Heterogeneity of the immunopathology in advanced multiple sclerosis

An autopsy cohort analysis

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CHAPTER 2

Inflammation is associated with demyelination and neurodegeneration in the brainstem of patients with progressive multiple sclerosis

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In prep
ABSTRACT

In progressive multiple sclerosis (MS), demyelinated lesion load in the brainstem strongly correlates with disease severity, but we know surprisingly little about the immunopathology of these lesions. Here we investigated the lesional activity, the extent of inflammation and neurodegenerative changes in 38 staged brainstem lesions from 9 progressive MS cases, compared to normal-appearing MS brainstem, 6 donors with other neurodegenerative diseases and 3 non-neurological controls. We found that 74% of MS brainstem lesions analyzed are active or mixed active/inactive (mixed). In mixed lesions, neurons and axons showed increased metabolic stress, disturbed axonal transport, reduced axonal density and loss of synapses. C1q deposits were detected at synapses while C3d was found on neuronal perikarya and focally on axons, a feature shared with other neurodegenerative diseases. MAC immunoreactivity, identified within phagocytic microglia/macrophages and on astrocytes, was a specific feature of MS. A dominance of CD8+ T cells was detected in mixed lesions but CD4+ T cells were also seen (CD8:CD4 ratio, ~3:1), and both infiltrated the parenchyma with some CD8+ T cells found in close association to neurons. CD20+ B cells were detected in mixed lesions but were more rare compared to T cells (CD3:CD20 ratio, ~7:1) and unlike T cells they localized perivascularly. CD138+ plasma cells, although scarce, infiltrated the tissue. Importantly, inflammation associated significantly with injury and loss of axons. Altogether, we report substantial inflammation in the brainstem of progressive MS patients which closely associates with demyelination and neurodegeneration. While deposits of early complement components in both neurodegenerative diseases and MS point to shared mechanisms of complement-mediated injury, MAC deposition may constitute a specific feature of the inflammatory response with pathogenic significance in MS.
INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory and demyelinating disease of the central nervous system (CNS) with a neurodegenerative component.\textsuperscript{16,17,30,66} Although MS has long been considered a disease of the white matter, several studies have now highlighted the involvement of grey matter demyelination in the supratentorial cortex\textsuperscript{10,38,41,59} and in the deep grey matter nuclei including the hippocampus,\textsuperscript{29} the hypothalamus\textsuperscript{35} and the brainstem.\textsuperscript{48} While the immunopathology of cortical demyelination has received widespread attention in recent years,\textsuperscript{6,33,34,47,51,52,67,68} we know surprisingly little about lesions in the deep grey matter nuclei, yet they are of interest since they appear to contribute considerably to the neurological disabilities of MS patients.\textsuperscript{14,19,36,46,53} The brainstem is a deep grey matter structure particularly relevant in MS because brainstem atrophy, a measure of neurodegeneration, occurs from the earliest stages of the disease and is an important predictor of clinical disability.\textsuperscript{19} Brainstem involvement in MS can be manifested with different symptoms, among which diplopia is the most common (68%) followed by facial sensory symptoms (32%), unstable gait (30.7%) and vertigo (18.7%).\textsuperscript{31} Notably, our recent study using the autopsy MS cohort of the Netherlands Brain Bank (NBB), has shown that the brainstem grey and white matter are heavily demyelinated, and the lesion load strongly correlates with disease severity.\textsuperscript{48} However, the lesional activity, the extent of inflammation and changes to neurons, axons and synapses in these lesions are currently unknown. It is also unknown whether demyelination and neurodegeneration in the MS brainstem progress independently from inflammation or whether these processes are linked.

Using post-mortem MS brainstem tissue collected at rapid autopsy, we first staged lesions based on demyelination – detected with the proteolipid protein (PLP) marker of myelin – and microglia/macrophage accumulation, distribution and morphology – detected with the human leukocyte antigen (HLA) marker of myeloid cells. Across all lesional stages of the MS brainstem, we analyzed neurodegenerative changes including metabolic stress (mtHSP70), axonal transection/impaired transport (SMI312, APP) and synaptic alterations (synaptophysin) as well as the localization and extent of deposits of early (C1q, C3d) and terminal (MAC) complement factors, and the localization and density of T (CD3\textsuperscript{+}, CD4\textsuperscript{+}, CD8\textsuperscript{+}) and B (CD20\textsuperscript{+}, CD138\textsuperscript{+}) lymphocytes. Findings in the MS brainstem were compared to brainstem tissue of non-neurological controls and controls with neurodegenerative diseases. Finally, we correlated the extent of inflammation with the extent of neurodegenerative changes at neurons, axons and synapses. Understanding the type of lesional activity, the extent of inflammation and neurodegeneration, and the relationship between these processes in the brainstem of progressive MS patients, will inform on the mechanisms leading to brainstem injury in progressive disease and could guide development of therapies aimed at slowing deterioration of clinical symptoms related to this region.
METHODS

Post-mortem brainstem tissue and inclusion criteria

All paraffin-embedded or fresh frozen brainstem tissue blocks were collected at rapid (average PMD 7h:19m) autopsy by the Netherlands Brain Bank (NBB, Amsterdam, The Netherlands) from 4 standardized fronto-caudal locations in the brainstem (nigra, pons/locus coeruleus, pons/colliculus inferior, medulla oblongata). Brainstem tissue was collected from 9 MS donors, 3 non-neurological control (NNCo) donors and 6 control donors with other neurodegenerative diseases (referred to as neurodegenerative controls, NDCo). The diagnoses for MS or neurodegenerative diseases were confirmed by a certified neuropathologist. Donors diagnosed with neuromyelitis optica, known to trigger complement activation and preferentially affect the brainstem, were excluded. NNCo included 3 cases without neurological diseases. NDCo included 3 cases of Alzheimer’s disease (AD, Braak 6, female: male 3:0; age 54–60 years), 2 cases of frontotemporal dementia (FTD, female: male 2:0; age 61–63 years) and 1 case of amyotrophic lateral sclerosis (ALS, female; age 64 years). The brainstem of donors with other neurological disease showed disease-associated pathological changes such as amyloid β plaque, neurofibrillary tangles (not shown). Multiple sclerosis samples comprised 6 cases with SPMS and 3 cases with PPMS. Since patients with PPMS and SPMS share very similar pathological changes in the brain and spinal cord, for the purpose of this study they were considered as one clinical group of progressive MS donors. Of note, the MS cases analyzed included a higher number of males compared to NDCo cases in which females predominated. Detailed clinical and demographic data of all donors are provided in Table 1 and Suppl. Table 1. NBB obtained permission from donors for brain autopsy and the use of tissue and clinical information for research purposes as part of a program approved by the Ethical Committee VU University Medical Center (Amsterdam, The Netherlands).

