REPORT

Reconstruction of single cortical projection neurons reveals primary spine loss in multiple sclerosis

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Grey matter pathology has emerged as an important contributor to long-term disability in multiple sclerosis. To better understand where and how neuronal damage in the grey matter is initiated, we used high resolution confocal microscopy of Golgi-Cox impregnated tissue sections and reconstructed single cortical projection neurons in autopsies from eight patients with long-standing relapsing-remitting or secondary progressive multiple sclerosis and eight control patients without neurological disease. Analysis of several hundred individual neurons located in the insular, frontotemporal and occipital lobe revealed a widespread and pronounced loss of dendritic spines in multiple sclerosis cortex that occurs independent of cortical demyelination and axon loss. The presence of a primary synaptic pathology in the normal-appearing cortex of multiple sclerosis patients challenges current disease concepts and has important implications for our understanding of disease progression.

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Abbreviation: NAGM = normal-appearing grey matter

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Introduction

Multiple sclerosis is the most common demyelinating disease of the CNS and an important cause of neurological disability. While inflammatory lesions in the white matter are the most obvious pathological feature of the disease, the degree of long-term disability shows only limited correlation to the observed numbers or volume of these lesions (Fisniku et al., 2008). This indicates that pathology in other anatomical compartments is likely to contribute to the disease process. Indeed, refined histopathological and neuro-imaging techniques have revealed grey matter alterations in multiple sclerosis that appear to be present from the early stages of the disease, expand as the disease progresses, and correlate with progression of disability in advanced stages (Chard et al., 2002; Fisniku et al., 2008; Calabrese et al., 2013). Grey matter lesions in the cortex and hippocampus that are characterized by widespread demyelination are a prominent aspect of grey matter pathology (Bo et al., 2003; Dutta et al., 2011; Lucchinetti et al., 2011) and neuronal loss as well as neurite transections and synapse loss have been reported in demyelinated grey matter lesions (Peterson et al., 2001; Wegner et al., 2006; Dutta et al., 2011; Michailidou et al., 2015). However, it is so far unclear in which sequence neuronal damage in these lesions is initiated and whether neuronal alterations can extend beyond the lesion area. To better understand the microanatomy of neuronal alterations in multiple sclerosis grey matter we reconstructed the dendrites of individual cortical layer IV–VI neurons in the demyelinated and normal-appearing grey matter (NAGM) of multiple sclerosis cortex by combining a modified Golgi-Cox impregnation technique with high resolution confocal microscopy. We found a widespread loss of dendritic spines in the cortex of patients with multiple sclerosis that was present in different cortical regions, similarly affected demyelinated and NAGM, and preceded loss of cortical axons.

Materials and methods

Human tissue collection

For histopathological analyses human tissue samples of eight brain autopsies of male (n = 3) and female (n = 5) individuals with long-standing relapsing-remitting or secondary progressive multiple sclerosis [duration > 10 years in most cases; mean age 56.75 ± 10.48 years; stored for 1.88 ± 1.33 months in 4% neutral buffered formalin; mean ± standard deviation (SD)] and eight control patients (n = 5 males, n = 3 females) without neurological disease (mean age 69.88 ± 11.33 years; stored for 2.38 ± 1.06 months in 4% neutral buffered formalin; mean ± SD) were used. As spine density at 200 and 250 μm distance to neuronal soma negatively correlated with age in control samples (Pearson r = 0.77 at 250 μm, P < 0.05 and 0.80 at 200 μm, P < 0.05), only age-matched control samples were used for spine density analysis (two males and two females; mean age 61.25 ± 7.27 years; mean ± SD). For all other analyses all control cases were included. According to the principles of the Declaration of Helsinki informed consent was obtained and the tissue sample collection was approved by local regulatory agencies.

Histological stainings

Individual cortical neurons were visualized using a modified Golgi-Cox impregnation method as previously described (Glaser et al., 1981). Briefly, after washing tissue blocks were incubated afloat in a solution containing 5% potassium dichromate, 5% mercury chloride and 5% potassium chromate in distilled water for 3 weeks protected from light at room temperature and postfixed in 10% formalin for 1 h at 37°C. Tissue was cut in 70-μm thick sections using a vibratome. Tissue slices were treated with 5% sodium carbonate for 2 min at room temperature, washed with distilled water, mounted in UltraKitt on gelatin (0.5%-)-coated slides and coverslipped. Immunostaining for cortical myelin (MBP), T cells (CD3) and macrophages/activated microglia (CD68) as well as staining to visualize axons (Bielschowsky impregnation) was carried out on 3-μm thin sections of paraffin-embedded tissue blocks as previously described (Rodriguez et al., 2014).

