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The topograpy of demyelination and neurodegeneration in the multiple sclerosis brain

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Multiple sclerosis is a chronic inflammatory disease with primary demyelination and neurodegeneration in the central nervous system. In our study we analysed demyelination and neurodegeneration in a large series of multiple sclerosis brains and provide a map that displays the frequency of different brain areas to be affected by these processes. Demyelination in the cerebral cortex was related to inflammatory infiltrates in the meninges, which was pronounced in invaginations of the brain surface (sulci) and possibly promoted by low flow of the cerebrospinal fluid in these areas. Focal demyelinated lesions in the white matter occurred at sites with high venous density and additionally accumulated in watershed areas of low arterial blood supply. Two different patterns of neurodegeneration in the cortex were identified: oxidative injury of cortical neurons and retrograde neurodegeneration due to axonal injury in the white matter. While oxidative injury was related to the inflammatory process in the meninges and pronounced in actively demyelinating cortical lesions, retrograde degeneration was mainly related to demyelinated lesions and axonal loss in the white matter. Our data show that accumulation of lesions and neurodegeneration in the multiple sclerosis brain does not affect all brain regions equally and provides the pathological basis for the selection of brain areas for monitoring regional injury and atrophy development in future magnetic resonance imaging studies.

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Introduction

Multiple sclerosis is a chronic disease of the CNS, specifically featured by inflammation, widespread primary demyelination and progressive neurodegeneration. A widely held concept of the pathogenesis of the disease is that tissue injury in the brain and spinal cord is initiated by T cell-mediated inflammation and that demyelination and neurodegeneration are driven by heterogeneous mechanisms, involving both adaptive and innate immune systems (Lassmann *et al.*, 2007). We and others recently proposed that microglia activation, production of reactive oxygen species and oxidative damage are key mechanisms driving demyelination and neurodegeneration, particularly in the progressive disease stage (Haider *et al.*, 2011; Fischer *et al.*, 2013). In addition, mitochondrial injury (Mahad *et al.*, 2008; Campbell *et al.*, 2011) may further propagate oxygen radical production (Murphy, 2009) and amplify demyelination and neurodegeneration by energy deficiency through histotoxic hypoxia (Trapp and Stys, 2009; Witte *et al.*, 2010). With disease progression, the intensity of the inflammatory response declines, but oxidative injury and mitochondrial damage are aggravated by additional factors related to ageing of the patients and to the accumulation of disease and lesion burden (Mahad *et al.*, 2015).

If this concept is valid, besides density of veins and meningeal inflammatory infiltrates also arterial anatomy of the brain should influence the topographical distribution of demyelinated lesions and neurodegeneration within the CNS due to varying basic levels of oxygen tension (Desai *et al.*, 2014). In our study we analysed predilection sites of demyelination and neurodegeneration and how these relate to arterial and venous anatomy and inflammation or demyelination in multiple sclerosis.

Materials and methods

Patient cohort

We performed our study on autopsy material from 51 patients with multiple sclerosis and 38 age-matched controls without neurological disease or focal brain lesions. The whole sample included two cohorts. In the first cohort we analysed the topographical distribution of demyelinated lesions and neurodegeneration. This cohort consisted of hemispheric or double hemispheric paraffin-embedded mid-thalamic sections from 19 multiple sclerosis cases and 20 age-matched controls (Supplementary Tables 1 and 2). The cohort included two cases with relapsing remitting multiple sclerosis, two cases with primary progressive and 15 cases with secondary progressive multiple sclerosis. All of these cases had long-lasting disease (median age 68, range 44–90 years); disease duration (median 32, range 7.25–51 years). In the second cohort we analysed the relation between inflammation, demyelination and neurodegeneration in routine sized sections. This cohort consisted of 46 cases and included 13 cases with acute multiple sclerosis (Marburg, 1906), one case with relapsing remitting multiple sclerosis, 13 cases with secondary progressive multiple sclerosis,

eight cases with primary progressive multiple sclerosis and one case with subclinical multiple sclerosis, diagnosed through neuropathology as well as 18 age-matched controls (Supplementary Tables 3 and 4). The respective tissue blocks were selected from an archival collection to provide sections with all types of cortical multiple sclerosis lesions and with normal-appearing grey matter as well as with subcortical or deep white matter lesions.

Neuropathological techniques and immunohistochemistry

All sections were screened for inflammation, demyelination and axonal damage in sections stained with haematoxylin/eosin, Luxol Fast blue and Bielschowsky silver impregnation. Cortical demyelinated lesions were identified in sections stained by immunohistochemistry for proteolipid protein. Iron accumulation within the brain was visualized by the DAB-enhanced Turnbull blue staining (Hametner *et al.*, 2013). We screened for the presence of white matter or cortical demyelinated lesions. Detailed lesion staging and lesion maps were made for each tissue block.

Immunohistochemistry was performed on paraffin sections using the primary antibodies and antigen retrieval techniques listed in Supplementary Table 5. For demonstration of retrograde neuronal degeneration we stained the sections with antibodies against phosphorylated neurofilaments (Koliatsos *et al.*, 1989). Neurons with oxidative injury were identified by the presence of oxidized phospholipids within the neuronal cell body and dendrites (Fischer *et al.*, 2013).

Quantification for topographical probability maps in hemispheric sections: Sample 1

Demyelination and leukoaraiosis

Each demyelinated lesion in the brain of individual patients was manually drawn in the patient's own brain section map. Lesions were identified by focal areas of myelin loss or sharply demarcated shadow plaques. All lesions identified in individual patients were then superimposed onto a virtual brain section of the same area (Supplementary Fig. 1). The probability of a lesion to occur in a specific brain area is provided by a colour code (white means that no lesions were present in these locations, while dark red colour means that a lesion was present in all 19 patients in this particular location; Fig. 1). Areas of leukoaraiosis in controls were identified in sections stained with Luxol Fast blue. In a normal brain of young individuals only diffuse differences in myelin density are seen between different white matter areas (Supplementary Fig. 2A) in comparison with the corpus callosum. Areas of leukoaraiosis were defined as those with at least 30% reduction of myelin density in comparison to the myelin density in the corpus callosum, measured by densitometry (Supplementary Fig. 2B).

