Activated Microglia Mediate Axoglial Disruption That Contributes to Axonal Injury in Multiple Sclerosis

Owain W. Howell, PhD, Jon L. Rundle, PhD, Anurag Garg, BSc, Masayuki Komada, PhD, Peter J. Brophy, PhD, and Richard Reynolds, PhD

Abstract

The complex manifestations of chronic multiple sclerosis (MS) are due in part to widespread axonal abnormalities that affect lesional and nonlesional areas in the central nervous system. We describe an association between microglial activation and axon/oligodendrocyte pathology at nodal and paranodal domains in normal-appearing white matter (NAWM) of MS cases and in experimental autoimmune encephalomyelitis (EAE). The extent of paranodal axogial (neurofascin-155/Casp1) disruption correlated with local microglial inflammation and axonal injury (expression of nonphosphorylated neurofilaments) in MS NAWM. These changes were independent of demyelinating lesions and did not correlate with the density of infiltrating lymphocytes. Similar axogial alterations were seen in the subcortical white matter of Parkinson disease cases and in preclinical EAE, at a time point when there is microglial activation before the infiltration of immune cells. Disruption of the axoglial unit in adjuvant-immunized animals was reversible and coincided with the resolution of microglial inflammation; paranodal damage and microglial inflammation persisted in chronic EAE. Axoglial integrity could be preserved by the administration of minocycline, which inhibited microglial activation, in actively immunized animals. These data indicate, that in MS NAWM, permanent disruption to axoglial domains in an environment of microglial inflammation is an early indicator of axonal injury that likely affects nerve conduction and may contribute to physiologic dysfunction.

Key Words: Demyelination, Multiple sclerosis, Neurofascin, Neuroinflammation, Node of Ranvier, Sodium channel.

INTRODUCTION

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) characterized by focal areas of inflammation, demyelination, gliosis, and axon loss (1). Neurological decline in the progressive stages of MS is suggested to result from axon loss that is dependent on compartmentalized inflammation and chronic activation of the innate immune system (2); this concept is supported by the observation that progressive MS patients are refractory to therapies that target the peripheral immune component (3). The normal-appearing white matter (NAWM) in progressive MS is often widely affected and contains areas of diffusely activated microglia, degenerating axons, and a compromised blood-brain barrier (BBB) (4–6). Furthermore, imaging data also suggest that permanent neurologic deficit may in part be attributed to these global and diffuse changes of the NAWM (7, 8). However, the nature and effects of this diffuse inflammation have been rarely studied.

The integrity of myelin-producing oligodendrocytes is crucial for ensuring correct organization of axonal domains required for action potential propagation and the maintenance of axon caliber and transport (9, 10). The glial 155-kd isoform of neurofascin (Nfasc155) is expressed at the leading edge of the myelin sheath and associates with Caspr1 and contactin of the axolemma to ensure the correct generation and maintenance of paranodal axogial junctions and facilitate effective saltatory conduction (11–13) (Figure, Supplemental Digital Content 1, http://links.lww.com/NEN/A174). Nfasc155, Caspr1, or contactin single-gene knockouts have disrupted axogial junctions and displaced K,1 channels that are normally expressed under the myelin sheath at the juxtaparanode. Such alterations in nodal architecture are accompanied by ataxia, muscle tremors and severely reduced nerve conduction velocities, despite normal myelin ensheathment (12–14).

Gene deletion of major oligodendrocyte and myelin proteins yields mice with morphologically normal myelin but with a striking axonal pathology, including organelle accumulation in nodal regions (15–17). The Nfasc155 null mouse lacks normal paranodal junctions and manifests an axonal pathology of disorganized neurofilaments and nodal/paranodal swellings (18), which suggests that changes to myelin composition or interruptions to glial-axonal communication can be catastrophic for normal axon function and survival.

Paranodal axogial components are lost earlier than nodal markers after myelin degeneration in rodents (19), and...
such changes are a sensitive indicator of early myelin abnormalities in MS (20–22). Here, we report how changes in paranodal and nodal components are apparent in MS NAWM and in the murine model of MS, EAE, at sites remote from lesions. These changes correlated with the incidence of activated microglia and were abolished in mice treated with minocycline, which inhibited microglial inflammation. Nodal alterations were also seen in adjuvant-only–treated animals and in Parkinson disease (PD) patient white matter (WM) that contained activated microglia, implying that generalized microglial inflammation is sufficient to induce these changes. This work suggests an important and novel mechanism of tissue injury in the inflamed CNS whereby the disruption of axoglial communication by activated microglia would impinge on saltatory conduction and normal axon function potentially contributing to the diversity of clinical manifestations of MS.

**MATERIALS AND METHODS**

**Human Postmortem Tissue Characterization**

Tissue for this study was provided by the UK Multiple Sclerosis Tissue Bank and the UK Parkinson’s Disease Society Tissue Bank at Imperial College London under ethical approval from the National Research Ethics Committee (08/MRE09/31 and 07/MRE09/72). Cases of neuropathologically confirmed MS (n = 18, 11 women; mean postmortem delay [PMD], 17.2 hours [range, 7–24 hours]; mean age, 55.6 years [range, 39–78 years]; and mean disease duration, 25.9 years [range, 12–47 years]), 6 cases of PD (3 women; PMD, 20.2 hours [range, 15–28 hours]; mean age, 79.2 years [range, 75–85 years]; and mean disease duration, 17.5 years [range, 10–27 years]), and 11 nonneurologic controls (3 women; PMD, 22.4 hours [range, 14–33 hours]; mean age, 71.9 years [range, 35–88 years]) were studied (Table). Tissue blocks (2 × 2 × 1 cm) dissected immediately on brain retrieval were fixed in 4% paraformaldehyde (Sigma-Aldrich, Dorset, UK) in phosphate-buffered saline (PBS) for a minimum of 12 hours, cryoprotected in 30% sucrose in PBS, and cryosectioned at 10 μm. Because of tissue availability, single-tissue blocks were examined from 11 MS cases (9 women) and 2 blocks were examined for each of the remaining 7 cases (2 women). Immunohistochemical and immunofluorescent staining was performed on sections from the same blocks.