Table 1. Overall clinical and demographic data of MS donors and controls.

<table>
<thead>
<tr>
<th>Cases (n)</th>
<th>Age (years)</th>
<th>Sex (M/F)</th>
<th>pH value</th>
<th>Brain weight (g)</th>
<th>Disease duration (years)</th>
<th>Disease course</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiple sclerosis</td>
<td>9</td>
<td>58 ± 11,5</td>
<td>8/1</td>
<td>6,5 ± 0,3</td>
<td>1260 ± 112,6</td>
<td>27,1 ± 13,2</td>
</tr>
<tr>
<td>Neurodegenerative controls</td>
<td>6</td>
<td>59,8 ± 3,8</td>
<td>0/6</td>
<td>6,25 ± 0,2</td>
<td>1008,8 ± 100,4</td>
<td>–</td>
</tr>
<tr>
<td>Non-neurological controls</td>
<td>3</td>
<td>51,7 ± 17,0</td>
<td>2/1</td>
<td>6,43 ± 0,1</td>
<td>1390,7 ± 192,5</td>
<td>–</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD (standard deviation).

F, female; M, male; PMD, post-mortem delay; PP, primary progressive; SP, secondary progressive.

Neuropathology techniques

Paraffin or frozen sections from each tissue block were stained by standard histological methods with hematoxylin and eosin (HE) and luxol fast blue (LFB) myelin stain and by immunohistochemistry.
for proteolipid protein (PLP) as a marker of myelin and human leukocyte antigen (HLA-DP-DQ-DR) as a marker of microglia/macrophages. Since brainstem lesions are almost always mixed grey/white matter lesions, the lesional activity was inferred from the white matter portion of the lesion and staged as reactive, active, mixed active/inactive and inactive, according to the classification routinely used at the NBB and the latest international consensus as previously published. Normal-appearing brainstem was defined as a region of the brainstem located at least 1 cm away from demyelinated plaques and reactive lesions. Lesions were staged by two independent observers (NLF and VR). All lesions and randomly selected areas of normal-appearing brainstem underwent immunohistochemical and quantitative analysis (see below).

Selection of markers for inflammation and neurodegeneration
To evaluate the involvement of innate immunity in the brainstem, we determined the accumulation, distribution and morphology of microglia/macrophages across the lesions and normal-appearing tissue using an antibody against the human leukocyte antigen (HLA). In addition, we evaluated the deposition of key components of the complement system, using antibodies against the recognition protein and initiator of the classical complement pathway (C1q), the cell-bound product of activation of the common pathway complement component C3 (C3d) and the neo epitope of C9 that is formed when a polymer of C9 is incorporated into the membrane attack complex (MAC) as a result of terminal complement activation. To determine the basic composition of adaptive immune cellular infiltrates we used antibodies against all T cells (CD3), against MHC class I restricted T cells (CD8α), MHC class II restricted T cells (CD4) and B cells (CD20). Plasma cells were identified by their enlarged cytoplasm and expression of CD138. To determine evidence of neurodegeneration, we used an antibody against a mitochondrial stress response molecule (mitochondrial heat shock protein 70, mtHSP70) because impaired energy metabolism has been detected in axons within active and inactive white matter lesions and has been proposed as a possible cause of neuronal injury in MS. In addition, we used an antibody against a fast axonal transport protein (amyloid precurson protein, APP) that accumulates focally in swollen axons and transected axons forming axonal spheroids or end-bulbs was used as measure of axonal injury since these features may persist at sites of damage for an extended period. An antibodies against a neurofilament (SMI312) was used to visualize axons, including axonal swelling. An antibody against a pre-synaptic element (synaptophysin) was used to visualize synapses as a measure of structural dis/connection of brainstem networks, since loss of synapses has been previously detected in the MS grey matter.

Immunohistochemistry
Seven micron thick paraffin or cryo sections were mounted on Superfrost Plus glass slides (Knittel Glass, Germany). Cryostat sections were fixed in formalin for 10 minutes and washed in phosphate-buffer saline (PBS, pH 7.4, Life technologies, Bleiswijk, The Netherlands). Paraffin sections were deparaffinated and rehydrated. Endogenous peroxidase activity was blocked with 0.3% H₂O₂ in methanol or incubation medium for 20 minutes as previously described. For some antigens,
epitopes were exposed by heat-induced antigen retrieval in either appropriate buffer solutions, depending on the primary antibody used (see Table 2). For both paraffin and cryostat sections, non-specific binding of antibodies was blocked using 10% Normal Goat or Horse Serum (DAKO, Glostrup, Denmark) in PBS for 20 minutes at room temperature. Primary antibodies (Table 2) were diluted in Normal Antibody Diluent (Immunologic, Duiven, The Netherlands) or incubation medium (SUMI) as previously described and incubated for 1 hour at room temperature. Detection was performed by incubating the sections in the secondary Poly-HRP-Goat anti Mouse/Rabbit/Rat IgG (Immunologic, Duiven, The Netherlands) antibodies diluted 1:1 in PBS for 30 minutes at room temperature followed by incubation in 3,3- diaminobenzidine tetrahydrochloride (DAB; Vector Laboratories, Burlingame, CA, USA) as chromogen and counterstaining with hematoxylin (Histolab, Gothenburg, Sweden) for 5 minutes. Sections stained with secondary antibody alone were included as negative controls with each test. After dehydration, slides were mounted in Pertex (Histolab). Images were captured with a light microscope (Olympus BX41TF, Zoeterwoude, The Netherlands) using the Cell D software (Olympus, Zoeterwoude, The Netherlands).

Immunofluorescence

For the immunofluorescence staining, primary antibodies were detected with FITC-conjugated goat anti-rabbit IgG or Cy3-conjugated goat anti-mouse IgG (Sigma-Aldrich, Saint Louis, MI, USA) diluted 1:200 in PBS and incubated for 1 hour at RT. After washing, sections were counterstained with 4,6-diamidine-2-phenylindole dihydrochloride (DAPI) (blue, 280nm) (Vector Laboratories), air dried and mounted in Vectashield (Vector Laboratories). Images were captured digitally with a fluorescence microscope (DM LB2; Leica Microsystems, Rijswijk, The Netherlands).