SMI31-positive neurons

Hemispheric sections were stained by immunocytochemistry for phosphorylated neurofilaments. Neurons with signs of retrograde neurodegeneration were identified by their cytoplasmic accumulation of phosphorylated neurofilaments, by their enlarged cell body and the eccentric location of their nucleus
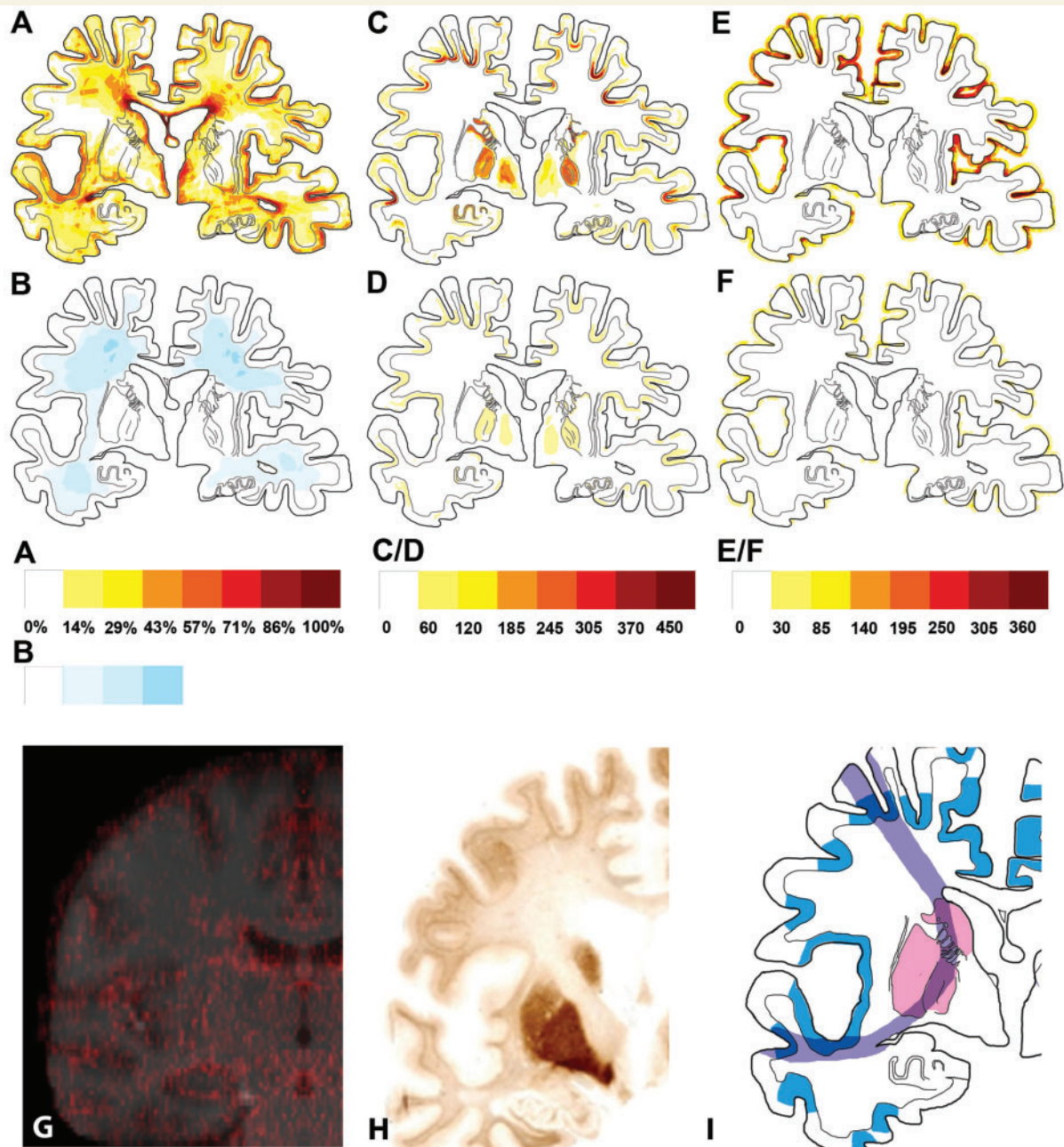
Figure 1 Probability maps of multiple sclerosis patients. Probability maps of multiple sclerosis patients (**A**, **C** and **E**) and age-matched controls (**B**, **D** and **F**). The colour codes indicate the probability of finding lesions (in % of cases **A** and **B**), the cumulative frequencies of neurons affected by retrograde neurodegeneration (**C/D**) and the cumulative frequencies of meningeal inflammatory cells (**E/F**) in a specific location of this virtual brain slice. (**A**) The probability of demyelinating lesions in the white and grey matter of multiple sclerosis patients. (**B**) The probability of leukoaraiosis in control subjects. (**C**) The cumulative number of neurons with retrograde degeneration in multiple sclerosis patients. (**E**) The accumulated number of inflammatory cells in the leptomeninges of multiple sclerosis patients. The highest incidence of demyelination (**A**) in the white matter is seen in the so-called watershed areas, which are located at the borders of the supply territories of the major cerebral arteries. In contrast, cortical lesions are mainly concentrated in invaginations of the cortical surface, such as the cortical sulci, in regions with high incidence of meningeal inflammatory infiltrates (**E**). Retrograde neurodegeneration is mainly seen in the deep cortical layers and the deep grey matter and in part follows the putative fibre projection from white matter lesions into the cortex (**C**). In age-matched controls no plaques of primary demyelination were present, but there were areas of diffuse white matter alterations (leukoaraiosis) in 43% of the cases investigated (blue areas in **B**). Cortical neurodegeneration was much less pronounced compared to that seen in multiple sclerosis (**D**), but showed a topographically similar distribution compared to that seen in multiple sclerosis patients. Inflammatory infiltrates were also seen in low numbers and incidence in the meninges of the age-matched controls (**F**). Definitions of regions of interest: (**G**) venous density atlas from Grabner *et al.* (2014) depicts the density of veins (red) in different brain areas. Brains were scanned in a 7 T MRI and a venous map of the normal human brain was created. (**H**) Turnbull staining showing the iron distribution throughout the brain. (**I**) Our regions of interest in one hemisphere of the virtual brain map. Areas of low CSF flow are indicated by blue, watershed area in purple and the basal ganglia in pink.