Multiple sclerosis tissue blocks from frontal and parietal regions were characterized as normal appearing or lesional by screening with a panel of histochemical and immunohistologic markers, as previously described (22). Multiple sclerosis NAWM was defined as being at least 10 mm from focal demyelinated plaques and avoiding neurofilament-labeled fiber tracts emerging from gray/WM lesions. Briefly, all cases were processed for hematoxylin/Luxol fast blue histology and immunohistochemistry/immunofluorescence for HLA-DR, ionized calcium binding adapter molecule 1 (IBA1), myelin/oligodendrocyte glycoprotein (MOG), glial fibrillary acidic protein, neurofilament-H protein, and CD3 antigens (Table, Supplemental Digital Content 2, http://links.lww.com/NEN/A175). Parkinson disease tissue blocks containing subcortical WM from frontal and parietal areas were screened for α-synuclein expression, microglial inflammation (HLA-DR”, IBA1”, CD68”), and axonal pathology (neurofilament-H”).

**Induction of EAE in C57BL/6 Mice**

Female C57BL/6 mice (6–8 weeks; Charles River Laboratories, Margate, UK) were immunized on days 0 and 7 with MOG35-55 peptide (200 μg; Advanced Biotechnol-ogy Centre, Imperial College London, UK) supplemented with 320 μg of Mycobacterium tuberculosis and 80 μg of Mycobacterium butyricum (Difco). Pertussis toxin (200 μg; Calbiochem, Nottingham, UK) was administered intraperitoneally on days 0, 1, 7, and 8. Myelin/oligodendrocyte glycoprotein EAE, adjuvant controls (the absence of MOG peptide), and naive mice (n = 5 per group) were weighed and assessed daily for clinical signs. All protocols for animal research conformed to UK Home Office Project Licence regulations.

Animals were terminally anesthetized and perfused intracardially with PBS followed by 4% paraformaldehyde at day 10 (before disease onset) and day 44 (chronic disease). Spinal cords were postfixed for 4 hours, cryoprotected in 30% sucrose in PBS, and sectioned at 10 μm. The spinal cord was cut into 3 pieces, the most rostral 3 mm of each portion was mounted for transverse sectioning, and the remaining 10-mm portions were sectioned longitudinally. Cellular infiltrates and myelin changes were assessed on the transverse and longitudinal sections of the cords by immunostaining for CD3, CD11b, IBA1, inducible nitric oxide synthase (iNOS), toll-like receptor 4 (TLR4), CD68 (ED1 antigen), neurofilament-H protein, nonphosphorylated neurofilaments (SMI32 antibody), and myelin basic protein. Quantification of CD11b immunoreactivity (IR) was performed on transverse sections (cervical, thoracic and lumbar) and paranodal/nodal quantifications on lateral WM tracts of the lumbar spinal cord.

**Minocycline Treatment**

MOG35-55–immunized animals (n = 5) were given minocycline hydrochloride intraperitoneally (Sigma-Aldrich) according to the dosing regimen of Brundula et al (23), from the day of EAE induction until the end of the experiment (day 13), when the first clinical signs were noted in vehicle-treated animals and inflammation was widespread. Vehicle control animals (n = 5) received saline at the same volume and frequency as the minocycline group. Spinal cord tissue was prepared as above.

**Immunohistochemistry**

Tissue sections were air dried, rehydrated in PBS and subjected to antigen retrieval when necessary (10 mmol/L sodium citrate buffer or methanol), before commencing with immunohistochemical or immunofluorescent staining (22). Primary antibodies are listed in Table, Supplemental Digital Content 2, http://links.lww.com/NEN/A175. Secondary antibodies were purchased from Vector Laboratories (Peterborough, UK), Molecular Probes (Invitrogen, Paisley, UK), and Jackson Immunoresearch (Stratech Scientific, Soham, Cambridgeshire, UK). Secondary antibody controls, processed using identical protocols except for the omission of primary antibodies, were devoid of staining.
Image Analysis and Experimental Details

Tissue sections were analyzed on a Nikon E1000M epifluorescence microscope (Nikon Instruments, Inc, Melville, NY) with a digital camera (QImaging, Staffordshire, UK) or by confocal laser scanning microscopy with a Leica SP5 MP inverted microscope (Leica Microsystems, Bucks, UK). All images were analyzed using Image ProPlus (Media Cybernetics, Bucks, UK) and ImageJ (http://rsb.info.nih.gov/ij/) and prepared in Photoshop CS2 (Adobe Systems, Uxbridge, UK). Quantification was performed with the observer blinded to case identification.

Analysis of Inflammation in MS NAWM

Microglial/macrophage density and the incidence of damaged axons were assessed by quantifying HLA-DR+/iNOS+ cells and SMI32+ (nonphosphorylated neurofilament+) axons from 4 randomly captured fields (magnification, 200×; 0.07 mm²) per region of NAWM per tissue block. Total perivascular and CD3+ infiltrates were determined from 4 to 6 vascular structures per block. The perivascular space was outlined to calculate perivascular area and the number of DAPI+ nuclei, and the number of CD3+/DAPI+ cells was calculated per squared millimeters of perivascular space. A composite measure of local inflammation and axonal stress was calculated using an empirical scoring system (0–10) to subgroup MS NAWM areas into low-level localized inflammation/axon pathology (MS low, 0–6) and high-level localized pathology (MS high, 7–10) for comparisons with respect to Kv1 distribution.

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C, control; DLB, dementia with Lewy bodies; F, female; M, male; n/a, not applicable; NAWM, normal appearing white matter; PD, Parkinson disease; PDD, Parkinson disease with dementia; PMD, postmortem delay; PP, primary progressive multiple sclerosis; SP, secondary progressive multiple sclerosis.

TABLE. Demographic and Pathologic Data on Control, Multiple Sclerosis, and PD Cases

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101–200, or 2 = 200+ cells), the absence or presence of microglial nodules (0 = none or 1 = nodules present) and parenchymal amoeboid HLA-DR+ macrophages (0 = none or 1 = present—albeit at very low densities of 1–2 cells per field), perivascular CD3+ T cells (0 = 0–500/mm², 1 = 501–1000, or 2 = 1001+), iNOS+ cells (0 = 0–100, 1 = 101–200, or 2 = 200+), and SMI32+ axons (0 = 0–10, 1 = 11–20, or 2 = 20+/mm²).

Quantifying Disruption of the Node of Ranvier

Nodal, paranodal, and juxtaparanodal domains were measured based on the extent of pan-Na, Na1.1, Na1.6, βIV spectrin, Nfasc155, Caspr1, or K1.1.2 expression on fluorescent images of regions of interest (ROIs) scored for inflammation. Images were captured with a 63× oil-immersion objective (captured field = 0.008 mm²), and only positive paranodal structures in focus were measured from a minimum of 8 sampled fields (range, 8–14), per ROI for each tissue block analyzed. Mean ± SEM values were calculated per ROI and the group means (e.g. control, MS) were plotted with PRISM (GraphPad Software, Inc, La Jolla, CA) as bar graphs or box plots (showing median, 25%–75% data, and whiskers that represent the minimum and maximum observation). Groups were compared using nonparametric Mann-Whitney t test or analysis of variance (ANOVA) and appropriate posttest (PRISM).