Quantification of immunohistochemistry

All sections were digitally scanned using a Philips scanner 3.1.1.2. Lesions and normal-appearing tissue were identified based on HLA and PLP staining and manually outlined using QuPath (Version: 0.2.0, University of Edinburgh, Scotland). CD3+ cells, CD4+ cells, CD8+ cells, CD20+ cells, CD138+ cells, APP+ axonal swellings and C1q+ neurons were manually counted using the points tool in QuPath. Values are expressed as counts per square millimeter. C3d+ and MAC+ deposits were manually counted on digitally photographed section using a light microscope (Olympus BX41TF, Zoeterwoude, The Netherlands) and the Cell D software (Olympus, Zoeterwoude, The Netherlands). Lesions and normal-appearing tissue were outlined and the size of the areas was measured using the “measurement” function of the Image Pro Plus 7.0 imaging software (MediaCybernetics, Rockville, MD, USA). Five to 20 fields per lesion (0.1-30mm²) and 20 to 100 fields per normal-appearing tissue (130-230mm²) were quantified. Values are expressed as counts per square millimeter. SMI312 and mtHSP70 staining were quantified as the percentage of immunopositive surface area using the positive pixel count tool in QuPath and dividing the positive pixel area in um² by the total area in um². Down sample factor was set at 5, gaussian sigma was set at 3, positive DAB threshold was set at 0.2 for SMI312 and 0.3 for mtHSP70. Values are expressed as percentage of stained area per area analyzed.
Table 2. Antibodies for immunohistochemistry.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Target</th>
<th>Host</th>
<th>Clone</th>
<th>Conc.</th>
<th>Tissue fixation</th>
<th>Product code, company</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLP (Proteolipid protein)</td>
<td>Myelin</td>
<td>Mouse</td>
<td>Plpc₁</td>
<td>1:1000</td>
<td>FFPE b</td>
<td>Bio-Rad Cat# MCA839G, RRID:AB_2237198</td>
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<tr>
<td>HLA-DP, DQ, DR (Human leukocyte antigen)</td>
<td>Microglia/macrophages</td>
<td>Mouse</td>
<td>CR3/43</td>
<td>1:100</td>
<td>FFPE b</td>
<td>Agilent Cat# M0775, RRID:AB_2313661</td>
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<tr>
<td>GFAP (Glial fibrillary acidic protein)</td>
<td>Astrocytes</td>
<td>Rabbit</td>
<td>Poly</td>
<td>1:4000</td>
<td>FF</td>
<td>Agilent Cat# Z0334, RRID:AB_10013382</td>
</tr>
<tr>
<td>CD3</td>
<td>All T cells</td>
<td>Rabbit</td>
<td>Poly</td>
<td>1:100</td>
<td>FFPE a</td>
<td>Agilent Cat# A0452, RRID:AB_2335677</td>
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<tr>
<td>CD4</td>
<td>MHC class II restricted T cells</td>
<td>Rabbit</td>
<td>EPR6855</td>
<td>1:500</td>
<td>FFPE a</td>
<td>Abcam Cat# ab133616, RRID:AB_2750883</td>
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<tr>
<td>CD8</td>
<td>MHC class I restricted T cells</td>
<td>Rabbit</td>
<td>Poly</td>
<td>1:500</td>
<td>FFPE a</td>
<td>Abcam Cat# ab4055, RRID:AB_304247</td>
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<td>CD20cy</td>
<td>B cells</td>
<td>Mouse</td>
<td>L26</td>
<td>1:100</td>
<td>FFPE a</td>
<td>Agilent Cat# M0755, RRID:AB_2282030</td>
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<td>CD138</td>
<td>Plasma cells</td>
<td>Mouse</td>
<td>B-A38</td>
<td>1:500</td>
<td>FFPE a</td>
<td>Bio-Rad Cat# MCA2459, RRID:AB_566507</td>
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<tr>
<td>IgM (Immunoglobulin M)</td>
<td>Immunoglobulins</td>
<td>Rabbit</td>
<td>Poly</td>
<td>1:16000</td>
<td>FFPE c</td>
<td>Agilent Cat# A042602, RRID:AB_578520</td>
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<tr>
<td>C1q</td>
<td>Classical pathway of complement</td>
<td>Mouse</td>
<td>34E2</td>
<td>1:100</td>
<td>FFPE c</td>
<td>Abcam, ab235454</td>
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<td>C3d</td>
<td>Common pathway of complement</td>
<td>Rabbit</td>
<td>Poly</td>
<td>1:2000</td>
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<td>Agilent Cat# A006302, RRID:AB_578478</td>
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<td>C9neo</td>
<td>Terminal pathway of complement</td>
<td>Mouse</td>
<td>aE11</td>
<td>1:100</td>
<td>FF</td>
<td>Agilent Cat# M0777, RRID:AB_2067162</td>
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<tr>
<td>mHSP70 (Mitochondrial heat shock protein 70)</td>
<td>Mitochondrial stress</td>
<td>Mouse</td>
<td>30A5</td>
<td>1:50</td>
<td>FFPE c</td>
<td>Enzo Life Sciences Cat# SPS-825E, RRID:AB_916897</td>
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<tr>
<td>APP (Amyloid precursor protein)</td>
<td>Impaired axonal transport</td>
<td>Mouse</td>
<td>22C11</td>
<td>1:300</td>
<td>FFPE c</td>
<td>Millipore Cat# MAB348, RRID:AB_94882</td>
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<tr>
<td>Synaptophysin</td>
<td>Synapses</td>
<td>Chicken</td>
<td>Poly</td>
<td>1:100</td>
<td>FFPE c</td>
<td>Synaptic Systems Cat# 101 006, RRID:AB_26222339</td>
</tr>
<tr>
<td>SMI312 (Pan-neurofilament)</td>
<td>Axons</td>
<td>Mouse</td>
<td>SMI 312</td>
<td>1:1000</td>
<td>FFPE d</td>
<td>BioLegend Cat# 837904, RRID:AB_2566782</td>
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</tbody>
</table>

a 10mM Sodium Citrate, pH 6.0; b 50 mM Tris base, 150 mM NaCl, pH 7.6; c 50mM Tris base, 150 mM NaCl, 1mM EDTA, pH 9.0; d 50 mM Tris base, 150 mM NaCl, pH 9.0; FF, fresh-frozen; FFPE, formalin-fixed paraffin-embedded; poly, polyclonal.
Statistical analysis

Comparison between MS brainstem lesions or normal-appearing tissue and brainstem from NNCo and NDCo were analyzed using Kruskal-Wallis test and Dunn’s post-hoc tests in GraphPad Prism 8.1.1. (©1992-2019 GraphPad Software, Inc.). Correlation analysis between measures of inflammation and measures of neurodegeneration in the MS brainstem was performed in R studio (Version 1.2.5033) using the psych and ggcorrplot package and using Spearman correlation coefficient. FDR multiple testing correction was applied on all correlation tests.