(Fig. 2). The entire grey matter of the individual section was screened by superimposing a grid of 5 mm^2. Each cortical area represented in the outlines of the grid was given four different scores: Score 0: no positive neurons; score 1: 1–10 positive neurons; score 2: 11–40 positive neurons and score 3: >40 positive neurons. The respective scores were inserted into the map of the representative section in their exact location in the grey matter. Having determined these values for each individual patient, the results for all patients were superimposed into the respective areas of a single virtual brain section. We then determined the average number of positive neurons for the individual scores and multiplied the incidence of the scores in each given brain area with this average number. This provided a summary score of immune-reactive neurons for each cortical region, derived from all patients included in this investigation. The summary scores were then graphically transferred into different colours from light yellow to dark red, as depicted in Fig. 1. The scales in the figure show the summative number of neurons with SMI31 reactivity in a given cortical region (from 0 to 450). Note that the numbers provided here are closely similar to the numbers given from the small tissue block analysis in Fig. 2 (e.g. average number of positive cells in highly affected regions of 4.7), considering the larger size of the grid (5 mm^2) and the summation of 19 patients (4.7 × 5 × 19 = 446).

Meningeal inflammation

To determine meningeal inflammation in essence we used the method described by Magliozzi *et al.* (2007) and Howell *et al.* (2011). The presence of leucocytes was analysed in haematoxylin and eosin stained sections in the microscope at an objective magnification of 20×, resulting in the determination of leucocyte infiltrates in meningeal stretches of 0.23 mm per microscope field. Inflammation was defined by the following scores: score 0: <5 leucocytes; score 1: 5–50 leucocytes and score 3: >50 leucocytes. Transformation of the data from individual patients into the virtual lesion map followed the same principles as described above for SMI31 positive neurons. The colour code represents the summation of the numbers of leucocytes/0.23 mm^2 seen in the entire sample of 19 patients. When calculated on the basis of single patients, the values described in our present study are similar to those reported by Howell and co-authors (2011) and in our previous study based on quantification of T cells, B cells and plasma cells by immunocytochemistry (Frischer *et al.*, 2009). A much lower extent of meningeal inflammation was reported previously in the study by Kutzelnigg *et al.* (2005). In this study, however, meningeal inflammation was assessed by the presence of large inflammatory infiltrates, representing meningeal follicle-like aggregates as defined by Magliozzi *et al.* (2007). Examples of meningeal inflammatory infiltration, as determined in this study, are provided in Supplementary Fig. 3.

Collation of the lesion map

Manually quantified counts from the microscope were plotted into patients' individual scans with Adobe Photoshop™. In the next step these results were manually transposed from the individual scans to an average brain (Supplementary Fig. 1). These average brain data were then quantified with ImageJ at a resolution of 10 000 pixels/image and imported into IBM SPSS™. The raw results were then corrected for the area that the regions of interest covered on each individual patient's Klüver-Pas scan, to control for variation of section level within the mid-thalamus

and interindividual variation. Venous density was determined in a 7 T MRI venous atlas of the normal human brain (Fig. 1G) (Grabner *et al.*, 2014), based on venous-sensitive susceptibility weighted imaging. A corridor of ~1 cm adjacent to border zones of the anterior-, medial-, posterior- cerebral and anterior choroidal arterial territory was considered as watershed area (Fig. 1I) (van der Zwan and Hillen, 1991). Iron content in different brain regions was depicted by Turnbull staining (Fig. 1H) (Hametner *et al.*, 2013). Structures in the fissura longitudinalis cerebri, in the sulcus lateralis and individual sulci were considered as areas with low CSF flow (Fig. 1I).

Quantification of retrograde neuro-degeneration and oxidative injury in standard tissue sections: Sample 2

To determine the relation between oxidative neuronal injury or retrograde neuronal degeneration with demyelination and meningeal inflammation, we analysed sections from small tissue blocks, counted the number of neurons with immunoreactivity for oxidized phospholipids (E06) or phosphorylated neurofilament. The immunostained sections were overlaid by a morphometric grid (á 0.0576 mm^2). Regions of interest were: normal-appearing grey matter (in controls/multiple sclerosis), leucocortical lesions (extending over the cortex-white matter border), subpial lesions and normal-appearing cortex adjacent to large subcortical white matter lesions. The activity of cortical lesions was characterized by the presence of PLP-reactive myelin degradation products in macrophages or microglia (Fischer *et al.*, 2013). Neuronal cells were defined by nuclear morphology in the haematoxylin counterstaining. Neurons with phosphorylated neurofilament-reactive somata were manually counted in 10 fields per region of interest. Neurons with signs of E06 reactivity were divided into three groups according to Fischer *et al.* (2013) (Fig. 2). Average counts per square millimetre were calculated for each region of interest per case and compared by statistical analysis.

Statistical analysis

Statistical analysis was performed with IBM SPSS™. Due to uneven distribution of our data, non-parametric tests were applied. Descriptive statistics included median value and range. Differences between two groups were assessed with Wilcoxon Mann Whitney U-tests. In case of multiple testing (comparison of more than two groups), significant values were corrected with the Bonferroni procedure. Interdependence of variables was evaluated by the Spearman non-parametric correlation test. The reported *P*-values are results of two-sided tests. A *P*-value $\leqslant 0.05$ was considered statistically significant.