Data were plotted as single paranode length, nodal length (the unstained region between Nfasc155+ paranodes), and Nfasc155+ paranodal width per experimental group. To investigate the association between Nfasc155+ paranodal profiles and axonal damage, the length of Nfasc155+ profiles associated with SMI32+ axons was assessed. Nfasc155+ structures on SMI32+ axons were compared with adjacent profiles not associated with SMI32+ fibers. Neurofilaments are often hypophosphorylated at nodes; therefore, we only measured Nfasc155+ profiles associated with long SMI32+ fibers (often 30–50 μm in length) to avoid including normal

FIGURE 1. Multiple sclerosis normal-appearing white matter (NAWM) contains activated microglia, damaged axons and perivascular lymphocytic infiltrates. (A, B) Luxol fast blue stain and MOG immunohistochemistry were used to identify regions of interest in forebrain NAWM (arrowhead, A). (C, D) There are HLA-DR+ microglia with an activated morphology throughout the NAWM (C); CD3+ T-cell infiltrates are restricted to perivascular cuffs (D). (E–E2) SMI32 antibody identifies stressed/damaged axons containing nonphosphorylated neurofilaments that are morphologically consistent with transections (asterisks) or closely apposed to IBA1+ microglia (arrows). (F, F1, G, H) Microglial activation is indicated by their altered morphology and expression of HLA-DR and iNOS in MS NAWM (F, F1) and was present at a greater density versus controls (G, H). Ctrl, control; LFB, Luxol fast blue; MOG, myelin oligodendrocyte glycoprotein. Scale bars = (A) 0.5 cm; (B, C, E–E2) 20 μm; (D) 100 μm; (F–F1) 10 μm; (G, H) 50 μm.
axons. Nodes were analyzed by measuring the axonal length of pan-Na$_1$+$^+$ profiles from Na$_1$+$^+$/Nfasc155$^+$ colabeled sections. Changes at the juxtaparanodal/paranodal domain were assessed by quantifying the number of overlapping K$_1.2^+$/Nfasc155$^+$ domains and the distance separating the juxtaparanode and node of Ranvier. The number of normal-appearing Na$_1.6^+^+$ nodes (strong Na$_1.6$-IR flanked by Nfasc155$^+$ expression) and the combined frequency of aberrant nodes (nodes with no detectable Na$_1.6$ reactivity or the presence of “split” or heminodal Na$_1.6^+^+$ associated with Nfasc155$^+$ staining) was calculated and expressed as percentage of total nodes quantified. To determine the aberrant nature of nodal Na$_1.6$ expression in EAE spinal cord, z stacks of images from areas of interest were captured from actively immunized mice at 10 days postinduction (dpi; n = 3 mice). A line selection of constant length was centered to the node and the intensity of Nfasc155 and Na$_1.6$ signal determined using a modified version of the RGB profiles tool (http://rsb.info.nih.gov/ij/macros/tools/RGBProfilesTool.txt), courtesy of Dr C. Liebig, Imperial College London (http://www3.imperial.ac.uk/imagingfacility/resources).

RESULTS

Paranodal Axoglial Junctions Are Disrupted in MS NAWM and PD WM

We first examined changes to the axoglial complex in MS NAWM and determined how they might be affected by local inflammation in comparison to subcortical WM from nonneurologic age-matched controls (Fig. 1). To determine the specificity of the structural alterations, we also studied PD brain tissue blocks that contained diffuse microglial inflammation without demyelination (Figure, Supplemental Digital Content 3, http://links.lww.com/NEN/A176).

Regions of NAWM were selected for analysis of inflammation and nodal/paranodal alterations from fixed

**FIGURE 2.** Microgliosis and paranodal abnormalities in multiple sclerosis (MS) and Parkinson disease (PD) white matter (WM). (A, B) Paranodal axoglial junctions identified by immunostaining for Nfasc155 normally show compact paranodal structures separated by Na$_1$ channels of the node (A); there are occasional elongated, disrupted structures (B, arrows). (C, D) Paranodal disruption that takes the form of an elongation along the length of the axon is identified by altered expression in Nfasc155$^+$ and Caspr$^+$ immunostaining (arrow, D) versus normal appearance (C). (E) Quantification of single Nfasc155$^+$ structures from control, MS, and PD WM revealed a significant increase in Nfasc155 mean paranode lengths in MS and PD samples in comparison to controls (minimum, maximum, interquartile, and median values are indicated). **p < 0.01; ***p < 0.001, ANOVA and Bonferroni multiple-comparison post test. Representative measurements are shown at the right. (F-H) Nfasc155$^+$ structure length correlates with HLA-DR$^+$ microglial density (F) and iNOS$^+$ microglia (G) but not with the presence of CD3$^+$ T lymphocytes in the MS tissue (H). Spearman nonparametric correlation. Ctrl, control. Scale bars = (A–D) 2 µm.
frozen and cryosectioned blocks of superior frontal and/or parietal subcortical WM (Fig. 1). Luxol fast blue histology and immunostaining for MOG confirmed myelin integrity (Figs. 1A, B). Normal-appearing WM typically contained scattered HLA-DR<sup>+</sup> microglia with an activated morphology (i.e. thicker and shorter processes than observed in control microglia; Fig. 1C), very few amoeboid macrophages, and T lymphocytes restricted to perivascular sites (Fig. 1D).

SMI32<sup>+</sup> axons displaying localized swellings and transections were associated with ramified microglia that frequently expressed iNOS (Figs. 1E, F). Microglial activation (activated morphology and increased expression of IBA1) was greater in MS NAWM than in age-matched nonneurologic control samples (Figs. 1G, H).