RESULTS

Staging of MS brainstem lesions

The collection of MS brainstem tissue blocks used in this study included a total of 38 lesions, comprising of 2 reactive, 5 active, 24 mixed active/inactive lesions (referred to in the text and figures as mixed lesions) and 7 inactive lesions, classified based on immunostaining for myelin (PLP) (Figure 1A) and microglia/macrophages (HLA) (Figure 1B). Compared to brainstem tissue of non-neurological controls, which showed normal PLP staining and HLA+ cells of resting, thinly-ramified appearance (Figure 1C-E), the normal-appearing MS brainstem showed PLP+ myelin but enhanced HLA staining (Figure 1F, G), suggesting increased microglia/macrophages reactivity in non-lesional brainstem compared to controls, as previously shown for normal-appearing white matter. In addition, we observed clusters of HLA+ microglia/macrophages throughout the normal-appearing MS brainstem, which we refer to as nodules. The nodules localized along segments of nerve fibers (Figure 1H) which, in transverse view, appear as clusters of ramified microglia/macrophages around a central core (Figure 1H’), similarly to profiles we and others previously observed in normal-appearing MS white matter. Reactive lesions were positive for PLP but showed obvious accumulation of HLA+ microglia/macrophages (Figure 1I-K). Active lesions showed profound loss of PLP staining, with microglia/macrophages infiltrating across the lesion (Figure 1L-N). In these lesions the morphology of microglia/macrophages varied from thickly ramified to foamy, indicating myelin phagocytosis (Figure 1N). Mixed lesions in the brainstem were characterized by a border of activated myeloid cells, whose morphology ranged from thickly ramified to amoeboid and foamy. The lesion core of mA/I brainstem lesions was usually hypocellular (Figure 1O-Q). Inactive lesions comprised a sharp PLP positive lesion border lacking activated microglia/macrophages and a gliotic acellular core (Figure 1R-T). Notably, in this cohort of progressive multiple sclerosis brainstem donors, we found predominantly active (n=4; 11% of all detected lesions) and mixed (n=24; 63% of all detected lesions) lesions, demonstrating that the disease remains active in the progressive stage, in line with previous reports.

Neurodegeneration in the MS brainstem

We first determined neurodegenerative changes in the MS brainstem compared to brainstem of NNCo and NDCo cases.
Figure 1. Multiple sclerosis brainstem lesions.
Representative sections of post-mortem brainstem from a donor with progressive multiple sclerosis, stained for (A) proteolipid protein (PLP) to visualize myelin, and (B) human leukocyte antigen (HLA-DP-DQ-DR) to visualize microglia. Examples of demyelinating lesions (lack of PLP staining, in A) with or without a rim of microglia (HLA-DP-DQ-DR staining, in B) are shown. (C-T) High magnification images of PLP, HLA-DP-DQ-DR or double PLP/HLA-DP-DQ-DR immunostaining of (C-E) brainstem sections from a non-neurological control and (F-T) multiple sclerosis donors. Examples of various stages of MS brainstem lesions, classified based on PLP and HLA-DP-DQ-DR staining as described in the text, are shown: (F-H) lesion free MS brainstem, (I-K) reactive site, (L-N) active lesion, (O-Q) mixed active/inactive lesion or (R-T) inactive lesion. In F, GM indicates grey matter; LWM indicates longitudinal white matter tract; TWM indicates transverse white matter tract. Scale bars in A and B, 10mm. Scale bars in the single staining PLP or HLA-DP-DQ-DR, 500μm. Scale bars in the double staining PLP/HLA-DP-DQ-DR, 50μm.
Mitochondrial heat shock protein 70 (mtHSP70)
While NNCo showed no mtHSP70+ signal (not shown), and normal-appearing brainstem showed only a few immunopositivity neurons and axons (not shown), mtHSP70+ neurons and axons were detected in NDCo (Figure 2A) and in mixed MS brainstem lesions (Figure 2B, zoom 2B), indicating metabolic stress in this cell type (PMID: 19591199).

Amyloid precursor protein (APP)
APP signal was detected in neurons of NNCo brainstem (not shown), NDCo brainstem (Figure 2C) and MS brainstem lesions (Figure 2D). In addition, mixed MS lesions showed staining of axonal swellings and axonal bulbs especially at the lesion rim (Figure 2D, zoom 2D), demonstrating impaired fast axonal transport and transected axons.8,20,26,39

SMI312
While we did not detect obvious changes of axonal density in the brainstem of NNCo (not shown) and NDCo (Figure 2E, zoom 2E), axonal density appeared reduced in most MS brainstem lesions (Figure 2F, zoom 2F) compared to brainstem of NDCo, NNCo and the normal-appearing brainstem. In addition, mixed MS lesions often showed SMI312+ axonal swelling and end-bulbs, demonstrating transected axons in the lesion rim (zoom 2F).

Synaptophysin
Compared to NNCo (not shown) and the NDCo, synaptophysin staining was evidently reduced in MS brainstem lesions (Figure 2G and H, zoom 2G and zoom 2H), indicating a loss of pre-synaptic elements.17
Quantitative analysis of mtHSP70+ staining, APP+ end-bulbs and SMI312+ axons across all lesion types compared to NNCo and NDCo demonstrated a robust axonal degenerative component to the pathology of the MS brainstem, which is most evident in the active rim of mixed lesions (normal-appearing vs mixed mean rank: mtHSP70: 16,43 vs 17,82 \( p=\text{n.s.} \); APP: 15,40 vs 32,61, \( p=0.01 \); SMI312: 35,40 vs 19,40, \( p=0.02 \); by Kruskal-Wallis and Dunn’s post-hoc test) (Figure 2I-K).

The extent of complement deposits is highest in active and mixed MS brainstem lesions
Next, we analyzed the localization and extent of complement deposits in the MS brainstem in relation to lesional activity and in comparison to NNCo and NDCo brainstem.

