Disruption of neurofascin localization reveals early changes preceding demyelination and remyelination in multiple sclerosis

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Saltatory conduction in the nervous system is enabled through the intimate association between the leading edge of the myelin sheath and the axonal membrane to demarcate the node of Ranvier. The 186 kDa neuron specific isoform of the adhesion molecule neurofascin (Nfasc186) is required for the clustering of voltage gated Na\(^+\) channels at the node, whilst the 155 kDa glial specific isoform (Nfasc155) is required for the assembly of correct paranodal junctions. In order to understand the relationship between these vital structures and how they are affected in multiple sclerosis we have examined the expression of Nfasc155 and Nfasc186 in areas of inflammation, demyelination and remyelination from post-mortem brains. Fourteen cases of neuropathologically confirmed multiple sclerosis (8 female and 6 male; post-mortem delay 7–24 h; age 37–77 years; and disease duration 15–40 years), comprising 20 tissue blocks with 32 demyelinating or remyelinating lesions, were used in this study. A significant early alteration in Nfasc155\(^+\) paranodal structures occurs within and adjacent to actively demyelinating white matter lesions that are associated with damaged axons. Shaker-type K\(_v\)1.2 channels, normally located distally to the paranode, overlapped with the disrupted Nfasc155\(^+\) structures. In the absence of Nfasc155, K\(_v\)1.2 channels abutted normally clustered Nfasc186\(^+\) nodes, indicating that complete disruption of the paranodal structure and movement of K\(_v\)1.2 channels precede alterations at the node itself. Within areas of partial remyelination, a number of atypical triple-Nfasc155\(^+\) structures were noted that may represent transient oligodendrocyte-axonal contacts during the process of myelin repair or aberrant interactions. Within shadow plaques discretely clustered Na\(_v\)^\(^+\), Nfasc186\(^+\) and Nfasc155\(^+\) domains indicated the restoration of normal nodal architecture. The alterations in oligodendrocyte Nfasc155 expression that accompany inflammation and demyelination suggest an ongoing disruption to the axonal–oligodendrocyte complex within newly forming as well as established lesions in multiple sclerosis, resulting in destruction of the Nfasc186\(^+\)/Na\(_v\)^\(^+\) nodal complex vital to successful fast neurotransmission in the CNS.

**Keywords**: multiple sclerosis; neurofascin; axo-glial junction; node of Ranvier, demyelination

**Abbreviations**: MOG = myelin oligodendrocyte glycoprotein

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**Introduction**

Oligodendrocyte derived myelin enwraps axons in the CNS allowing the rapid propagation of action potentials from node to node by saltatory conduction. The excited node depolarizes mainly through the opening of voltage gated sodium channels (Na\(_v\)) that are present at a high density and subsequently re-polarizes by, in part, the movement of K\(^+\) via inwardly rectifying K\(_v\) channels concentrated at the juxtaparanode. Na\(_v\) channels assemble at high
concentrations at the node through interactions with proteins including the 186 kDa isofrom of neurofascin (Nfasc186). K\textsubscript{v} channels of the juxtaparanode are separated from the node by the specialized paranodal junctions that require the interaction of an alternatively spliced glial-specific 155 kDa isoform of neurofascin (Nfasc155) in the end loops of the oligodendrocyte with proteins of the axonal membrane. The correct assembly and maintenance of paranodal junctions are vital to the integrity of the node and juxtaparanodal regions, permitting fast neurotransmission along myelinated fibres (Salzer, 2003; Poliak and Peles, 2003; Sherman and Brophy, 2005).

The correct expression of Nfasc155 and Nfasc186 is integral to the formation of paranodal junctions and the node of Ranvier (Sherman et al., 2005). Oligodendroglial Nfasc155 is believed to associate with a complex of Caspr-1 and the GPI-linked protein contactin in the axonal membrane, to form the septate-like paranodal junctions that flank the node of Ranvier (Charles et al., 2002; Sherman et al., 2005). Caspr-1 and contactin are required for their mutual targeting to the axonal membrane for paranodal assembly, although glial Nfasc155 is still detectable at the paranodes in their absence (Bhat et al., 2001; Boyle et al., 2001). In contrast, Nfasc155 appears necessary for Caspr-1 and contactin to be detectable at the axonal membrane, allowing correct paranodal structures to be generated (Sherman et al., 2005). In the absence of these individual paranodal components cytoplasm rich glial end loops are disorganized and paranodal junctions compromised, resulting in severely reduced conduction velocities contributing to muscle paresis and tremors in knockout animals (Bhat et al., 2001; Boyle et al., 2001; Sherman et al., 2005). The importance of the paranodal junction to the integrity of the node of Ranvier has been elegantly demonstrated in a number of different animal models. Dysmyelinating mutants or animals unable to synthesize major myelin constituents have disrupted paranodes and juxtaparanodal K\textsubscript{v} ion channels, which are normally insulated from the node by the paranode, that are displaced to paranodal and nodal domains; whereby efficient nerve conduction is impeded (Dupree et al., 1998; Dupree et al., 1999; Tait et al., 2000; Mathis et al., 2001; Arroyo et al., 2002).