Immunostaining of NAWM for components of paranodal axoglial junctions such as Nfasc155<sup>+</sup> normally reveals discrete pairs of structures separated by Na<sub>v</sub> 1<sup>+</sup> channels at the node (Fig. 2A). Elongated and disrupted Nfasc155<sup>+</sup> paranodes that contrasted with the more regular, discrete pattern of paired Nfasc155<sup>+</sup> paranodes were seen in MS NAWM and PD (Fig. 2B; Figure, Supplemental Digital Content 3, http://links.lww.com/NEN/A176). Double immunofluorescence for Nfasc155<sup>+</sup> in the glial end loops and Caspr1 in the axolemma revealed elongated paranodes with a disrupted profile that was identical for these proteins (Figs. 2C, D). This colocalization indicates that either protein can be used as an indicator of changes at paranodal axoglial junctions and implies that both oligodendrocyte and axonal compartments are similarly affected. To confirm that paranodal junctions were altered in MS and PD, we measured axonal lengths and widths of single Nfasc155<sup>+</sup> structures and the lengths of the unstained nodal gaps between pairs of Nfasc155<sup>+</sup> paranodes. In total, 928 individual Nfasc155<sup>+</sup> paranodal profiles from 18 MS cases, 311 Nfasc155<sup>+</sup> paranodes from 6 PD cases, and 583 paranodes from 11 controls were quantified and plotted as mean paranodal length per case (Fig. 2E). Nfasc155<sup>+</sup> paranodal lengths were 23% longer on average in the MS NAWM (2.9 ± 0.1 μm, p < 0.001) and 19% longer in PD tissues (2.8 ± 0.1 μm, p < 0.01) versus those in nonneurologic controls (2.7 ± 0.1 μm). Almost a fifth of all quantified Nfasc155<sup>+</sup> structures in MS (170/928, 18%) and an eighth (39/311, 12%) of all PD-quantified paranodes were longer than the 95th percentile of quantified control paranodes (2.74 μm). In contrast, the lengths of nodes of Ranvier (1.4 ± 0.17, 1.61 ± 0.06, and 1.62 ± 0.05 μm) and the widths of Nfasc155<sup>+</sup> paranodes (0.75 ± 0.07, 0.89 ± 0.02, and 0.78 ± 0.04 μm) were not different among control, MS, and PD groups, respectively. There was no correlation between postmortem delay, age or cause of death with our measures of paranodal integrity, microglial activation, and axonal pathology (Table, Supplemental Digital Content 4, http://links.lww.com/NEN/A177).

**Paranodal Axoglial Damage Correlates With Microglial Inflammation in MS**

To examine the relationship between WM inflammation and paranodal alterations in MS, we quantified the numerical density of microglia, macrophages, iNOS<sup>+</sup> cells, and CD3<sup>+</sup> T cells within the same fields of NAWM used for quantification of Nfasc155<sup>+</sup> paranodal profiles. There was a significant positive correlation between the mean Nfasc155<sup>+</sup> paranodal length and the density of HLA-DR<sup>+</sup> ramified microglia and iNOS<sup>+</sup> microglia (Figs. 2F, G). In contrast, paranodal disruption did not correlate with the presence of total perivascular infiltrates (data not shown) or the number of perivascular CD3<sup>+</sup> T cells (Fig. 2H), indicating an influence of the local microglial response rather than peripheral immune infiltrates on paranodal integrity.

**Disrupted Paranodes Are Associated With SMI32<sup>+</sup> Stressed/Damaged Axons**

Paranodal disruption was greatest in cases with the highest density of SMI32<sup>+</sup> stressed/damaged axons (p < 0.001; Fig. 3A). Most disrupted and elongated Nfasc155<sup>+</sup> profiles were directly associated with SMI32<sup>+</sup> axons in the MS NAWM (40/52 paranodes with a length >75 percentile were associated with SMI32<sup>+</sup> axons). Nfasc155<sup>+</sup> paranodes were significantly elongated on SMI32<sup>+</sup> paranode length, 4 ± 0.2 μm) in comparison to paranodes associated with SMI32- axons (2.8 ± 0.1 μm; Figs. 3B, C). SMI32<sup>+</sup> axons in control tissues (albeit at a much reduced density in comparison to MS) were also associated with alterations in Nfasc155<sup>+</sup> paranodal domains (2.3 ± 0.1 and 2.9 ± 0.1 μm, for Nfasc155<sup>+</sup> profiles associated with SMI32-negative and SMI32<sup>+</sup> axons in control tissues), indicating the close relationship between neurofilament phosphorylation and axoglial junction integrity. SMI32<sup>+</sup> axonal swellings were noted at disrupted nodes (Fig. 3D), and accumulations of amyloid precursor protein occasionally associated with disrupted nodal profiles in the NAWM (Fig. 3E), supporting previous reports of nodal/paranodal domains as frequent sites of early axonal pathology (10).

**Juxtaparanodal Disruption Is Accompanied By Increased Microglial Inflammation**

A primary role of the organized paranodal axoglial junction is the segregation of the ion channel–rich domains of the node and juxtaparanode, which become displaced on disruption of the paranode. To determine whether the elongated Nfasc155<sup>+</sup> paranodal profiles represented disrupted

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**FIGURE 3.** Paranodal disruption is associated with underlying axonal pathology. (A) Nfasc155<sup>+</sup> paranode length correlates with the incidence of SMI32<sup>+</sup> axons per normal-appearing white matter (NAWM) region of interest (Spearman nonparametric correlation). (B) Quantification of individual Nfasc155<sup>+</sup> paranodes reveals an increase in length of those associated with SMI32<sup>+</sup> axons versus Nfasc155<sup>+</sup> paranodes on SMI32-negative profiles (Box plot of mean paranodal lengths per case showing minimum, maximum, interquartile, and median values). Analysis of variance and Bonferroni multiple-comparison post tests (*p < 0.05; **p < 0.001). (C-C2, D) SMI32<sup>+</sup> axons associated with disrupted Nfasc155<sup>+</sup> paranodes and SMI32<sup>+</sup> axonal swellings are noted at nodes of Ranvier. (E) Amyloid precursor protein accumulation, an indicator of axonal abnormality, was noted in NAWM axons that were occasionally associated with disrupted nodal profiles. Scale bars = (C-E) 2 μm.
structures and if this was associated with local inflammation, we examined the distribution of nodal (pan Na\textsubscript{1,1}\textsuperscript{+}) and juxtaparanodal (K\textsubscript{v1,1,2}\textsuperscript{+}) domains in NAWM samples with low or high levels of inflammation (Table) versus controls (Fig. 4). There was significant displacement of K\textsubscript{v1,1}\textsuperscript{+} domains closer to the node of Ranvier in MS NAWM. The displacement was greatest in the MS group with the most pronounced inflammation in the NAWM (separation from node to juxtaparanode for control = 4.6 ± 0.2 μm, MS low = 4.2 ± 0.2 μm, MS high = 3.7 ± 0.1 μm; Fig. 4D). The occurrence of overlapping Nfasc155\textsuperscript{+}/K\textsubscript{v1,1}\textsuperscript{+} domains was also greatest in this group (percent overlapping juxtaparanodal/paranodal domains for control = 10.8%, MS low = 34.6%, and MS high = 46%; Fig. 4E).