Image acquisition and analyses of cortical dendrites from projection neurons

Golgi-Cox-impregnated dendrites of cortical layer IV–VI neurons were classified to be located within a demyelinated lesion or in the NAGM based on the MBP staining of respective tissue blocks. The basal segments of the apical dendrite were imaged after 488 nm excitation using a confocal laser scanning microscope (Zeiss LSM510Meta) equipped with a 63 × 1.4 oil immersion objective by detecting the reflected laser light (Mancuso et al., 2013). Image stacks were obtained using a ×3 zoom and deconvolved using Huygens Essential software. Dendrite width was measured and the densities of dendritic spines and branches were determined for segments centred at defined distances from the neuronal cell body using Fiji open source software. Gamma was adjusted non-linearly to enhance low-intensity objects. A blinded investigator performed all evaluations. Quantification of spine density by an independent evaluator revealed a strong inter-rater reliability of the analysis (Pearson r = 0.9020, P < 0.0001; n = 531 images).

Quantification of cortical axon density

The number of horizontal axons that crossed a line drawn perpendicular to the cortex (maximum length of 3000 μm) was counted on paraffin-embedded sections after Bielschowsky impregnation. Axons were quantified in 100-μm segments and counts were related to NAGM and lesion areas as well as cortical layers (I, II, III–IV and V–VI) based on adjacent sections immunostained for MBP followed by Nissl counterstaining. CaseViewer software was used for displaying and optimizing the brightness and contrast of the images. Axonal density was averaged for each case and
normalized to the mean of control cases in the corresponding lobe. The normalized data were pooled for the final data representation.

**Statistical analysis**

Data illustration and statistical analysis was performed using Prism 5.01 (GraphPad). Data are presented as mean ± SEM unless indicated otherwise and analysed using one-way or two-way analysis of variances followed by Bonferroni post hoc tests. Significance levels are indicated as follows: *P < 0.05; **P < 0.01; ***P < 0.001.

**Results**

**Processing and characterization of cortical multiple sclerosis samples**

For the evaluation of dendritic alterations in cerebral cortex we compared autopsies of multiple sclerosis patients with long-standing disease (see ‘Materials and methods’ section) and control patients without neurological affection. Cortical brain tissue was sampled from the insular, frontotemporal, and occipital lobe of both hemispheres (Fig. 1A) and tissue blocks were split into two parts of which one was processed for Golgi-Cox impregnation (Fig. 1B) and the other was embedded in paraffin for histopathological evaluation (Fig. 1C). Cortical tissue specimens from multiple sclerosis patients were then categorized into NAGM and demyelinated cortex based on MBP immunostaining (Fig. 1C). In total, we analysed 88 tissue blocks and found cortical lesions with demyelination extending from the pial surface into the cortical layers IV–VI in five samples from multiple sclerosis cortex, while all control cases displayed intact neocortical myelin. We did not detect enhanced numbers of CD68+ macrophages/activated microglia cells (30.65 ± 3.99 in control cortex, 26.38 ± 6.49 in NAGM and 26.67 ± 10.35 CD68+ cells/mm² in demyelinated cortex, n = 3–5 cases, values are averaged for different cortical layers of each case) or the infiltration of CD3+ T cells in multiple sclerosis cortex suggesting that the cortical lesions were at a chronic, inactive stage.

**Reconstruction of single projection neurons reveals widespread spine loss in multiple sclerosis cortex**

To assess where along the neuron pathological changes are initiated we reconstructed Golgi-Cox stained dendrites of single pyramidal neurons located in layers IV–VI of the cerebral cortex by confocal laser scanning microscopy (Fig. 2A and B). For each apical dendrite we measured its width, the number of branches emerging from it and the number of spines it carries at defined distances from the neuronal soma. We evaluated dendrites located in control brains and in the normal-appearing as well as in the demyelinated multiple sclerosis cortex to differentiate lesion-independent and lesion-associated changes in the multiple sclerosis brain and compared results from different cortical lobes to assess the regional spread of these changes. Our results showed that the dendritic shafts generally get thinner as they extend further from the cell soma; however, there were no significant differences in the width of the dendrites located in either demyelinated cortex, NAGM or control cortex in any of the cortical lobes analysed (Fig. 2C and D). Branches of the main apical dendrite were most commonly observed close to the neuronal cell body in the control cortex and their numbers were significantly reduced in all regions of the demyelinated multiple sclerosis cortex and to a lesser extent also in the NAGM (Fig. 2E and F). The density of dendritic spines—the sites where these neurons receive synaptic input—generally increased with the distance to the neuronal soma. However, compared to control cortex a marked reduction of spine density was observed primarily in the insular and frontotemporal lobe of the multiple sclerosis brain (Fig. 2G and H). Of note, spine density in these regions was equally reduced by more than 50% in the demyelinated and normal-appearing areas of the cortex suggesting a widespread, lesion-independent loss of synaptic input in the multiple sclerosis brain.