Nfasc186 is localized to the axonal membrane of the axon initial segment and at the node of Ranvier where it associates with ankyrin G, neuronal cell adhesion molecule (NrCAM) and a high density of Na\textsubscript{v} channels (Lambert et al., 1997; Van Wart et al., 2005). The interplay between NrCAM, Nfasc186 and Ankyrin G is important in correct assembly of Na\textsubscript{v} channel clusters and Nfasc186 appears pivotal to this process (Lambert et al., 1997; Custer et al., 2003). A pioneering role for Nfasc186 in node assembly was demonstrated by the complete lack of nodal clustering in Nfasc186 null animals. NrCAM, ankyrinG, βIV-spectrin and Na\textsubscript{v} channels were completely undetectable implying a need for Nfasc186 protein in recruiting the other components of the mature node of Ranvier required for rapid nerve conduction to take place (Sherman et al., 2005).

Perivascular inflammation, myelin and oligodendrocyte damage, axonal loss and astrocytic scarring characterize multiple sclerosis. Many of the earliest clinical symptoms of multiple sclerosis are suggested to be attributable to a reduction in axonal conduction velocity due to the inflammatory milieu and disruption of the myelin-axonal complex at the node of Ranvier (Compston and Coles, 2002) before segmental demyelination occurs. However, little is known about the early changes to the axon–myelin unit in multiple sclerosis lesions. Loss of paranodal junctions and remyelination is accompanied by the slow diffusion of nodal components, in particular the Na\textsubscript{v} channels, in order to restore the ability to conduct an action potential along the denuded axon, albeit at a far reduced rate (Moll et al., 1991; Felts et al., 1997; Waxman, 1998; Craner et al., 2003; Waxman et al., 2004). Remyelination is frequently seen at the edge of multiple sclerosis lesions, whereby newly generated oligodendrocytes wrap myelin sheaths around denuded axons (Prineas and Connell, 1979; Franklin, 2002). Remyelination contributes to remission of clinical symptoms through the restoration of fast conduction (Smith et al., 1981) and results in the clustering of previously disrupted nodal, paranodal and juxtaparanodal protein assemblies accompanied by the return of normal fast nerve transmission (Dugandzija-Novakovic et al., 1995; Rasband et al., 1998; Craner et al., 2003).

Paranodal domains are believed to be disrupted prior to nodal domains during demyelination and the disruption of axonal Caspr-1 localization has been demonstrated to be a sensitive indicator of myelin abnormalities at sites near chronic demyelinated lesions (Wolsiwik and Balesar, 2003). However, little is known concerning how areas of inflammatory demyelination or remyelination in the multiple sclerosis brain influence the composition and integrity of the other nodal components vital to successful fast nerve conduction. Due to the proposed requirement for glial and axonal neurofascin in the assembly of nodal and paranodal domains during myelination (Sherman et al., 2005), we have undertaken an analysis of the expression pattern of Nfasc155 and Nfasc186 to determine the changes that occur at the node of Ranvier during the formation and repair of multiple sclerosis lesions, in comparison to normal appearing brain tissue.

**Material and methods**

**Human post-mortem tissue and lesion characterization**

Tissue blocks for this study were provided by the UK Multiple Sclerosis Tissue Bank at Imperial College. All tissues were collected following fully informed consent by the donors via a prospective donor scheme following ethical approval by the London Multicentre Research Ethics Committee (MREC 02/2/39). Fourteen
cases of neuropathologically confirmed multiple sclerosis (Table 1: 8 female and 6 male; post-mortem delay 7–24 h; age 37–77 years and disease duration 15–40 years), comprising 20 tissue blocks with 32 demyelinating or remyelinating lesions, were used in this study. Tissue was fixed for a minimum of 4 h in 4% paraformaldehyde, cryoprotected in 30% sucrose in phosphate buffered saline (PBS) and cryosectioned at 8 μm. Tissue sections were stored at −20°C until required.