Quantification of Na\textsubscript{1,1}\textsuperscript{+} nodal profiles revealed no size differences in these structures between control and MS (Fig. 4F). These data are in agreement with our previous observations of an unchanged nodal gap between paired Nfasc155\textsuperscript{+} paranodes. Na\textsubscript{1,6} channels are the primary Na\textsubscript{1} channel subtype revealed instances of attenuated or undetectable staining of nodes with normal and disrupted Caspr1\textsuperscript{+} paranodes. Scale bars = (A–C) 2 μm; (G–I, K, L) 1 μm; (J) 0.5 μm.

FIGURE 4. Microgliosis and paranodal disruption result in displacement of juxtaparanodal domains (JPN) and loss of Na\textsubscript{1,6} expression at nodes. (A–C) K\textsubscript{v1,1,2}\textsuperscript{+} that demarcate the JPN domains underneath the myelin sheath are normally separated from the node by Nfasc155\textsuperscript{+} paranodes. Nfasc155\textsuperscript{+} paranodal junctions with a disrupted appearance frequently coincide with apparent encroachment of JPN K\textsubscript{v1,1}\textsuperscript{+} channels (arrows, B, C). (D–F) Multiple sclerosis normal-appearing white matter (MS NAWM) was categorized as containing relatively low (MS "low") NAWM inflammation/axonal pathology or relatively high inflammation/axonal pathology (MS "high") (Table). K\textsubscript{v1,1}\textsuperscript{+} JPN profiles were displaced nearer to the node of Ranvier in MS NAWM, with the JPN nodal separation smallest in the MS high group (D). Within the same group, the occurrence of overlapping Nfasc155\textsuperscript{+}/K\textsubscript{v1,1}\textsuperscript{+} domains was greatest (E). Box plot representing mean values/case showing minimum, maximum, interquartile, and median values. Analysis of variance and Bonferroni post test (D, E; *p < 0.05; **p < 0.01). Quantification of Na\textsubscript{1,1}\textsuperscript{+} nodal profiles associated with paranodal Nfasc155\textsuperscript{+} immunolabeling revealed these structures to be unchanged in size between control and MS (F). (G–I) Na\textsubscript{1,1} at the node associate with cytoskeletal proteins such as βIV spectrin, which labels nodes of Ranvier. (J–L) Immunostaining for the Na\textsubscript{1,6} channel subtype revealed instances of attenuated or undetectable staining of nodes with normal and disrupted Caspr1\textsuperscript{+} paranodes.
component of mature nodes and are tethered at the axolemma through interactions with axonal neurofascin (Nfasc186), contactin, ankyrin G, and βIV spectrin (24). Immunolabeling revealed that most Nfasc155+ paranodal axoglial junctions were associated with βIV spectrin+ (Figs. 4G–I) or Na1+,1+ (Figs. 2A, B, and E) nodes, although the expression of Na1,1.6 was attenuated and sometimes undetectable between Caspr1+ paranodes (Figs. 4J–L).

**Microglial Activation Precedes the Onset of Clinical Disease in EAE**

To study the relationship between inflammation and paranodal disruption, we used a murine MS model (25). Microglial activation, quantified by the area of CD11b-IR in transverse sections of spinal cord, was increased in MOG-immunized animals at 10 dpi and in long-term CFA and MOG groups at 44 dpi (Figs. 5A, B). At 10 dpi in MOG EAE (before the appearance of clinical signs and the influx of peripheral immune cells), CD11b-IR was increased, and double immunofluorescence for iNOS and TLR4 indicated the presence of reactive microglia in perivascular and parenchymal locations in lumbar spinal cord WM (Fig. 5C). Adjuvant-only (CFA dpi 10) animals contained activated (TLR4+/IBA1+) microglia that were not found in long-term CFA animals, suggesting that their activation state (as assessed by TLR4-IR) was reduced (Figs. 5D, E); however, CD11b-IR was increased (p < 0.01; Fig. 5B). Thus, by analyzing paranodal changes at 10 dpi, we determined the effects of microglial activation on the integrity of the axoglial unit in the absence of parenchymal lymphocytic infiltration and demyelination.

**Disruption of Nfasc155+ Paranodes in MOG and CFA-Immunized Animals**

In preclinical MOG-immunized mice at 10 dpi, there were discrete and elongated Nfasc155+ paranodes in the lateral lumbar spinal cord (some distance from potentially confounding meningeal infiltrates; Fig. 5F). Measurement of paranodal length, width, and nodal gap in MOG-immunized mice revealed significant elongation of Nfasc155+ paranodes by 10 dpi (3.0 ± 0.3 μm; 17% longer than controls, 2.6 ± 0.1 μm), which further increased in long-term MOG-immunized mice (4.3 ± 0.2 μm; 67% longer). The CFA-immunized animals at 10 dpi not only exhibited microglial activation (TLR4+) but also had significantly elongated paranodal profiles (3.1 ± 0.1 μm). However, paranodal disruption in the CFA group appeared transient because Nfasc155+ paranodal length returned to control levels in the long-term animals (Fig. 5G), when microglial TLR4 expression had resolved. In agreement with data from the human tissue, paranodal Nfasc155+ width and the length of the unstained nodal gap between pairs of Nfasc155+ structures were unchanged in all groups compared with naive controls (data not shown).

**Nfasc155+ Paranodal Disruption in EAE Is Associated With Juxtapanodal Rearrangements and Axonal Stress**

Nfasc155+ paranodal elongation in MOG-immunized animals likely indicates disruption of axoglial junctions because it was accompanied by encroachment of juxtapanodal K1.2 channels toward the node. There was a significantly increased incidence of overlapping K1.2+/Nfasc155+ expression in both MOG- (38.3% ± 2.5%, p < 0.001) and CFA- (28.9% ± 0.6%, p < 0.05) immunized animals versus naive controls (Figs. 6A, B, and D). SM32+ axons in both experimental groups were associated with elongated Nfasc155+ paranodes (Figs. 6C, E). In MOG-immunized animals, paranodal lengths were 32% greater for paranodes associated with SM32+ axons (3.0 ± 0.2 μm) in comparison to those not associated with SM32+ axons (2.3 ± 0.1 μm). A similar relationship was found for CFA animals (Fig. 6E). Therefore, at presymptomatic stages, axonal stress and microgliosis are accompanied by paranodal axoglial alterations similar to that seen in MS NAWM.