**Cortical axon numbers are reduced in the demyelinated but not in the normal-appearing multiple sclerosis cortex**

Pyramidal neurons in the cortex receive synaptic input from local collaterals and from extracortical projections such as thalamocortical axons (Nieuwenhuys et al., 1994). We thus wondered if the observed widespread loss of dendritic spines in multiple sclerosis cortex is related to a general reduction of cortical axon numbers. For this purpose, we quantified the density of tangentially oriented cortical fibres that mostly represent afferent intra- and extra-cortical axons (Nieuwenhuys et al., 1994) in different cortical layers of the insular and frontotemporal lobes (Fig. 3A). We found that a significant reduction of cortical axon density is only observed in the demyelinated multiple sclerosis cortex while cortical axon density was unchanged in all cortical layers of the normal-appearing multiple sclerosis brain (Fig. 3B). Together, these findings indicate that spine loss in the NAGM does not result from local inflammation or axon loss but represents a primary synaptic pathology in the multiple sclerosis brain.

**Discussion**

Our reconstruction of individual cortical projection neurons and their dendrites reveals distinct types of neuronal
pathology in the grey matter of multiple sclerosis patients. In focal grey matter lesions all neuronal compartments are affected and spine loss is accompanied by a reduction of dendritic branches and cortical axons, while a selective spine loss is observed in the NAGM where cortical axon density and overall dendrite morphology remain mostly unaltered. This suggests the presence of a diffuse primary synaptic pathology that can affect cortical projection neurons in the entire grey matter of the multiple sclerosis cortex (although with some regional variability) and that is focally accentuated by the loss of dendrites and axons in demyelinated cortical lesions. Different pathomechanisms acting alone or in concert could account for such a primary synaptic pathology: first, spine loss could represent a direct consequence of reduced afferent synaptic input. While the density of axons was only reduced within demyelinated cortical areas, it is conceivable that longstanding functional alterations, including the reduction of axonal transport in
Figure 2  Reconstruction of dendritic compartments of individual cortical neurons. (A and B) Confocal images of proximal (A) and distal (B) segments of Golgi-Cox impregnated dendrites of frontotemporal cortical layer IV–VI neurons located in a control cortex (‘Control’, left images) and normal-appearing (‘NAGM’, middle images) and demyelinated (‘Lesion’, right images) areas of multiple sclerosis cortex. Arrowheads in A indicate branches of the main dendrite, hollow circles in B indicate spines along the apical dendrite. (C) Scheme of a cortical dendrite with dendritic shaft highlighted in blue. (D) Quantification of the width of the main apical dendrite measured at different distances from the neuronal soma in layer IV–VI projection neurons located in the insular (left: control, n = 69–109 neurons from eight cases; NAGM, n = 49–119 neurons from eight cases; lesion, n = 1–10 neurons from two cases), frontotemporal (middle: control, n = 52–95 neurons from eight cases; NAGM, n = 37–
Figure 3  Axon density is reduced in demyelinated but not in normal-appearing areas of the multiple sclerosis cortex. (A) Bielschowsky’s silver stained sections of cortical layer I of the insular lobe in control (‘Control’, left) brains and normal-appearing (‘NAGM’, middle) and demyelinated (‘lesion’, right) areas of multiple sclerosis cortex. Arrowheads indicate axons that cross a vertical line used for quantification. (B) Quantification of relative axon density in different cortical layers is shown for control, NAGM and demyelinated multiple sclerosis cortex. Axon density was quantified in the insular lobes of eight control and eight multiple sclerosis cases (cortical lesions were present in three cases) and in the frontotemporal lobes of seven control and eight multiple sclerosis cases (a cortical lesion was present in one case). The data were normalized to the average control of each region and pooled for the graph. ***P < 0.001, two-way analysis of variance with Bonferroni post hoc test. Images were adjusted to improve brightness and contrast. Scale bar: A = 10 µm.