Tissue was characterized as normal appearing or lesion by screening with a panel of histochemical and immunohistological markers. Multiple sclerosis plaques were grouped as: (i) actively demyelinating; (ii) chronically demyelinated and (iii) remyelinating/shadow plaque according to published criteria (Lucchinetti et al., 1996; Lassmann et al., 1998). Areas of active demyelination were characterized by the presence of large numbers of HLA-DR+ macrophages throughout the lesion, decreased luxol fast blue (LFB) and myelin oligodendrocyte glycoprotein (MOG) staining, myelin vacuolization and evidence of myelin breakdown products within macrophages. Immunologically active lesions were sub-grouped as early active or late active demyelinating lesions according to the presence of MOG and LFB positive degradation products within macrophages (Lucchinetti et al., 1996). Lesions with hypocellular and fully demyelinated centres with little immune activity, but with evidence of ongoing demyelination at the lesion edge were termed chronic active lesions. Hypocellular lesions with no evidence of ongoing demyelination or immune activity were termed chronic inactive. Areas of remyelination were identified by the presence of thinly labelled MOG+ myelin sheaths, shorter internodes and small round MOG+ cells with a compact nucleus that were often associated with adjacent areas of late active demyelination and HLA-DR+ macrophages and microglia (Lassmann and Wekerle, 2005). Completely remyelinated plaques were characterized by thin myelin sheaths and macroscopically by well-defined areas of LFB+ myelin pallor (shadow plaques). Such areas had evidence of gliosis and only residual numbers of HLA-DR+ cells.

### Immunohistochemistry

Tissue sections were pretreated with 0.5 mg/ml sodium borohydride in PBS for 5 min or with a 0.3% solution of H2O2, prior to immunodetection by fluorescence or peroxidase techniques, respectively. Sections to be probed with anti-melanin protein antibodies or with antibodies to neurofascin were pretreated with cold methanol for 8 min prior to 30 min in a 3% solution of normal goat serum (Sigma) in PBS containing 0.1% Triton X-100 (NGS-Tx buffer). Tissue sections were exposed to primary antibodies in PBS or in NGS-Tx buffer overnight before detection with biotinylated goat anti-mouse or goat anti-rabbit antibodies, or fluorochrome labelled secondary antisera (alexafluor-488 or Cy3). Streptavidin conjugated horseradish peroxidase or streptavidin conjugated alexafluor-488 were used for labelling biotin-conjugated secondary antisera for immunohistochemical or immunofluorescent detection, respectively. All sections for double immunofluorescence were subsequently probed with appropriate secondary antibody and secondary conjugate prior to counter-staining with the nuclear dye 4’,6’-diamidino-2-phenylindole (DAPI; Sigma), rinsed in dH2O and coverslipped in aqueous mountant (Vectashield, Vector Laboratories, Peterborough, UK). Immunoperoxidase labelled sections were developed using diaminobenzidine as chromogen and counterstained with 0.1% cresyl violet. Sections were dehydrated through alcohol, cleared in xylene and coverslipped in DPX mountant (VWR International, Lutterworth, UK). All secondary antibody controls, processed using identical protocols to that noted above, except for the omission of primary antibodies, were negative for staining.

### Antibodies

Primary antibodies: mouse anti-MOG (clone Z12, S. Piddlesden, University of Cardiff, UK); mouse anti-neurofilament-H protein (clone RT97; Chemicon, Chandlers Ford, UK); mouse anti-dephosphorylated neurofilament protein (clone smi32;
Results

Pattern of Nfasc155 and Nfasc186 expression in normal appearing brain

Immunohistochemistry with isoform specific polyclonal antibodies against Nfasc155 revealed regions of Nfasc155 immunoreactivity with the expected distinct paranodal distribution (hereafter referred to as Nfasc155+ paranodes) aligned along MOG+ and neurofilament-H+ (NFil) processes in normal appearing subcortical white matter from multiple sclerosis brain (Fig. 1A and B). A similar pattern of Nfasc155 immunostaining was seen in non-pathological control brain sections (data not shown). Nfasc186+ structures were observed as narrow node-like bands (hereafter referred to as Nfasc186+ nodes) in association with MOG+ myelin sheaths or NFil+ axons in subcortical NAWM (Fig. 1C and D). Similar observations were obtained from all the post-mortem cases included in this study (Table 1).