Results

Topographical distribution of demyelinated lesions in the multiple sclerosis brain

Demyelinated lesions in the cerebral cortex

As reported previously, cortical demyelination was dominated by widespread subpial lesions (Bo *et al.*, 2003;
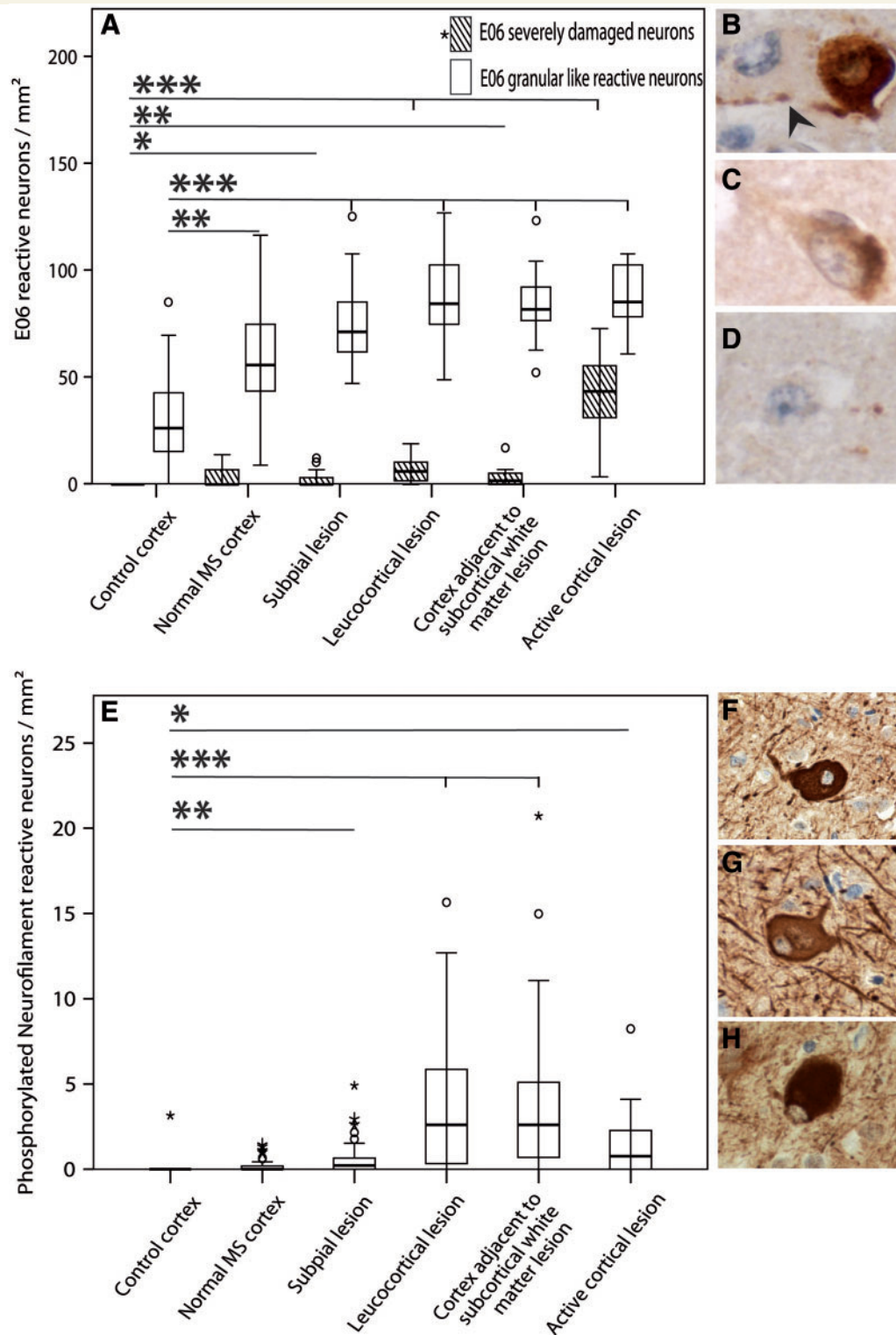
Figure 2 Neurodegeneration in the multiple sclerosis cortex. Oxidative injury in cortical neurons is reflected by two different neuronal changes. (**A–D**) The first is the intense and diffuse immunoreactivity for oxidized phospholipids (E06 immunoreactivity) in neurons (**B**), associated with beading and fragmentation of cell processes (indicated by the arrowhead) and neuronal apoptosis (Fischer *et al.*, 2013). This is mainly seen in active cortical lesions, but in lower frequency also in inactive lesions and the normal-appearing multiple sclerosis cortex (grey bars in **A**). The second is reflected by a granular, lipofuscin-like immunoreactivity in neurons (**C**). This is significantly more frequent (white bars in **A**) in the multiple sclerosis cortex compared to controls, but within multiple sclerosis its incidence is not significantly different between active and inactive lesions. (**D**) A normal neuron without immunoreactivity for oxidized phospholipids in the multiple sclerosis cortex. (**E–H**) Retrograde neuro-degeneration is reflected by the cytoplasmic accumulation of phosphorylated neurofilaments (**F**). This is frequently associated with ballooning of neurons and an eccentric location of the nerve cell nucleus (**G** and **H**). Retrograde neurodegeneration is most prominently seen in the cortex adjacent to demyelinated lesions with axonal transection in the white matter (**E**). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Table 1 Quantitative evaluation of demyelination and neurodegeneration in multiple sclerosis and control brains in relation to watershed areas and cortical invaginations

	Watershed	Non-watershed	Invaginations	Surface
MS cortex DM	15.5 (3.4–71.3)	8.2 (2–30)	**17.3 (4.9–49.5)**	5.5 (2.7–27.3)
MS cortex ND	**43 (16.3–51.1)**	8 (4.7–12.1)	**20.3 (12.4–33.4)**	2.3 (1.4–3.6)
MS WM DM	**31.3 (18.4–62)**	12.1 (4.9–22.6)	n.a.	n.a.
MS DGM DM	**25 (11.6–55.8)**	11.7 (7.4–16.5)	n.a.	n.a.
MS DGM ND	**83.7 (28.9–115.8)**	11 (4.3–24.3)	n.a.	n.a.
CO cortex ND	0.42 (0–3.1)	0.14 (0–1.6)	0.7 (0–3.4)	0 (0–0.3)
CO DGM ND	0 (0–1.6)	0 (0–0.4)	n.a.	n.a.