Figure 2  Continued
121 neurons from eight cases; lesion, n = 3–25 neurons from two cases) and occipital (right: control, n = 58–121 neurons from eight cases; NAGM, n = 41–125 neurons from eight cases; lesion, n = 3–4 neurons from one case) lobes. (E) Scheme of a cortical dendrite with dendritic branches highlighted in blue. (F) Quantification of the branch density along the main apical dendrite measured at different distances from the neuronal soma in layer IV-VI pyramidal neurons located in the insular (left: control, n = 69–109 neurons from eight cases; NAGM, n = 49–119 neurons from eight cases; lesion, n = 1–10 neurons from two cases), frontotemporal (middle: control, n = 52–95 neurons from eight cases; NAGM, n = 37–121 neurons from eight cases; lesion, n = 3–25 neurons from two cases) and occipital (right: control, n = 58–121 neurons from eight cases; NAGM, n = 41–125 neurons from eight cases; lesion, n = 3–4 neurons from one case) lobe. (G) Scheme of a cortical dendrite with spines emerging from the dendritic shaft highlighted in blue. (H) Quantification of the spine density along the main apical dendrite measured at different distances from the neuronal soma in layer IV–VI projection neurons located in the insular (left: control, n = 42–56 neurons from four age-matched cases; NAGM, n = 49–119 neurons from eight cases; lesion, n = 1–10 neurons from two cases), frontotemporal (middle: control, n = 25–44 neurons from four age-matched cases; NAGM, n = 37–121 neurons from eight cases; lesion, n = 3–25 neurons from two cases) and occipital (right: control, n = 39–62 neurons from four age-matched cases; NAGM, n = 41–125 neurons from eight cases; lesion, n = 3–4 neurons from one case) lobe. Graphs represent mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, two-way analysis of variance with Bonferroni post hoc test. Images were adjusted to improve brightness and contrast. Scale bars: A and B = 5 µm.
those axons that run through inflamed areas, could lead to a distal synaptic dystrophy without affecting proximal axon numbers (Sorbara et al., 2014). Second, it is conceivable that altered spine density could mirror the consequence of retrograde degeneration of efferent pyramidal axons that are damaged within the white matter. Indeed, such retrograde spine loss has been described to occur in layer V neurons after an experimental spinal cord injury. Interestingly, spine loss in this model did not only affect axotomized neurons but also their non-transected neighbours, possibly reflecting the local remodelling of motor circuits (Ghosh et al., 2012). Finally, synapses could be a susceptible target of widespread inflammation in the CNS compartment and spine loss could result e.g. from the long-range action of secreted inflammatory mediators that cause synaptic pathology such as IFN-γ (Kreutzfeldt et al., 2013) and TNF-α (Yang et al., 2013) or the diffuse activation of microglia cells induced, for example, by the local activation of the complement cascade that has been recently shown to occur both in demyelinated and myelinated hippocampi of multiple sclerosis patients (Michailidou et al., 2015). Such inflammatory mechanisms could be further enhanced by the presence of meningeal follicle-like structures that lead to enhanced neuronal pathological in a subset of patients with progressive multiple sclerosis (Magliozi et al., 2010) and their effects amplified by the compromised state of cortical neurons in multiple sclerosis that results, for example, from the progressive accumulation of dysfunctional mitochondria (Mahad et al., 2015). The concept that widespread but low grade inflammatory processes in grey matter and/or meninges drive primary synaptic pathology independent from focal white and grey matter lesions would also help explain why over time disease symptoms in multiple sclerosis appear to disconnect from focal pathology (Fisniku et al., 2008; Calabrese et al., 2015).

As spines play a central role in the reception and active dendritic integration of synaptic inputs (Grienberger et al., 2015) it is further likely that their widespread loss in the multiple sclerosis cortex could be at least one of the contributors to the cognitive deficits that affect a substantial proportion of multiple sclerosis patients (Chiaravalloti and DeLuca, 2008). Notably, such cognitive impairments have been found to correlate with MRI measures of grey matter pathology including widespread cortical thinning (Calabrese et al., 2009, 2010). These findings further offer an interesting parallel to classical neurodegenerative conditions such as Alzheimer’s disease, in which prominent synaptic pathology occurs as a harbinger of progressive cognitive dysfunction (Spires-Jones and Hyman, 2014). Taken together, the primary synaptic pathology we observe here might thus help to better understand multiple sclerosis progression and provide a new therapeutic target for strategies that aim to halt the insidious accumulation of physical and cognitive disability in patients, who have entered advanced stages of the disease.

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