Nfasc155+ paranodes and Nfasc186+ nodes are disrupted in demyelinating lesions

Lesions undergoing demyelination in subcortical white matter were identified using a panel of histological and immunological stains for myelin proteins, axons, microglia and macrophages (Fig. 1E–G). Nfasc155+ structures with an elongated or disrupted profile were clearly evident at the edges of chronic active white matter lesions. These structures were always associated with MBP+ or MOG+ myelin sheaths that were still present within the lesion or near the border of the expanding lesion (Fig. 1H). Disrupted Nfasc155+ paranodes were always associated with NFil+H+ axons (Fig. 1I). Nfasc186+ profiles were disrupted in demyelinated white matter lesions with highly elongated Nfasc186+ structures observed that were not associated with myelin staining (Fig. 1J and K).

Nfasc155+ paranodes are already disrupted in areas of inflammation and close to active demyelinating lesions

We examined the length and diameter of Nfasc155+ structures within and adjacent to active demyelinating lesions to assess early changes at the axo-glial junction during ongoing inflammation and demyelination. The total Nfasc155+ paranodal length (the presumptive paranodes based upon the pattern of Nfasc155+ immunoreactivity), the length of the unstained nodal gap (the presumptive node) and the diameter of the Nfasc155+ structures were determined from tissue blocks from nine multiple sclerosis cases. Nfasc155+ structures were analysed from NAWM, peri-plaque WM (10–20 mm from lesion edge), lesion edge and remyelinating areas that were not associated with myelin staining (Fig. 1J and K).
was increased in peri-plaque areas and at the lesion edge and was maximal within demyelinating lesion centres in comparison to NAWM. The greater range of data in the lesion border and lesion centre groups is likely to reflect the differing and ongoing damage to myelin sheaths and oligodendrocytes occurring in those areas (Table 2 and Fig. 2C–F). Typically Nfasc155+ paranodes increased in length with a spreading of Nfasc155 immunoreactivity towards the juxtaparanodes. Only the most disrupted Nfasc155+ structures appeared as a continuous domain without an obvious unlabelled node-like gap. Such Nfasc155+ structures were commonplace at the lesion edge and lesion centre (Table 2).

Within chronically demyelinated lesion centres, the only detectable Nfasc155 labelling was found in association with MOG+ or MBP+ myelin fragments rather than intact sheaths. Therefore, Nfasc155 measurements from chronically demyelinated lesions were not included in these data because

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**Fig. 1** Expression of neurofascin 155 and neurofascin 186 in normal appearing white matter and demyelinating lesions. Anti-Nfasc155 (green) bound small rod-like structures with the appearance of paranodes (A and B). These associated with MOG+ (A) myelin sheaths and neurofilament-H+ axons (red) (B). Nfasc186 (green) was present as discrete clusters of immunostaining bordered by MOG+ myelin staining or in association with neurofilament+ axons (C and D). Chronic active lesions, with macrophages (HLA-DR+) containing myelin degradation products (LFB) at a high density at the demyelinated border were analysed for Nfasc155 and Nfasc186 immunostaining (E–G). Nfasc155+ profiles in association with MOG+ myelin or neurofilament+ axons at the border of a chronic active lesion were disrupted along the axonal length (H and I). Nfasc186 structures not associated with myelin had a disrupted expression pattern (J and K). Scale bar: 10 μm, except (E and G).
it was not apparent that they represented paranodal structures.

Disruption of Nfasc155 expression associates with early axonal pathology

Dephosphorylated neurofilaments are a hallmark of axon stress that can occur as a result of inflammation and/or myelin disturbance and are present in large numbers in active multiple sclerosis lesions (Trapp et al., 1998). Using the smi32 monoclonal antibody we scored the length and diameter of Nfasc155+ structures associated with smi32+ axons in comparison to those present in the absence of smi32 immunolabelling (all Nfasc155+ structures associate with phosphorylated Nfil-H+ axons, see Figs 1 and 3). Both the length and diameter of Nfasc155+ structures associated with smi32+ axons was significantly increased in comparison to Nfasc155+ structures in the same region that were not

Table 2 Nfasc155+ profiles are significantly disrupted in length and diameter in demyelinating and remyelinating lesions