Demyelination and neurodegeneration are significantly more pronounced (bold numbers in the table) in watershed areas and within the cortex in invaginations of the brain surface, as defined in Fig. 1I.

Demyelination is expressed as the number of pixels with demyelination in a region of interest, divided by the total area of this region of interest of the same patient.

Neurodegeneration is expressed as the number of pixels with retrograde neurodegeneration (correcting for the levels low, intermediate and severe), divided by the total grey matter area of the same patient.

All P-values result of a two-sided Man-Whitney U-test and were subjected to Bonferroni correction for multiple testing.

MS = multiple sclerosis; CO = controls; WM = white matter; DGM = deep grey matter (basal ganglia, thalamus, hypothalamus); DM = demyelination; ND = retrograde neurodegeneration.

Kutzelnigg *et al.*, 2005), while intracortical and cortico-subcortical lesions were less frequent (Fig. 1A). Demyelinated lesions were seen along the entire cortical ribbon, but their incidence and size were significantly larger in cortical sulci, and in deep invaginations of the brain surface, such as the cingulate gyrus and in the insular cortex (Fig. 1A and Table 1). The topographical distribution of meningeal inflammatory infiltrates, which were increased in cerebral sulci and in deep invaginations of the brain surface (Supplementary Fig. 3), was significantly associated with subpial cortical demyelination (R = 0.646; P = 0.003) (Fig. 1A and E).

Demyelinated lesions in the white matter

The highest frequency of lesions was seen in the periventricular white matter, but in addition the lesions accumulated in a fan-shaped pattern expanding from the periventricular region towards the deep and juxta-cortical white matter (Fig. 1A). As it can be seen from the venous density map of the brain (Fig. 1G), areas with a high venous density are likely to harbour demyelinated white matter lesions, but not all brain areas with high venous density are equally affected. However, precipitation of focal white matter lesions occurs in the border areas of the territories of blood supply from the major cerebral arteries (the so-called watershed areas) (Fig. 1A and I; Table 1).

No focal demyelinated lesions were observed in the white or grey matter of control patients. However, 43% of control patients showed areas of variable size with diffuse reduction of myelin, representing leukoaraiosis or small vessel disease of the white matter, which is typically present in a subset of aged control patients (Fig. 1B and Supplementary Fig. 2).

Oxidative injury in cortical neurons

In our study we distinguished between two different patterns of neurodegeneration. The first was oxidative injury, reflected by the cytoplasmic accumulation of oxidized phospholipids (Fig. 2A–D). They were either present in granular form, resembling lipofuscin granules (Fig. 2C), or in a diffuse distribution within the entire cytoplasm of neurons and dendrites. The latter was associated with dendritic beading and fragmentation, suggesting active neurodegeneration in the course of oxidative stress (Fig. 2B). The highest incidence of neurons with diffuse cytoplasmic accumulation of oxidized phospholipids was seen in actively demyelinating cortical lesions (Fig. 2A), characterized by the presence of abundant meningeal inflammation, microglia activation at the lesion edges and the presence of macrophages with early myelin degradation products at sites of active demyelination in the cortex (Fischer *et al.*, 2013). In addition, we found neurons with diffuse or granular accumulation of oxidized phospholipids also in inactive cortical lesions, irrespective of their type and independent from the presence of subcortical demyelinated lesions (Fig. 2A). Neurons with accumulation of oxidized phospholipids were also present in the normal appearing grey matter of multiple sclerosis patients (Fig. 2A). The incidence of neurons with oxidative injury was significantly higher in the multiple sclerosis cortex in comparison to controls and correlated with inflammation in the meninges, covering the respective cortical areas (R = 0.469; P = 0.008).

Retrograde neurodegeneration in cortex and deep grey matter nuclei

The second pattern of neuronal pathology represents retrograde neurodegeneration (Fig. 2E–H). When axons are transected, their associated neuronal cell bodies react with a morphological change called central chromatolysis. This process is characterized by the accumulation of phosphorylated neurofilament protein within the perinuclear and dendritic neuronal cytoplasm, an increase in cytoplasmic volume and an eccentric dislocation of the neuronal nucleus (Fig. 2F–H) (Koliatsos et al., 1989; Martin et al., 1999).

Overall, we found an increased number of neurons with retrograde degeneration in the multiple sclerosis cortex in comparison to the cortex of age-matched controls (Fig. 2E). The highest incidence of neurons with cytoplasmic accumulation of phosphorylated neurofilaments was present within cortico-subcortical lesions and in normal-appearing cortex adjacent to subcortical white matter plaques. In contrast, only very few neurons with central chromatolysis were seen in subpial lesions (Fig. 2E).

We then used a similar approach in entire double hemispheric brain sections to evaluate the global incidence of retrograde degeneration in the brain (Fig. 1C). The highest incidence of neurons with cytoplasmic accumulation of phosphorylated neurofilaments was present in deep grey matter nuclei (mainly in the thalamus and the globus pallidus) and in the depth of cortical sulci, mainly affecting neurons in the fifth and sixth cortical layer. Neurons with signs of retrograde degeneration were topographically related to the location of demyelinated lesions in the white matter (Fig. 1A and C) and, thus, also accumulated in watershed areas (Table 1). In addition, we found a much higher incidence of neurons with retrograde neurodegeneration in cortical sulci compared to cortical gyri (Fig. 1C and Table 1) and a substantial number of these neurons also contained oxidized phospholipids in their cytoplasm (Fischer et al., 2013).