C1q
C1q is a pattern recognition molecule that together with the proteases C1r and C1s (the C1 complex) can trigger the classical pathway of complement activation by binding to immunoglobulins13,37 In addition, antibody-independent binding of C1q to myelin76 and to synapses56 have been described. In line with previous reports which described C1q deposits at synapses during normal ageing77 and neurodegeneration,32 we found staining for C1q (although weak) around neurons in the brainstem of NNCo (not shown) and NDCo (Figure 3A). In the MS brainstem, C1q immunoreactivity was marked around neurons in mixed lesions and showed a punctate staining pattern that is consistent with a synaptic localization (Figure 3B). To verify whether C1q deposits in mixed brainstem lesions
Figure 2. Neurodegeneration in multiple sclerosis brainstem.
Representative brainstem sections from progressive multiple sclerosis cases and neurodegenerative disease. (A and B and zoom B) Mitochondrial heat shock protein 70 (mtHSP70) visualizes metabolic stress in neurons and axons in both MS and neurodegenerative disease. (C and D and zoom D) Amyloid precursor protein (APP) visualizes disturbed axonal transport, specifically in the active rim of mixed active/inactive lesions where they show axonal end-bulbs. (E and F and zooms) Pan-neurofilament marker of axons (SMI312) visualizes axons demonstrating variable axonal loss in mixed active/inactive lesions, demonstrating also axonal end-bulbs in the active rim of the mixed active/inactive lesion. (G and H and zooms) Synaptophysin visualizes synapses, in neurodegenerative disease and mixed active/inactive lesions, showing synapse loss in MS lesions. Asterisks indicate neurons. Hematoxylin stains nuclei in blue. Staining patterns are representative of those seen in the brainstem lesions from 9 donors with progressive multiple sclerosis. Isotype-matched antibodies were used as negative controls. (I-K) Quantification of mtHSP70, APP* axons and bulbs and SMI312 in MS, neurodegenerative disease and non-neurological controls.
localized at synapses, we performed double immunofluorescent studies using the anti-C1q antibody together with an antibody against synaptophysin to label synapses (Figure 3C and 3D). We found punctate C1q deposits that localized at synaptophysin+ synapses (Figure 3 arrows in zoom C and zoom D). Although we also detected synaptophysin+ synapses that where negative for C1q (Figure 3 asterisk in zoom C and zoom D), additional C1q deposits that did not co-localize with synaptophysin+ synapses was also noted (Figure 3 arrows head in zoom C and zoom D), possibly indicating local production of C1q by neurons or microglia as we previously reported. These data suggest that C1q tags a subset of pre-synaptic terminals in the mixed lesions of the MS brainstem. Since antibodies are known to recruit C1q to the targeted tissue, we tested whether immunoglobulins (Ig) deposition could be detected in the MS brainstem. Staining for IgM and IgG (not shown) was negative in all cases except for one MS case where IgM deposits were found in the vicinity of a few neurons at the edge of a mixed lesions (Suppl. Figure 1A and zoom A). These data suggest that immunoglobulins are likely not involved in the tagging of synapses by C1q.

Quantitative analysis of C1q deposition on the perikarya of neurons across all lesion types compared to NA brainstem, NNCo and NDCo demonstrated increased deposition of C1q on neurons in the mixed MS brainstem lesions compared to the normal appearing brainstem in MS (normal-appearing vs mixed mean rank: C1q: 11,43 vs 23,46 p=0,04.; by Kruskal-Wallis and Dunn’s post-hoc test). (Figure 3E)

C3d
C3d is the end-product of the activation of the complement component C3, which is a common factor to all (classical, alternative and lectin) complement pathways. Deposition of C3d in tissue indicates C3 activation with release of opsonins (molecules that enhance the ability of macrophages and neutrophils with complement receptors to phagocytose material – C3b, iC3b, C4b etc.) and anaphylatoxins (peptides that induce local and systemic inflammatory responses, increasing the permeability of blood vessels and attracting neutrophils through their chemotactic properties – C3a, C4a and C5a). While NNCo showed little or no evidence of C3d deposits in the brainstem (not shown), we found C3d deposits on short stretches of nerve fibers in NDCo brainstem (Figure 4A) and normal-appearing MS brainstem (Figure 4B, C). In some cases the C3d deposits also appeared as enlarged punctate profiles (Figure 4D). Double staining for C3d and PLP showed that C3d immunoreactivity localized on axons with disrupted PLP+ myelin profiles (Figure 4E, F). Double staining for C3d and SMI312 showed that C3d immunoreactivity localized on swollen axons and end-bulbs (Figure 4G, H). These data are in line with earlier findings from our group showing that C3 focally tags injured axons in normal-appearing white matter, possibly as part of a physiological mechanism to remove irreversibly damaged axons in chronic disease.

In mixed MS brainstem lesions and brainstem of NDCo, we found in addition C3d deposits on the perikarya of neurons (Figure 4I), which in some cases showed morphological evidence of injury (i.e. decentrated nucleus) (Figure 4J), and on demyelinated axons (Figure 4K and L) with signs of impaired energy metabolism, as shown by the colocalization with mtHSP70 (Figure 4M and N). NDCo also showed C3d staining associated with specific neuropathological features of the neurodegenerative disease, including amyloid $\beta$ deposits or hyperphosphorylated tau (not
Figure 3. Complement activation products C1q are deposited on synapses in both neurodegenerative disease and multiple sclerosis brainstem lesions.

(A) Representative immunohistochemical staining for the initiator of the classical pathway C1q in neurodegenerative disease, showing positive but faint staining on a neuronal perikarya. (B) Representative C1q immunohistochemical staining in mixed active/inactive lesions showing a dark punctuated staining pattern on neurons, indicated with arrows. (C and D) Representative C1q and synaptophysin confocal images of mixed active/inactive brainstem lesions from progressive multiple sclerosis donors. (Zoom C and D) Staining of C1q localizes at synaptophysin-positive synapses. In zoom C and zoom D, open arrows show synaptophysin-positive synapses also positive for C1q; arrow heads indicate C1q staining on synapses with reduced synaptophysin immunoreactivity; asterisks indicate synaptophysin-positive synapses negative for C1q. DAPI stains nuclei in blue. Isotype-matched antibodies were used as negative controls. E. Quantification of number of C1q positive deposits in non-neurological controls, neurodegenerative disease and MS lesions. Results are expressed as median and interquartile range. Kruskal-Wallis and Dunn’s post-hoc test p-values are shown.
shown). Quantification analysis showed that the most pronounced density of C3d deposition occurred in NDCo and mixed MS lesions which was significantly increased compared to the normal appearing MS brainstem. (normal-appearing vs mixed mean rank: C3d: 9,80 vs 41,63 \( p=0.0002 \); normal-appearing vs NDCo mean rank: 9,80 vs 50,5 \( p<0.0001 \) by Kruskal-Wallis and Dunn’s post-hoc test) (Figure 4O).