<table>
<thead>
<tr>
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<th>NAWM</th>
<th>ppWM</th>
<th>Lesion edge</th>
<th>Lesion centre</th>
<th>Remyelinating</th>
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<tbody>
<tr>
<td></td>
<td>Mean ± SEM (µm)</td>
<td>n</td>
<td>Mean ± SEM (µm)</td>
<td>n</td>
<td>Mean ± SEM (µm)</td>
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<tr>
<td>Total length</td>
<td>5.91 ± 0.14</td>
<td>139</td>
<td>6.64 ± 0.18</td>
<td>96</td>
<td>7.58 ± 0.27</td>
</tr>
<tr>
<td>Node length</td>
<td>1.40 ± 0.05</td>
<td>135</td>
<td>1.40 ± 0.07</td>
<td>96</td>
<td>1.19 ± 0.08</td>
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<tr>
<td>Diameter</td>
<td>0.78 ± 0.02</td>
<td>139</td>
<td>0.95 ± 0.03</td>
<td>96</td>
<td>1.03 ± 0.03</td>
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The total length (µm), unstained nodal gap (µm) and maximum diameter (µm) of Nfasc155+ structures was determined for normal appearing white matter (NAWM), peri-plaque white matter (ppWM), active lesion edge and active lesion centre profiles from 7 multiple sclerosis cases. Total Nfasc155+ profile length and diameter was significantly increased above NAWM values in those areas highlighted (yellow) (ANOVA and Dunnett’s post-test comparing NAWM to all other samples, P < 0.001).

Fig. 2 The dimensions of Nfasc155 profiles are disrupted at areas adjacent to actively demyelinating lesions. Discrete Nfasc155+ structures (green, arrows) were seen in NAWM areas near microglia expressing the HLA-DR antigen (red) (A). HLA-DR+ macrophages within peri-plaque white matter near a number of Nfasc155+ paranodes with an elongated or disrupted structure (B, arrow). The length and diameter of Nfasc155+ structures was determined for NAWM, lesion edge, actively demyelinating white matter and remyelinating areas (C–F). The NAWM data range is shaded to highlight the increased number of enlarged Nfasc155+ profiles found within lesion border, lesion centre and remyelinating areas. 498 Nfasc155+ structures were quantified from nine multiple sclerosis subjects. Scale bar: 10 µm in all panels.
Nodal Nfasc186 expression is disrupted following demyelination

The formation of nodes of Ranvier requires close association between myelinating oligodendrocytes and the axon. Demyelinating lesions contained both discrete and disrupted Nfasc186+ clusters (Fig. 1). We noted that Nfasc186 expression appeared neatly clustered and similar to NAWM profiles when nodal Nfasc186 was bounded by paranodal myelin in and close to active lesions. Nfasc186 expression often appeared more diffuse when Nfasc186 was associated with attenuated or absent myelin, although discrete Nfasc186+ clusters in the absence of myelin were also observed (Fig. 4A and B). Therefore, we quantified the length of linear Nfasc186+ profiles to assess nodal disruption in association with myelinated and demyelinated axons (Fig. 4). Nfasc186+ structures bounded by myelin immunolabelling had a mean axonal length of 1.27 ± 0.06 μm (n = 95) compared to 7.92 ± 0.81 μm (n = 58; P < 0.0001, unpaired Student’s t-test) for Nfasc186+ structures with no association with myelinated fibres.

Disrupted Nfasc186+ structures associate with axon pathology

In order to determine if axonal damage was associated with disrupted expression of nodal Nfasc186, we examined nodes identified by Nfasc186+ immunoreactivity in sections co-labelled with smi32 antibody (Fig. 4E and F). Both
discrete and disrupted Nfasc186 structures were seen in association with axons containing dephosphorylated neurofilaments (smi32+). Quantification of these profiles revealed a huge variation in the length of Nfasc186 structures associated with smi32+ immunoreactive axons (data range 0.6–25.6 μm, mean 8.3 ± 1.7 μm; compared to smi32/C0 data range of 0.6–6.7 μm, mean 1.6 ± 0.3 μm; P < 0.01). The median Nfasc186 nodal length did not differ between normal and stressed axons (Nfasc186+/smi32+/C0 = 1.34, Nfasc186+/smi32+ = 1.62).