In comparison to age-matched controls, we found a significantly increased incidence of neurons with retrograde degeneration in multiple sclerosis patients, but the topographical distribution of affected neurons was similar in controls compared to that seen in multiple sclerosis patients (Fig. 1C and D). In controls, retrograde neurodegeneration was topographically related to diffuse periventricular white matter abnormalities (Fig. 1B and D).

Discussion

Our data on lesion topography in the multiple sclerosis brain suggest that several different factors contribute to their formation (Mahad et al., 2015). In line with current concepts, inflammation, microglia activation, oxidative injury and energy deficiency due to mitochondrial damage may be key factors, but their relative contribution may differ depending upon type and location of the lesions.

Earliest studies on multiple sclerosis pathology have already established that white matter lesions are formed around inflamed veins (Rindfleisch, 1863) and this is confirmed by recent studies using high field MRI (Tallantyre et al., 2008). Similarly, cortical lesions are associated with perivenous inflammation in early and aggressive multiple sclerosis cases (Lucchinetti et al., 2011) and active subpial demyelination was found to be related to inflammation in meninges (Kutzelnigg et al., 2005; Howell et al., 2011; Choi et al., 2012) and were most frequent in invaginations of the brain surface (Kutzelnigg and Lassmann, 2006). Their location, shape and patterns of active demyelination are compatible with the view that they are driven by a soluble factor, produced in the inflammatory infiltrates of the meninges, which diffuses into the cortex and triggers demyelination either directly or indirectly through microglia activation. Demyelinating and/or cytotoxic activity has been observed in the CSF of multiple sclerosis patients and has been ascribed to specific autoantibodies or other cytotoxic molecules (Lisak et al., 2012; Vidaurre et al., 2014). In addition, essentially similar cortical lesions have been induced in experimental models in rats and primates, associated with meningeal inflammation and the presence of demyelinating antibodies directed against myelin oligodendrocyte glycoprotein (Pomeroy et al., 2005; Storch et al., 2006). There is a flow of CSF within the arachnoid compartment of the meninges at the outer surface of the brain hemispheres (Abbott, 2004), which is likely to be dynamically restricted in the cerebral sulci and deep invaginations of the brain surface in the insular and cingulate cortex. This may explain the preferential accumulation of inflammation and subpial cortical demyelination at these sites.

Recent data suggest that oxidative injury at least in part mediates demyelination and neurodegeneration in the multiple sclerosis brain. Oxidative injury can trigger mitochondrial dysfunction and subsequent energy failure, a process termed histotoxic or 'virtual' hypoxia (Aboul-Enein et al., 2003; Trapp and Stys, 2009). This process may be amplified by genuine hypoxia through increased energy consumption around inflamed vessels, and the consequence of energy failure is amplified in areas with low arterial perfusion and oxygen supply (Davies et al., 2013). This may explain the accumulation of lesions in the multiple sclerosis brain in the so-called watershed areas (Brownell and Hughes, 1962). Our findings are in parallel with previous in vivo imaging studies showing that in patients with SPMS, lesions tend to accumulate in areas of low perfusion, while this is not seen in early disease stages (Holland et al., 2012). These data indicate that in early multiple sclerosis lesions are formed at any site of the brain, but that some of these early lesions disappear due to resolution of oedema and remyelination, while those located in areas of low blood perfusion persist due to the higher degree of tissue damage (Holland et al., 2012). In addition, oxidative injury and mitochondrial damage is amplified in the progressive stages of the disease by factors unrelated to the

inflammatory process, such as the age and disease burden-related microglia activation and mitochondrial gene deletions or accumulation of iron in the ageing human brain (Mahad *et al.*, 2015). Thus, low blood perfusion and oxygen tension may amplify tissue damage more severely in lesions formed in the progressive stage than in those arising at earlier phases of the disease. However, different patterns of tissue injury have been seen in patients with early (acute) multiple sclerosis (Lucchinetti *et al.*, 2000) and one of those is associated with profound oxidative and mitochondrial injury (Aboul-Enein *et al.*, 2003; Mahad *et al.*, 2008). It is likely that in such cases a similar relation between brain blood perfusion and lesion topography is present, but so far the low number of autopsy cases available for pathological analysis precluded a systematic comparison of lesion location between these cases and those following different patterns of demyelination.

Regarding neurodegeneration, we identified two different patterns in the multiple sclerosis brain. Acute nerve cell injury, characterized by dendritic and axonal fragmentation and cell changes of apoptosis or necrosis, was associated with accumulation of oxidized lipids and DNA in affected cells (Fischer *et al.*, 2013). In our study this was mainly seen in actively demyelinating lesions, less frequently in inactive cortical lesions and rarely in the normal appearing cortex. In addition, we found profound accumulation of lipofuscin-like granules, which mainly contain oxidized lipids and proteins (Höhn and Grune, 2013), within neurons and glia in the entire multiple sclerosis cortex. The significant correlation between the number of cortical neurons with accumulation of oxidized lipids and meningeal inflammation indicates that this type of neurodegeneration may be in part driven by oxidative stress mediated by activated macrophages and microglia.