**Disruption of Nodal Expression of Voltage-Gated Sodium Channels**

Disrupted paranodes were often accompanied by changes in the composition (Na1,1 channel subtype), but not morphology, of nodes in both MS NAWM and EAE (Figs. 4 and 7). To determine how nodal components were affected in early disease in relation to changes at the paranodal axoglial junction, we investigated the expression of Na1,1.6 channel subtype in combination with Nfasc155 expression. Paranodes were almost always separated by βIV spectrin+ nodes (Fig. 7A; βIV spectrin+ nodes per pair of Caspr1+ paranodes from naive (94% ± 1%) and MOG-immunized animals (95% ± 1%), respectively; 1070 paranodes examined) and pan-Na1+,1+ nodes per Nfasc155+ paranodes in naive (94% ± 1%) and MOG-immunized animals (95% ± 1%), respectively; 800 paranodes examined). In contrast, there were numerous instances of Na1,1.6-negative nodes (Figs. 7B–E). Confocal analyses of image stacks were used to generate line histograms of signal intensity through the nodal region to reveal this to be a true loss of detectable Na1,1.6-IR at these nodes (Figs. 7F, G). We measured the number of normal-appearing nodes (strong Na1,1.6-IR; Fig. 7C) and the combined frequency of nodes with no Na1,1.6 reactivity (Na1,1.6-negative nodes), or abnormal morphology, in association with Nfasc155+ structures. At 10 dpi, both MOG- and CFA-immunized animals had a reduction in the number of normal-appearing nodes compared with naive (27% and 33.3% reduction, respectively; Figs. 7H, I). At 44 dpi, MOG-immunized animals showed a significant reduction in normal-appearing Na1,1.6+ nodes compared with naive (46.4% reduction). The loss of Na1,1.6 expression in CFA animals at 10 dpi was reversed at 44 dpi, indicating the transient nature of nodal/paranodal abnormalities in the absence of a prolonged antigenic stimulus (Fig. 7H) and microglial activation (Fig. 5).

**Minocycline Reduces Microglial Activation and Nodal/Paranodal Disruption**

We next examined paranodal and nodal domains in MOG-immunized mice that had been treated with minocycline, a potent inhibitor of systemic and CNS inflammation. After 13 days of prophylactic minocycline treatment (when vehicle-treated MOG-immunized mice first displayed signs), microgliosis had a resting morphology (Figs. 8D–I), and the density of CD11b-IR (Figs. 8A–C; 4.87% ± 0.3%) was...
significantly reduced compared with that in vehicle-treated MOG-immunized mice (11.4% ± 2.4%). Therefore, administration of minocycline reduces the level of CNS parenchymal inflammation at this time point.

The average paranodal length in minocycline-treated animals (2.87 ± 0.58 μm) was reduced in comparison to vehicle controls (3.55 ± 1.2 μm; Fig. 8F) to a level comparable to that of naive animals (Fig. 5G). Minocycline-treated animals

FIGURE 6. Paranodal axoglial disruption in experimental autoimmune encephalomyelitis is associated with juxtaparanodal (JPN) rearrangements and axon stress. (A) Longitudinal sections of lumbar spinal cord white matter containing K_v1.2^+ JPN and Nfasc155^+ paranodal domains located in a nonoverlapping conformation. (B) Myelin oligodendrocyte glycoprotein (MOG)-immunized mice at 10 days postimmunization (dpi) frequently had K_v1.2^+ encroachment into disrupted paranodal domains. (C) SMI32^+ stressed/damaged axons associated with disrupted Nfasc155^+ profiles in both complete Freund adjuvant (CFA)- and MOG-immunized groups at 10 dpi (example from a MOG 10 dpi animal). (D) The incidence of overlapping K_v1.2/Nfasc155 domains was significantly increased in CFA- and MOG-immunized 10 dpi mice versus naive controls (proportion of overlapping K_v1.2^+ Nfasc155^+ domains of total K_v1.2^+ Nfasc155^+ domains analyzed; n = 5 per group; ANOVA with Bonferroni post test). (E) Damaged (SMI32^+) axons associated with elongated Nfasc155^+ paranodal profiles versus Nfasc155^+ paranodes on SMI32-negative fibers in both CFA and MOG 10 dpi groups. (Box plot of mean paranodal lengths per case showing minimum, maximum, interquartile, and median values; CFA or MOG group means compared by Mann-Whitney t test.). n = 5 animals per group. *p < 0.05; **p < 0.01; ***p < 0.0001. Scale bars = (A–C) 2 μm.

FIGURE 5. Microglial inflammation associated with the disruption of paranodal junctions in experimental autoimmune encephalomyelitis. (A, B) Immunohistochemistry for CD11b revealed significant increases in microglial density in transverse sections of spinal cord of myelin oligodendrocyte glycoprotein (MOG)_{35-55}-immunized mice at day 10 after immunization (MOG 10 dpi) and long-term complete Freund adjuvant (CFA) and MOG groups (44 dpi). Analysis of variance with Bonferroni post test (n = 5 animals/group). (C–E) MOG-immunized mice at 10 dpi contained meningeal, perivascular, and parenchymal toll-like receptor 4 (TLR4)^+ and iNOS^+ monocytes/microglia in lumbar spinal cord. TLR4+ microglia are noted in CFA 10 dpi tissue but not in the CFA long-term animals, indicating temporary microglial activation in this group. (F, G) Elongated Nfasc155^+ paranodes are present in preclinical MOG 10 dpi tissue (inset); paranodal elongation was significantly different from naive mice in CFA 10 dpi, MOG 10 dpi, and MOG 44 dpi lumbar spinal cord. Nfasc155 changes were reversed in the long-term CFA group. Box plot of mean paranodal lengths per case showing minimum, maximum, interquartile, and median values. Analysis of variance with Bonferroni post test (n = 5 mice/group). *p < 0.05; **p < 0.01; ***p < 0.001. Scale bars = (A) 300 μm; (C–E) 20 μm; (F) 2 μm.