**MAC**
The membrane attack complex (MAC) is the terminal effector complex of the complement system. It occurs downstream of C3 and C5 convertase activity and forms from the binding of C5b to C6, C7, C8 and a polymer of C9. In lytic levels it forms pores in the phospholipid bilayer of the target cell whereas sublytic levels can activate the NLRP1 inflammasome. While MAC deposits were absent in NNCo (not shown) and were almost undetectable in NDCo (Figure 5A and zoom A), active and mixed MS brainstem lesions showed substantial MAC immunoreactivity (Figure 5B). In the active white matter part of the MS lesions, MAC positive inclusions were seen inside myelin laden microglia/macrophages, suggesting phagocytosis of MAC-coated myelin debris (Figure 5 zoom B, white matter) in line with earlier observations of MAC deposits in myeloid cells within active demyelinating white matter lesions. Notably, in the grey matter part of active or mixed MS brainstem lesions MAC immunoreactivity appeared predominantly on astrocytes, particularly those located adjacent to neurons (Figure 5 zoom B grey matter, and C-D). These astrocytes showed no signs of necrosis, indicating resistance to MAC-induced lysis. These observations point to the possibility that MAC is deposited at sub-lytic amount on astrocytes in the grey matter portion of MS active brainstem lesions, suggesting a potential role in activating the inflammasome on the targeted astrocyte. Quantification analysis showed that the most pronounced density of MAC deposits was seen in both active and mixed lesions and this was significantly higher compared to the NDCo. (NDco vs active mean rank: C9neo: 2,50 vs 20,11 \( p=0.0087 \); NDCo vs mixed mean rank: 2,50 vs 22,56 \( p=0.0015 \) by Kruskal-Wallis and Dunn’s post-hoc test) (Figure 5E).

Altogether these findings indicate that in the MS brainstem the extent of complement deposits correlates with lesion activity. In addition, the MS brainstem shares features of early complement activation in common with classic neurodegenerative diseases, suggesting a convergence of neurodegenerative processes, whereas MAC deposition may constitute a specific feature of the inflammatory response with pathogenic significance in MS.

The density of inflammatory infiltrates is highest in active and mixed MS brainstem lesions
Next, we analyzed the localization and density of inflammatory infiltrates in the MS brainstem in relation to lesional activity, and in comparison to NNCo and NDCo brainstem.

**T cells**
In NNCo and NDCo CD3+ T cells were present and localized around blood-vessels, as expected in virtue of their role in immune surveillance at steady state. In the MS brainstem CD3+ T cells were more frequently seen in active and mixed lesions. They infiltrated the parenchyma and were found
Figure 4. C3d deposits are localized and neuronal perikaryal and axons in both neurodegenerative disease and multiple sclerosis brainstem lesions.

(A-D) In neurodegenerative controls and in normal appearing brainstem C3d, the final activation product of the central component of the complement pathways, C3, localizes stretches of axons. (E-F) C3d localized on myelined axons showing degenerating myelin. (G-H) C3d localized on SMI312 positive axonal bulbs in the normal appearing white matter. In mixed active/inactive lesions C3d is localized on the neuronal perikarya (I-J) and on stretches of axons (K-L). (M-N) The axons deposited with C3d in mixed active/inactive lesions showed impaired energy metabolism since they were positive for mHSP70. (O) Quantification of C3d in non-neurological controls, neurodegenerative disease and MS brainstem lesions demonstrated both increased C3d deposits in MS lesions and neurodegenerative brainstem. Results are expressed as median and interquartile range. Kruskal-Wallis and Dunn’s post-hoc test p-values are shown.
neurodegenerative control  

active lesion

Figure 5. Membrane attack complex, MAC, is specifically deposited in multiple sclerosis brainstem lesions in myelin laden microglia/macrophages and peri-neuronal astrocytes. (A) Staining for the terminal effector of the complement system, membrane attack complex (MAC, C9neo epitope), shows in neurodegenerative controls a light staining of potentially blood vessels. (B and zoom B white matter) In active MS lesions C9neo deposits are shown inside microglia/macrophages throughout the lesion, indicating phagocytosis of MAC opsonized myelin debris. (Zoom B grey matter) MAC deposits are also localized on astrocytes in close proximity to neurons in the grey-matter part of the lesions (arrows indicate MAC-positive astrocytes). (C and D) Confocal images of MS lesions shows colocalization of GFAP and C9neo. Isotype-matched antibodies were used as negative controls. (E) Quantification of MAC in neurodegenerative disease and MS brainstem lesions shows it is specifically deposited in MS lesions. Results are expressed as median and interquartile range. Kruskal-Wallis and Dunn’s post-hoc test p-values are shown.

to localize along the active rim and in close proximity to neuronal cell bodies (Figure 6A-C) (NDco vs active mean rank: 8,30 vs 38,0 p=0.043.; NDco vs mixed mean rank: 8,3 vs 35,5 p=0,002; NA vs mixed mean rank: 15,6 vs 35,5 p=0,010 by Kruskal-Wallis and Dunn’s post-hoc test). Consistent with
prior studies, further analysis of the CD4+ and CD8+ T cell subtypes revealed that the majority of T cell infiltration in the MS brainstem lesions consisted of CD8+ T cells, with a median CD8:CD4 ratio of ~3:1 in mixed lesions. This ratio was in line with findings in NNCo and NDCo (Suppl. Figure 2). The density of CD8+ T cells was highest in active and mixed lesions where they localized at the active rim and in close proximity to neuronal cell bodies (Figure 6D-F) (NDco vs mixed mean rank: 15,0 vs 34,1 p=0,03; by Kruskal-Wallis and Dunn’s post-hoc test). Although in lower numbers, CD4+ T cells were also detected in the MS brainstem, with the highest density in active and mixed lesions. Unlike CD8+ cells, CD4+ cells rarely infiltrated the brainstem parenchyma and localized mostly in close proximity to blood vessels (Figure 6G-I) (NDco vs active mean rank: 7,83 vs 37,50 p=0.030; NDco vs mixed mean rank: 7,83 vs 34,32 p=0,002; normal-appearing vs mixed mean rank: 14,20 vs 34,32 p=0,010 by Kruskal-Wallis and Dunn’s post-hoc test).

**B cells**

CD20+ B cells were not found in the brainstem of NNCo and NDCo, as expected. In the MS brainstem they were found in clusters localized in enlarged perivascular spaces around blood vessels, with the highest density in active and mixed lesions (Figure 6J-L) (NDco vs mixed mean rank: 6,0 vs 32,2 p=0,001; by Kruskal-Wallis and Dunn’s post-hoc test). Of note, the density of CD20+ B cells was highly variable between MS donors, and only 44% (4/9 cases) of the MS cases showed >4/mm² CD20+ B cells in the lesions, reflecting the heterogeneity of MS lesion pathology.

**Plasma cells**

CD138+ plasma cells were not observed in the brainstem of NNCo and NDCo. They were identified only in 22% (2/9) of MS cases analyzed but, when detected, they were found to infiltrate the parenchyma, localizing at the rim of both active and inactive lesions (Figure 6M-O).

