage thereby limiting the role of such indices as a treatment outcome in clinical trials. New techniques that are more specific for the underlying tissue damage are warranted [16].

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Table legends

Table 1 Clinical details of all cases.

Table 2 Area measurements [mm^2], relative and percent difference across spinal cord levels between multiple sclerosis and controls.

Table 3 Differences in (a) axonal density between normal control and multiple sclerosis, and (b) percent demyelination between grey and white matter in multiple sclerosis

Table 4 Mean axonal density in a) the lesion site and the two sites corresponding to the normal-appearing white matter directly above and below the lesion site on the ipsilateral side of the lesion and the corresponding contralateral sites on the same blocks, and b) differences in axonal density on the ipsilateral side of the lesion compared between the three positions, above the lesion, inside the lesion and below the lesion.

Figure legends

Fig. 1 Tissue sampling and quantification of cross sectional area and demyelination

(A) Spinal cord dissection: after identifying and numbering nerve roots, pictures were taken and the distance between thoracic level 2 and lumbar level 5 recorded. Axial blocks were then dissected at each nerve root level. At the thoracic level an additional block was dissected between nerve roots (see examples T5A and T5B). Scale bar: 1.5cm. (B) Control cervical cord section immuno-stained for SMI-31 at x4 magnification. The grey matter (GM) is outlined in red and the cross-sectional area (CSA) in blue. White matter (WM) was calculated as the difference between CSA and GM area. (C) Control upper thoracic cord section at x4 magnification stained for myelin basic protein (MBP). GM borders are outlined in red. (D) Multiple sclerosis upper thoracic cord section stained for MBP. Four WM (WM Lesions 1-4) and one GM lesions (GM Lesion 1, green contour) were manually outlined. Scale bar: 1mm

Fig. 2 Assessment of the lateral cortico-spinal tract area and axon counting

(A) The area of the lateral cortico-spinal tract was manually outlined on SMI-31 immuno-stained sections (blue) at x4 magnification as previously described by DeLuca and colleagues. An area of interest (AOI, black) was then placed well inside the anatomical boundaries of the cortico-spinal tract. Within the AOI four frames (yellow squares) were then randomly cast and images acquired at x40 magnification. Scale bar: 1mm. These SMI-31 images of control (b) and multiple sclerosis (c) spinal cord were opened using ImageJ. One counting field (120µm x 120µm, red squares) was placed inside each image. Scale bar: 50µm. Magnified counting frames were converted into black & white (8 bit) of control (d) and multiple sclerosis (e) cortico-spinal tract, and axons counted at x40 magnification. Scale bar: 25µm. Orange and green arrows indicate large and small diameter axons, respectively

Fig. 3 Focal demyelination and axonal loss in the spinal cord cortico-spinal tract

Myelin basic protein (MBP) stained sections showing the non-lesional CSTs on the blocks directly above (a) and below (c) the isolated lesion, as well as the demyelinated CST on the level of the plaque (b). Sequential sections stained with SMI-31 are shown for the three levels in g to i respectively in low magnification. Red circles show the CST boundaries on the homotopic, ipsilateral side of the lesion, while black circles show the CST boundaries on the contralateral, functionally identical, side of the lesion. The four yellow squares in each CST represent the four counting fields cast in each CST as described in the methods and Figure 2, where axonal density was counted. Scale bar: 1mm. Examples of SMI-stained images from one of the four counting fields on the contralateral side of the lesion are shown on high magnification in d, e and f for each of the three regions. K shows one example counting field from the lesional CST, while j and l correspond to the counting field

examples directly above and below the lesional CST respectively, on the ipsilateral side of the lesion. Scale bar: 50μm

Fig. 4 Assessment of cellularity and inflammation in control, non-lesional and lesional CSTs

Low magnification images of MBP (a and h), H&E (c and j) and CD68 (f and m) show the staining patterns in MS and control tissue respectively, while a lesional CST is shown on the left highlighted in the red circle and a non-lesional CST is shown in black on the right side of the MS image on panel a. Scale bar: 1mm. High magnification images of H&E are shown b and d for the lesional and non-lesional CSTs in MS respectively, while control images are shown in i and k on the left and the right normal-appearing CST tissue. Examples of CD68 staining in high magnification for the plaque and normal-appearing CST are shown in e and g respectively, while control staining is shown in l and n. Scale bar: 25μm.

Fig. 5 Area indices across spinal cord levels in multiple sclerosis and controls

(**A**) Cross sectional area (CSA) in multiple sclerosis was reduced at the cervical, thoracic and lumbar ($p= 0.055$) levels. (**B**) The area of the lateral cortico-spinal tract (aCST) was reduced at the cervical and thoracic levels, with trend difference at the lumbar level ($p= 0.099$). (**C**) The grey matter (GM) area was reduced in multiple sclerosis at the cervical and lumbar levels. (**D**) The white matter (WM) area was reduced at the cervical and thoracic levels. Each dot represents one tissue block. Error bars represent standard deviations and stars indicate significance levels (***$p<0.001$; **$p< 0.01$; *$p<0.05$). See also Table 2

Fig. 6 Axonal loss and demyelination in the multiple sclerosis spinal cord

Axonal density was reduced in multiple sclerosis spinal cord when including all (a), small diameter (≤ 3μm) (b) and large diameter (>3 μm) (c) axons across all levels. (D) The degree of GM demyelination was higher at the thoracic level when compared to the cervical and lumbar levels. (E) No significant difference was detected in the extent of WM demyelination between spinal cord regions. Each dot represents one tissue block. Error bars represent standard deviations and, stars indicate significance (****$p<0.0001$; *$p<0.05$)

Fig. 7 Association between axonal density and focal CST demyelination, axonal density and total white matter demyelination and cross-sectional area

No correlation was detected in multiple sclerosis spinal cord between axonal density and (a) aCST or (b) CSA. Focal demyelination affected axonal density locally ($p<0.0001$) and on the non-lesional CST area directly below the plaque ($p<0.0001$), while it had no effect on either the non-lesional CST above the lesion, or the normal appearing white matter on the contralateral side of the lesion in any level (c). Although axonal loss was affected

by focal demyelination, it did not have a significant correlation with overall WM demyelination (d). Error bars represent standard deviations and, stars indicate significance.