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displayed significantly greater expression of nodal Na$_{v}$.1.6$ \text{+}$ associated with Nfasc155$^+$ paranodes (75.3$ \% \pm 4.1$%) versus vehicle-MOG animals (65$ \% \pm 5$%; Fig. 8I). These data demonstrate that an attenuation of inflammation is sufficient to reduce nodal paranodal pathology in vivo.

**DISCUSSION**

We have demonstrated that disruption of paranodal and nodal domains correlates with axonal pathology and the degree of local microglial inflammation in MS NAWM and EAE spinal cord and suggests that this could provide a novel focus of tissue injury in acute and chronic CNS diseases. These alterations likely would 1) disrupt normal neurotransmission along the affected fibers, 2) disrupt axoglial communication, 3) disrupt the axonal cytoskeleton, thereby affecting normal axon transport, and 4) expose new targets to immune attack that could further exacerbate damage to the axon or ensheathing oligodendrocyte.

**Paranodal/Nodal Disruption Is Closely Associated With Axonal Pathology**

Paranodal disruption manifests as a loss of the electron dense transverse bands that are believed to represent the protein-protein interactions of the axoglial unit with a concomitant widening of the axoglial space (12, 13, 26). The increased separation at the axoglial junction is accompanied by elongation of the longitudinal paranodal profile, as shown by alterations in Nfasc155$^+$ and Caspr1$^+$ immunostaining. This could represent the progressive loss or disrupted contact of the innermost turns of the myelin sheath, as seen in the Nfasc155 null mouse (18), which is accompanied by encroachment of juxtaparanodal constituents into paranodal domains, as the molecular fence to protein diffusion is removed (27). A greater incidence of juxtaparanodal K$_{v}$.1$'$ in Nfasc155$^+$ domains was noted in both MS and EAE tissues, indicating that paranodal sealing was reduced, with predictable consequences for nerve conduction dynamics.

We observed that disrupted Nfasc155$^+$ paranodes were nearly always associated with stressed/damaged axons as visualized using the SMI32 antibody, a marker of early axonal pathology in MS, Alzheimer disease, and amyotrophic lateral sclerosis (28, 29). We only considered Nfasc155$^+$ profiles in association with long SMI32$^+$ axons because hypophosphorylated neurofilaments are a feature of some normal nodes. Although it is not possible to determine the relative timing of paranode disruption and that of neurofilament dephosphorylation in the postmortem material, the frequency of Nfasc155$^+$/SMI32$^+$ structures suggests that such changes may occur concurrently. How might axoglial disruption be concurrent with axon damage? Nfasc155 is required for the proper targeting of Caspr1/contactin to the axonal membrane, and together, they form protein complexes stabilized by interactions with the axon cytoskeleton via the cytoplasmic tail of Caspr1 bound to protein 4.1B (11, 30). Ankyrin B, 4.1B, and αII- and βII-spectrin accumulate subsequent to Caspr1, generating a specialized cytoskeleton at the paranode that may be linked to axonal neurofilaments (31). Therefore, axoglial disruption may be the cause or the consequence of axonal paranodal cytoskeletal alterations associated with localized abnormalities (32). Consistent with this, axonal swellings occur at the node in the Nfasc155 null animal (18) and we noted many SMI32$^+$ focal swellings and end bulbs, together with accumulations of amyloid precursor protein at nodal regions, indicating alterations in the axon cytoskeleton and normal fast transport in areas of paranodal disruption.

**Paranodal Disruption as a Consequence of Microglial Inflammation**

Multiple sclerosis NAWM is characterized by microglial activation, lymphocyte infiltration, myelin damage, and axonopathy that do not manifest as frankly demyelinated lesions (6, 33–36). Activated microglia contribute to the inflammatory milieu by the secretion of cytokines and free radicals, and they sustain inflammation by presenting antigen to lymphocytes (37). We confirmed the presence of reactive microglia based on their morphology and expression of markers induced by proinflammatory agents (38). Microglial/macrophage derived glutamate is an important mediator of cell damage in MS, and extracellular glutamate causes paranodal disruption, juxtaparanodal rearrangements, and eventual axonal degeneration (39, 40).

Although there was no correlation between perivascular T-cell infiltrates and changes in Nfasc155 expression, it is likely that T-cell infiltrates could sustain parenchymal inflammation, thereby indirectly affecting axoglial domains. Axonal pathology caused by distant lesions might also lead to axoglial disruption in the NAWM. By visualizing myelin and axons using immunohistochemistry, we ensured that the NAWM samples did not contain axons emanating from lesions from the same tissue block; however, because of the heterogeneity of lesion sites, we cannot be sure that sampled axons had not been damaged as a consequence of distant demyelination.

**FIGURE 7.** Disruption of nodal Na$_{v}$.1.6$ expression in experimental autoimmune encephalomyelitis. (A) βIV Spectrin$^+$ nodes associated with most Caspr1$^+$ paranodes in myelin oligodendrocyte glycoprotein (MOG)-immunized 10 dpi animals. (B) Nodal abnormalities took the form of Na$_{v}$.1.6-negative nodes (asterisks) and heminodes (arrows, B) at Nfasc155$^+$ paranodes. (C–E) Na$_{v}$.1.6-negative nodes were associated with normal-appearing and disrupted paranodal profiles. (F, G) The intensity of Nfasc155 (green line) and Na$_{v}$.1.6 (red line) immunostaining from regular and Na$_{v}$.1.6-negative nodes was calculated by plotting the mean of 20 normal and 20 Na$_{v}$.1.6-negative nodes (pixel intensity, 0–255 U), normalized, and averaged from single confocal images. These data confirmed the absence of detectable Na$_{v}$.1.6 at the node. (H, I) The proportions of aberrant (Na$_{v}$.1.6-negative or Na$_{v}$.1.6 heminodes) nodes were significantly different in MOG- and CFA-immunized animals at 10 dpi compared with naïve animals. This trend was reversed in long-term CFA animals (H); there was an increased prevalence of aberrant nodes in long-term MOG-immunized animals versus naïve controls (I). Analysis of variance and Bonferroni post test (**p < 0.001). n = 5 animals per group. dpi, days postinduction. Scale bars = (A, B) 5 μm; (C–E) 2 μm.
Nevertheless, in nearly all instances of paranodal alterations that are associated with SMI32 positivity, the pattern of axonal nonphosphorylated neurofilament expression was adjacent to affected paranodes, suggesting that most axonal changes were local and at the site of paranodal dysfunction.