Altogether these findings indicate that in the MS brainstem the extent of inflammatory infiltrates correlates with lesional activity. In line with previous studies in active and mixed white matter lesions, the relative higher abundance of CD8+ T cells and B cells may underline the potential importance of these immune cells in the inflammatory response in established multiple sclerosis lesions.

**Inflammation in the MS brainstem correlates with neurodegeneration**

To analyze the potential link between inflammation and neurodegeneration in brainstem lesions from progressive MS patients, we performed correlation analyses between the density of inflammatory infiltrate or complement deposits and neurodegenerative changes detected on serial sections from each MS brainstem tissue analyzed.

Spearman correlation and FDR multiple testing correction was performed on the measures for SMI312, APP, mthHSP70, C3d, C1q, CD4, CD8, CD20, CD138, and a correlation plot is shown in Suppl. Figure 3. Reduced fast axonal transport as shown by APP immunoreactivity, which was specifically seen in the inflammatory active rim of mixed lesions, positively correlated with the number of
Figure 6. In mixed active/inactive lesions CD8 T cells are found in the brain parenchyma in close contact with neurons and B cells are present in the perivascular space. (A-C) In active and mixed active/inactive lesions the number of T cells is increased compared to neurodegenerative brainstem and they are located in the brain parenchyma in the inflammatory active lesion rim. (D-F) T cells are mostly CD8+, in both the control, neurodegenerative and MS brainstem. In mixed active/inactive lesions CD8+ T cell number is increased, and in the GM part of MS lesions CD8+ T cells are found in close proximity to neuronal cell bodies. (G-I) The number of CD4+ T cells in the brainstem is lower compared to CD8+, however in both active and mixed active/inactive brainstem lesions the number is increased. CD4+ T cells are less often encountered in the brain parenchyma. (J-L) The number of CD20+ B cells is low and highly variable between MS brain donors, B cell number is increased in mixed active/inactive lesions and they are most often encountered in clusters around blood vessels. (M-O) The number of plasma cells was low and only in 2/9 MS cases they were encountered in the brain parenchyma, the number of plasma cells was not associated with the inflammatory activity of the lesions. Scale bars 50 μm, in the zoom scale bars are 20μm.
CD3+ T cells (Spearman’s Rho = 0.40, FDR p=0.04) and CD8+ T cells (Spearman’s Rho = 0.37 and FDR p=0.05) (Figure 7A). Axonal loss as measured by the surface area of SMI312 negatively correlated with the number of C1q deposited neurons (Spearman’s Rho = -0.49, FDR p=0.04) and negatively correlated with the number of CD20+ B cells (Spearman’s Rho = -0.49, FDR p=0.01). There was a trend for a positive correlation with the number of CD138+ plasma cells (Spearman’s Rho = 0.36 and FDR p=0.056) (Figure 7B). The extent of C3d deposits positively correlated with the number of CD8+ T cells (Spearman’s Rho = 0.36 and FDR p=0.05) and CD4+ T cells (Spearman’s Rho = 0.42 and FDR p=0.03) (Figure 7C).

These data show that in patients with progressive MS injury to neurons, axons and synapses is associated with inflammation. This is different from what we found in the brainstem of NDCo, where signs of metabolic stress in neurons shown by the immunoreactivity for myHSP70 was not associated with the density of T cells, B cells and plasma cells and no deposits of the terminal complement complex MAC were identified within the tissue.

Figure 7. Correlation of reduced fast axonal transport, axonal loss and C3d depositions with lymphocytes in MS brainstem lesions.
(A) The number of CD3+ and CD8+ T cells positively correlated with the number of APP+ bulbs. (B) The number of CD20+ B cells and C1q+ neurons negatively correlated with the axonal density, while the number of CD138+ plasma cells positively correlated with the axonal density. (C) The number of CD8+ and CD4+ T cells positively correlated with the number of C3d deposits.
Here we show that in the brainstem of progressive MS patients neurodegeneration and inflammation are extensive. In addition, we show that the extent of microglia/macrophages activation, complement deposition and lymphocyte infiltration are linked to demyelination and neurodegeneration in this tissue, suggesting a role for inflammation in the pathology of the progressive MS brainstem.

A possible link between inflammation and neurodegeneration in progressive MS has been largely debated, since magnetic resonance imaging (MRI) data show only rare Gadolinium (Gd)-enhancing lesions in patients with advanced and progressive MS, dissociating BBB-leakage (which is the entry venue for serum proteins and peripheral immune cells) from progressive damage of the grey and white matter. In addition, clinical findings have shown that current anti-inflammatory treatments, although effective in modulating peripheral immunity, do not prevent or resolve neurodegenerative changes in the brain (reviewed in 15). One theory supporting a key role of inflammation in neurodegeneration, while reconciling imaging and clinical findings of progressive MS, proposes that this injury may be driven by a compartmentalized immune response within brains that have a relatively intact BBB. In support, several studies have identified aggregates of lymphoid cells of variable size and organization, referred to as Tertiary Lymphoid Tissues (TLT), in the leptomeninges lining the cortex. Importantly, these leptomeningeal TLT are associated with underlying neuronal loss and rapid disease progression, suggesting that these structures can shape pathological processes into the CNS tissue. Recent studies reported that in active white matter lesions of progressive MS patients a subset of the infiltrating CD8+ T cells with features of tissue-resident memory cells show loss of the surface molecules S1P1 or CCR7, which are involved in the egress of leucocytes from inflamed tissue, possibly reflecting the compartmentalization of the inflammatory response also in the subcortical white matter.

Here we show that in the deep grey matter of progressive MS patients, parenchymal inflammation is extensive, further supporting a role for inflammation in the pathology of MS lesions during the progressive phase of the disease. The brainstem of patients with progressive MS displayed a substantial number of inflammatory active and mixed lesions (74% of total lesions analyzed) determined by the abundance, distribution and morphology of microglia/macrophages, classified according to criteria previously described for classical MS white matter lesions. Importantly, foamy macrophages, thought to be involved in ongoing demyelination, were also detected. Our data is in line with reports of active and mixed lesions in another deep grey matter structure, the hypothalamus. Interestingly, the abundance of microglia/macrophages in and around brainstem lesions of progressive MS patients is unlike what is often observed in cortical grey matter lesions of patients with a similar advanced stage of MS. While foamy macrophages
can be found in cortical grey matter lesions of patients with early MS, cortical grey matter demyelination and neuronal injury in patients with progressive MS do not typically associate with microglia/macrophage activation.