The second process of neurodegeneration in the multiple sclerosis brain revealed cellular changes which are typical for neurons after axonal transection (Koliatsos *et al.*, 1989; Martin *et al.*, 1999). They were mainly present in the cortex overlying subcortical demyelinated lesions, while they were rare within intracortical subpial lesions. Furthermore, their global distribution in the brain suggests that neurons with signs of retrograde degeneration were most frequent in cortical areas, which are topographically related to areas with a high probability to harbour white matter lesions (Kolasinski *et al.*, 2012). The low incidence of such changes in subpial cortical lesions can be explained by the low incidence of axonal transection in comparison to that in white matter lesions (Frischer *et al.*, 2009) and by the parallel loss of neurons and axons within these lesions (Schmierer *et al.*, 2010; Klaver *et al.*, 2015). Interestingly, a similar topographical distribution of neurons with retrograde degeneration, although much less numerous, was present in the cortex of controls. Aged controls often show diffuse periventricular white matter damage (leukoaraiosis), which is associated with axonal transection (Brown and Thore, 2011). Thus, periventricular white matter damage in patients with progressive multiple sclerosis is not only

due to axonal transection in plaques, but also to age-related injury. It is currently not possible to differentiate the contribution of each of these mechanisms to the global extent of damage in the periventricular normal-appearing white matter. It has, however, to be noted that the white matter injury in leukoarayosis also occurs at sites of low physiological blood perfusion in the brain and appears to be driven by age-related pathological changes in the long penetrating arteries providing the blood supply in these areas (Brown and Thore, 2011).

Neurons with retrograde degeneration tended to be located in the deeper cortical layers (layers 5 and 6), and they were mainly present in cortical sulci. We have shown previously that neurons with signs of retrograde degeneration frequently contained high amounts of oxidized phospholipids (Fischer *et al.*, 2013) and neurons with mitochondrial gene deletions are also predominantly seen in the same cortical layers (Campbell *et al.*, 2011). Retrograde neurodegeneration is accomplished by apoptosis and associated with oxidative damage of neuronal proteins and nucleic acids (Martin *et al.*, 1999). In addition, it has been suggested that oxidative injury in white matter plaques leads to mitochondrial gene deletions and that the defective mitochondria are transported in a retrograde manner into the cell body of cortical neurons. This process may further amplify oxidative injury by electron leakage from defective mitochondria (Mahad *et al.*, 2015).

Our results may have consequences for MRI studies focusing on brain atrophy. Brain atrophy is thought to be a useful paraclinical outcome measure for the evaluation of the effect of neuroprotective treatments (Filippi *et al.*, 2014). Careful analysis of regional atrophy in the brain may further improve the diagnostic and predictive value of MRI. Our current study may provide the neuropathological basis for regional stratification of atrophy analysis.

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Supplementary material

Supplementary material is available at *Brain* online.

References

Abbott NJ. Evidence for bulk flow of brain interstitial fluid: significance for physiology and pathology. Neurochem Int 2004; 45: 545–52.

Aboul-Enein F, Rauschka H, Kornek B, Stadelmann C, Stefferl A, Bruck W, et al. Preferential loss of myelin-associated glycoprotein reflects hypoxia-like white matter damage in stroke and inflammatory brain diseases. J Neuropathol Exp Neurol 2003; 62: 25–33.

Bo L, Vedeler CA, Nyland H, Trapp BD, Mork SJ. Intracortical multiple sclerosis lesions are not associated with increased lymphocyte infiltration. Mult Scler 2003; 9: 323–31.

Brown WR, Thore CR. Review: cerebral microvascular pathology in ageing and neurodegeneration. Neuropathol Appl Neurobiol 2011; 37: 56–74.

Brownell B, Hughes JT. The distribution of plaques in the cerebrum in multiple sclerosis. J Neurol Neurosurg Psychiatry 1962; 25: 315–20.

Campbell GR, Ziabreva I, Reeve AK, Krishnan KJ, Reynolds R, Howell O, et al. Mitochondrial DNA deletions and neurodegeneration in multiple sclerosis. Ann Neurol 2011; 69: 481–92.

Choi SR, Howell OW, Carassiti D, Magliozzi R, Gveric D, Muraro PA, et al. Meningeal inflammation plays a role in the pathology of primary progressive multiple sclerosis. Brain 2012; 135 (Pt 10): 2925–37.

Davies AL, Desai RA, Bloomfield PS, McIntosh PR, Chapple KJ, Linington C, et al. Neurological deficits caused by tissue hypoxia in neuroinflammatory disease. Ann Neurol 2013; 74: 815–25.

Desai R, Davies A, Kasti M, Laulund F, Smith K. Demyelinating lesions in multiple sclerosis: experimental evidence indicating why, how and where they may form. Mult Scler J 2014; 20 (Suppl 1): 56.

Filippi M, Preziosa P, Rocca MA. Magnetic resonance outcome measures in multiple sclerosis trials: time to rethink?. Curr Opin Neurol 2014; 27: 290–9.

Fischer MT, Wimmer I, Hoftberger R, Gerlach S, Haider L, Zrzavy T, et al. Disease-specific molecular events in cortical multiple sclerosis lesions. Brain 2013; 136 (Pt 6): 1799–815.

Frischer JM, Bramow S, Dal-Bianco A, Lucchinetti CF, Rauschka H, Schmidbauer M, et al. The relation between inflammation and neurodegeneration in multiple sclerosis brains. Brain 2009; 132 (Pt 5): 1175–89.

Grabner G, Dal-Bianco A, Hametner S, Lassmann H, Trattnig S. Group specific vein-atlasing: an application for analyzing the venous system under normal and multiple sclerosis conditions. J Magn Reson Imaging 2014; 40: 655–61.

Haider L, Fischer MT, Frischer JM, Bauer J, Hoftberger R, Botond G, et al. Oxidative damage in multiple sclerosis lesions. Brain 2011; 134 (Pt 7): 1914–24.

Hametner S, Wimmer I, Haider L, Pfeifenbring S, Bruck W, Lassmann H. Iron and neurodegeneration in the multiple sclerosis brain. Ann Neurol 2013; 74: 848–61.

Höhn A, Grune T. Lipofuscin: formation, effects and role of macroautophagy. Redox Biol 2013; 1: 140–4.