In PD, dying neurons, alterations in the BBB, and a proinflammatory milieu (among other factors) activate microglia with a resting morphology that lacked toll-like receptor 4 expression, in contrast to microglia in vehicle MOG-immunized mice. Nfasc155+ paranode length was increased in vehicle-treated animals in comparison to the minocycline group (box plot of mean paranodal lengths per case showing minimum, maximum, interquartile, and median values); the incidence of nodal abnormalities was greater in vehicle-dosed mice. n = 5 animals per group. *p < 0.05; **p < 0.01; Mann-Whitney test. dpi, days postinduction. Scale bars = (A, B) 200 μm; (D, E, G, H) 30 μm.

FIGURE 8. Administration of minocycline reduced the density of microglial inflammation and abolished disease-induced changes at paranodal and nodal units. (A–C) The density of CD11b immunoreactivity (mean signal intensity per animal ± SEM) in transverse spinal cord was greatly reduced in minocycline-treated mice versus vehicle-dosed myelin oligodendrocyte glycoprotein (MOG)-immunized animals. (D–I) Minocycline-treated animals showed microglia with a resting morphology that lacked toll-like receptor 4 expression, in contrast to microglia in vehicle MOG-immunized mice. Nfasc155+ paranode length was increased in vehicle-treated animals in comparison to the minocycline group (box plot of mean paranodal lengths per case showing minimum, maximum, interquartile, and median values); the incidence of nodal abnormalities was greater in vehicle-dosed mice. n = 5 animals per group. *p < 0.05; **p < 0.01; Mann-Whitney test. dpi, days postinduction. Scale bars = (A, B) 200 μm; (D, E, G, H) 30 μm.
add further evidence of an important role for a diffuse innate inflammatory process in neurodegeneration in PD. These data suggest that nodal pathology may occur in the absence of an overt adaptive immune response and be a consequence of the microglial inflammation that is common to a number of neurodegenerative/neuroinflammatory conditions.

Central nervous system alterations are apparent before the onset of clinical signs in MOG35-55 induced EAE (45–47). This is in part due to the presence of TLR ligands (e.g. mycobacteria) in CFA that can stimulate the proinflammatory signaling and microglial inflammation necessary for disease induction (48, 49). The preclinical CNS inflammatory response is initiated by the early induction of the innate immune system, which precedes the influx of peripheral immune cells (50). Changes in the expression of proinflammatory gene products, such as CD11b, myeloperoxidase, and cyclooxygenase 2, are noted as early as 3 dpi and occur to a similar extent in MOG35-55–immunized and CFA-only mice. Changes in proinflammatory gene expression were also noted in Rag1−/− mice that lack the adaptive arm of the immune system due to the absence of B and T lymphocytes (50). Here, we show that MOG-immunized animals at 10 dpi (before the influx of inflammatory cells) showed activation of microglia (iNOS+, TLR4+) that was associated with axoglial disruption and axonal injury. Similar but transient changes in nodal/paranodal domains were noted in CFA-only–treated animals, supporting the theory that an activated innate immune system is the likely source of factors that are capable of disrupting these domains at this early time point. In the absence of a sustaining CNS antigen (i.e. MOG peptide) in the long-term CFA group, microglial expression of TLR4 resolved, and the “resting” phenotype was associated with nodal domains unchanged in comparison to age-matched naive animals. These observations suggest that proinflammatory activation of microglia is sufficient to induce nodal/paranodal abnormalities and associated axonal pathology. Minocycline, a semi-synthetic tetracycline, freely crosses the BBB and inhibits CNS inflammation through direct effects on resident microglia/macrophages and on the peripheral immune system by inhibiting the activation of autoreactive T cells (51–53), which reduces neuroinflammation and may be neuroprotective in animal models and various neurodegenerative diseases as well as MS (54). Minocycline reduced microglial density and nodal/paranodal disruption, thereby providing further evidence for the damaging potential of inflammation at nodal and paranodal domains.

Reduced Expression of Na1.6 at Affected Nodes

Maintenance of the correct molecular architecture of the node of Ranvier is vital for continued rapid propagation of electrical impulses (24). Myelination and the formation of paranodal axoglial junctions are important for the transition from Na1.2 to Na1.6 as nodes mature (55, 56), and reduced expression of Na1.6 results in slowed conduction (57, 58). Most nodes of myelinated axons in MS NAWM expressed βIV spectrin and the pan-Na1 marker. Nevertheless, there were numerous Na1.6-negative nodes between paranodes, analogous to what has been reported in experimental allergic neuritis and that correlate with a reduced nerve conduction (59). The loss of detectable Na1.6 was investigated in pre-clinical and long-term EAE, in which a significant proportion of WM nodes lacked measurable Na1.6 expression. These changes persisted in MOG-immunized animals with chronic disease but were not seen in the long-term CFA controls, indicating that the regulation of Na1.6 expression at the node was directly affected by the inflammatory milieu. Loss of the major Na1 channel at nodes, disruption of paranodal axoglial junctions, and displacement of K1, channels would likely contribute to changes in nerve conduction along affected axons, which warrants further investigation.

It is not possible to determine from analysis of postmortem tissues at which point paranodal alterations become clinically relevant. However, similar structural changes are seen in neurofascin null mice that, despite the presence of normal compact internodal myelin, display significant conduction deficits (11). Further electrophysiological studies using a recently described glial cell–specific knockdown of Nfasc155 (18) will help to determine the clinical significance of paranodal disruption.

Changes at the Paranodal Axoglial Junction May Be an Early Marker of Cell Stress and a Precursor to Demyelination and Neurodegeneration

Subtle myelin abnormalities in MS NAWM may allow T-cell– or antibody-mediated attack to occur more readily by the exposure of internodal oligodendrocyte and axonal membranes to the inflammatory milieu. Such a scenario is supported by data from the myelin proteolipid protein–overexpressing rat that exhibits exacerbated EAE that is facilitated by mild microglial inflammation and unstable myelin (60). Furthermore, autoimmunity to neurofascin and contact1 has recently been described in some MS patients (61, 62), and accessibility of such antigens might be enhanced in areas of paranodal abnormalities.

Demyelinated axons in MS are vulnerable to further injury that culminates in their destruction. The denuded axon, in an attempt to restore conduction, re-expresses Na1, along its membrane (63, 64), thereby contributing to an elevated energy demand on a structure exposed to damaging inflammation that is likely to result in its degeneration (65). Therefore, in addition to expected effects on saltatory conduction, changes at the paranode in the presence of activated microglia could be the prelude to demyelination and neurodegeneration in many pathological scenarios, thereby contributing to an array of diffuse clinical manifestations.

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