We also report substantial lymphocytes infiltration within the brainstem parenchyma of progressive MS patients. Similar to what we observed for microglia/macrophages, while T cells have been observed at early MS biopsy in intracortical lesions and to a lesser extent in subpial lesions, lymphocytes are rarely found to infiltrate the subpial parenchyma in cortical grey matter lesions of progressive MS patients. Instead, lymphocytes aggregate in the meningeal compartment from where they are thought to contribute to pathology by producing cytotoxic mediators that diffuse across the injured glial limitans into the underlying subpial cortex (reviewed in1). The difference in the localization of leukocytes and extent of microglia/macrophage activation in cortical versus deep grey matter lesions of progressive MS patients may reflect tissue-specific differences in the inflammatory response between these two sites. For example, perivascular fibroblastic cells, which are known to remodel during inflammation participating in the scaffolds that supports immune cells in local niches, may exhibit inherent differences in their immune-stimulating potential at postcapillary venules compared to perivascular fibroblastic cells in the meninges.

Specifically, we found an abundance of CD8+ T cells in active lesions and at the rim of mixed lesions suggesting a role in demyelination. We also found few CD8+ T cells in close contact with neurons, which may reflect immunosurveillance or a cytotoxic CD8+ T cell response against neuronal antigens. CD4+ T cells although less abundant were also present at sites of demyelination but localized more perivascularly, in line with previous reports in MS lesions of the white matter. In terms of B cells, the density of CD20+ B cells although variable across donors was generally higher in enlarged perivascular spaces within active and mixed lesions, suggesting that B cells could be involved in antigen presentation and reactivation of CD4+ and CD8+ T cells in the brainstem of progressive MS patients. The density of CD20+ B cells in the brainstem was inversely correlated with the axonal density, suggesting that increased involvement of B cells is related to a decreased number of axons in this tissue. Interestingly, we found a positive correlation between the plasma cell density and the axonal density, suggesting that an increased number of plasma cells relates to more preserved axonal density in progressive MS brainstem lesions. Furthermore, the number of CD138+ plasma cells appeared to be enriched (although not significantly likely due to low power) in inactive lesions compared to normal-appearing tissue or active and mixed lesions. This accumulation of CD138+ plasma cells in the brainstem at a later lesional stage (i.e. inactive lesions) and concomitant with a decrease in CD20+ B cells number compared to more active lesional stages is in line with findings in MS white matter lesions and suggests the existence of a population of long-lived plasma cells, which may accumulate in the CNS during chronic inflammation. The significance of these potentially long-lived plasma cells is currently unknown, but recent studies have shown that they may exert regulatory roles during neuroinflammation.
In addition to lymphocytes, we also report substantial complement activation within the brainstem of progressive MS patients. While classical complement activation is generally linked to antibody-mediated demyelination which occurs in cases of myelin oligodendrocyte glycoprotein (MOG) antibody-associated inflammatory demyelinating disease or neuromyelitis optica, over the past decade several studies have identified early proteins of the complement system as key components of synaptic “pruning” during normal ageing, development, viral infection and neurodegeneration. In line with previous studies from our team showing that in the hippocampus of progressive MS patients C1q/C3 tags synapses for elimination by microglia in the absence of antibodies and terminal MAC activation, we show that C1q (but not antibodies or MAC) deposits at synapses also in the MS brainstem suggesting a C1q-dependent mechanism of synaptic pruning. What recruits C1q at synapses is unknown but a recent study proved that local pruning of dendritic spines is initiated by the mitochondrial production of reactive oxygen species and/or by the activation of the N-methyl-D-aspartate receptor (NMDA) receptors locally in dendrites. Both, mitochondrial oxidative stress and changes in the glutamate neurotransmitter system have been reported in progressive MS. Therefore it is possible that similar pathways of synapse elimination are at play in the MS brainstem. C1q deposition on neuronal perikarya was significantly increased in the mixed MS brainstem lesions compared to the normal appearing MS brainstem. Furthermore the extend of C1q deposition negatively correlated with the extend of axonal loss suggesting that the deposition of C1q is related to axonal degeneration in chronic MS lesions.

In line with our previous work which identified C3d deposits on short stretches of axons with impaired transport in the periplaque white matter, we found C3d on axons with disrupted myelin profiles, swollen axons and axonal end-bulbs also within the normal-appearing brainstem and brainstem from NDCo. These profiles are also found in advanced stroke lesions, therefore they likely represent a shared mechanisms aimed at removal of irreversibly damaged axons in chronic disease.

The terminal complement pathway, culminating in the formation of the MAC, is undoubtably the most inflammatory of complement axes. Lytic levels of MAC can directly disrupt the membrane integrity of a target cells, which is then engulfed by phagocytes recruited to the site of inflammation by anaphylatoxins. In addition, sublethal amounts of MAC can induce NLRP3 inflammasome activation on the target cell, resulting in the secretion of pro-inflammatory cytokines. Our finding of MAC deposits within foamy macrophages in the white matter portion of active brainstem lesions and on the surface of astrocyte on the grey matter portion of the active lesions is in line with these functions. In summary, while we show that early complement activation components (C1q and C3d) are a common feature between MS and classic neurodegenerative diseases, suggesting a convergence of neurodegenerative processes, deposition of the terminal complement component MAC may constitute a specific feature of the inflammatory response with pathogenic significance in progressive MS.
Altogether, while our work does not answer the question of causality or direction of causality, it does establish a link between an innate and adaptive immune responses, demyelination and neurodegeneration in the brainstem of progressive MS patients. Understanding the relation between inflammation, demyelination and neurodegeneration is critical to guide future therapeutic strategies aimed at slowing deterioration of clinical symptoms related to this region in progressive MS patients.

Acknowledgments
We thank the MS brain donors for donating their brain to research and the team of the Netherlands Brain Bank for their excellent service.

Disclosures
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REFERENCES


### Supplementary Table 1. Donor information per autopsy case.

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</table>

ALS, Amyotrophic Lateral Sclerosis; FTD, Fronto-temporal dementia; AD, Alzheimers disease; NNC, non-neurological control; F, female; M, male; PMD, post-mortem delay; PP, primary progressive; SP, secondary progressive.
Supplementary figure 1. Immunoglobulin staining in multiple sclerosis brainstem. Sections from the multiple sclerosis donor stained for immunoglobulin M (IgM), showing deposition on the perikarya of neurons near a mixed active/inactive lesions. Hematoxylin stains nuclei blue. Scale bars in the low-magnification images, 100μm. Scale bars in the high magnification, 50μm.

Supplementary figure 2. CD8/CD4 T cell ratio in MS lesions.

Supplementary figure 3. Spearman correlation coefficients for the significant correlations after FDR multiple testing correction.