Holland CM, Charil A, Csapo I, Liptak Z, Ichise M, Khoury SJ, et al. The relationship between normal cerebral perfusion patterns and white matter lesion distribution in 1 249 patients with multiple sclerosis. J Neuroimaging 2012; 22: 129–36.

Howell OW, Reeves CA, Nicholas R, Carassiti D, Radotra B, Gentleman SM, et al. Meningeal inflammation is widespread and linked to cortical pathology in multiple sclerosis. Brain 2011; 134 (Pt 9): 2755–71.

Klaver R, Popescu V, Voorn P, Galis-de Graaf Y, van der Valk P, de Vries HE, et al. Neuronal and axonal loss in normal-appearing gray matter and subpial lesions in multiple sclerosis. J Neuropathol Exp Neurol 2015; 74: 453–8.

Kolasinski J, Stagg CJ, Chance SA, Deluca GC, Esiri MM, Chang EH, et al. A combined post-mortem magnetic resonance imaging and quantitative histological study of multiple sclerosis pathology. Brain 2012; 135 (Pt 10): 2938–51.

Koliatsos VE, Applegate MD, Kitt CA, Walker LC, DeLong MR, Price DL. Aberrant phosphorylation of neurofilaments accompanies transmitter-related changes in rat septal neurons following transection of the fimbria-fornix. Brain Res 1989; 482: 205–18.

Kutzelnigg A, Lassmann H. Cortical demyelination in multiple sclerosis: a substrate for cognitive deficits?. J Neurol Sci 2006; 245: 123–6.

Kutzelnigg A, Lucchinetti CF, Stadelmann C, Bruck W, Rauschka H, Bergmann M, et al. Cortical demyelination and diffuse white matter injury in multiple sclerosis. Brain 2005; 128 (Pt 11): 2705–12.

Lassmann H, Bruck W, Lucchinetti CF. The immunopathology of multiple sclerosis: an overview. Brain Pathol 2007; 17: 210–18.

Lisak RP, Benjamins JA, Nedelkoska L Barger JL, Ragheb S, Fan B, et al. Secretory products of multiple sclerosis B-cells are cytotoxic to oligodendroglia in vitro. J Neuroimmunol 2012; 246: 85–95.

Lucchinetti C, Brück W, Parisi J, Scheithauer B, Rodriguez M, Lassmann H. Heterogeneity of multiple sclerosis lesions: Implications for the pathogenesis of demyelination. Ann Neurol 2000; 47: 707–17.

Lucchinetti CF, Popescu BF, Bunyan RF, Moll NM, Roemer SF, Lassmann H, et al. Inflammatory cortical demyelination in early multiple sclerosis. N Engl J Med 2011; 365: 2188–97.

Magliozzi R, Howell O, Vora A, Serafini B, Nicholas R, Puopolo M, et al. Meningeal B-cell follicles in secondary progressive multiple sclerosis associate with early onset of disease and severe cortical pathology. Brain 2007; 130 (Pt 4): 1089–104.

Mahad D, Ziabreva I, Lassmann H, Turnbull D. Mitochondrial defects in acute multiple sclerosis lesions. Brain 2008; 131 (Pt 7): 1722–35.

Mahad DH, Trapp BD, Lassmann H. Pathological mechanisms in progressive multiple sclerosis. Lancet Neurol 2015; 14: 183–93.

Marburg O. Die sogenannte akute multiple Sklerose. Jahrb Psychiatrie; 1906; 27: 211–312.

Martin LJ, Kaiser A, Price AC. Motor neuron degeneration after sciatic nerve avulsion in adult rat evolves with oxidative stress and is apoptosis. J Neurobiol 1999; 40: 185–201.

Murphy MP. How mitochondria produce reactive oxygen species. Biochem J 2009; 417: 1–13.

Pomeroy IM, Matthews PM, Frank JA, Jordan EK, Esiri MM. Demyelinated neocortical lesions in marmoset autoimmune encephalomyelitis mimic those in multiple sclerosis. Brain 2005; 128: 2713–21.

Rindfleisch E. Histologisches Detail zu der grauen Degeneration von Gehirn und Rückenmark. (Zugleich ein Beitrag zu der Lehre von der Entstehung und Verwandlung der Zelle.). Virchows Arch 1863; 26: 474–83.

Schmierer K, Parkes HG, So PW, An SF, Brandner S, Ordidge RJ, et al. High field (9.4 Tesla) magnetic resonance imaging of cortical grey matter lesions in multiple sclerosis. Brain 2010; 133 (Pt 3): 858–67.

Storch MK, Bauer J, Linington C, Olsson T, Weissert R, Lassmann H. Cortical demyelination can be modeled in specific rat models of autoimmune encephalitis and is major histocompatibility complex (MHC) haplotype related. J Neuropath Exp Neurol 2006; 65: 1137–42.

Tallantyre EC, Brookes MJ, Dixon JE, Morgan PS, Evangelou N, Morris PG. Demonstrating the perivascular distribution of MS lesions in vivo with 7-Tesla MRI. Neurology 2008; 70: 2076–8.

Trapp BD, Stys PK. Virtual hypoxia and chronic necrosis of demyelinated axons in multiple sclerosis. Lancet Neurol 2009; 8: 280–91.

van der Zwan A, Hillen B. Review of the variability of the territories of the major cerebral arteries. Stroke 1991; 22: 1078–84.

Vidaurre OG, Haines JD, Katz Sand I, Adula KP, Huynh JL, McGraw CA, et al. Cerebrospinal fluid ceramides from patients with multiple sclerosis impair neuronal bioenergetics. Brain 2014; 137 (Pt 8): 2271–86.

Witte ME, Geurts JJ, de Vries HE, van der Valk P, van Horssen J. Mitochondrial dysfunction: a potential link between neuroinflammation and neurodegeneration?. Mitochondrion 2010; 10: 411–18.