Changes to Nfasc155+ paranodes precede disruption of the node of Ranvier and Nfasc186 expression

The voltage gated sodium channels Na,1.6, and to a lesser extent Na, 1.2, are clustered at the nodes of Ranvier (in association with Nfasc186) and are redistributed along the axon in multiple sclerosis lesions (Craner et al., 2004), whilst inwardly rectifying K+ gated voltage channels are concentrated at the juxtaparanode and separated from the node by the tightly adhered septate-like junctions of the paranode (Rasband and Shrager, 2000). Therefore, we investigated the fate of Na, channels at the node (using a pan-Na, channel antibody) in relation to Nfasc155+ paranodes (Fig. 5). In the absence of Nfasc155+ paranodes/heminodes, Na,+ structures were greatly disrupted in comparison to normal appearing Na, node-like structures (1.08 ± 0.08 μm and 10.5 ± 1.4 μm, respectively; Fig. 5E). The dimensions of the diffuse Na, stained structures in the complete absence of Nfasc155+ paranodes was similar to that of Nfasc186+ profiles on demyelinated fibres reported earlier (Fig. 4C).

Tissue sections containing active demyelinating lesions were immunostained with anti-K,1.2 and anti-Nfasc155 antibodies to investigate the respective distribution of these juxtaparanodal and paranodal constituents (Fig. 5F–I). Within areas of normal appearing white matter and adjacent to lesion borders, the majority of Nfasc155+ paranodes were associated with K,1.2+ channels in a non-overlapping paranode-juxtaparanodal distribution, as typically seen in other species (Fig. 5F) (Rasband, et al., 1998, 1999b; Rasband and Shrager, 2000). Nfasc155+ paranodes with an atypical, elongated, appearance had a pattern of overlapping K,1.2 channel immunostaining (Fig. 5G–I), indicating that disruption of the paranodal junction leads to the displacement of the juxtaparanodal K,1.2 channels to nearer the node of Ranvier. Complete displacement of K,1.2 channels, giving large, weakly immunoreactive profiles, noted at the centre of demyelinated lesions that did not co-label for Nfasc155 (data not shown).
We also investigated the relationship between juxtaparanodal Kv1.2 channels and nodal Nfasc186 in demyelinating lesions. Within NAWM areas discrete nodal Nfasc186 was seen associated with juxtaparanodal Kv1.2 channels separated by an unstained region corresponding to presumptive paranodes (Fig. 5J). Other discrete Nfasc186^+ nodes appeared in close association to Kv1.2 profiles with a striated appearance (Fig. 5K and L), reminiscent of Kv1.2 structures seen overlapping with disrupted Nfasc155^+ paranodes (Fig. 5G and H). Finally, Kv1.2 immunostaining was seen to overlap with discrete Nfasc186 nodes (Fig. 5M).

These observations imply that disruption of paranodal junctions, accompanied by the displacement of juxtaparanodal Kv1.2 channels, precedes alterations in nodal Nfasc186 and Na\textsubscript{v} channels.

**Neurofascin expression in partially and fully remyelinated white matter areas**

Areas of partial remyelination, characterized by thin MOG^+ myelin sheaths with short internodes (Fig. 6D and E), were analysed with respect to the distribution of nodal proteins. Quantification of Nfasc155^+ structures revealed a mixed population of normal appearing and atypical paranodes in areas of remyelination (Fig. 2F and Table 2). Total paranodal length of Nfasc155^+ structures in partially remyelinated plaques was increased significantly over NAWM values but also significantly reduced in comparison to the maximally disrupted Nfasc155^+ structures present in actively demyelinating lesions (Table 2 and Fig. 2F), with a mean paranode length and scatter of data similar to lesion border paranodes. The heterogeneous dimensions of Nfasc155^+ paranodes in remyelinating lesions is likely to reflect the ongoing generation of new paranodal junctions and the possible regeneration of diffusely distributed Nfasc155^+ structures into concentrated paranodal clusters. A characteristic of all remyelinating lesions analysed (12 lesions from seven multiple sclerosis cases) was the presence of unusual triple-Nfasc155^+ structures, whereby each discrete Nfasc155^+ structure associated with MOG^+ myelin (Fig. 6B). The small spaces between the Nfasc155^+ bands were of a similar magnitude to nodes of Ranvier previously noted (Figs 1, 2, 4 and 5). We double-labelled remyelinating tissue sections with antibodies to Nfasc155 and pan-Na\textsubscript{v} channels to examine nodal Na\textsubscript{v} channel expression in relation to triple-Nfasc155^+ structures in remyelinating white matter lesions (Fig. 6C). Triple-Nfasc155^+ paranodes that had Na\textsubscript{v} channel clusters located in the two intervening nodal regions were found. Binary nodes are a feature of developmental myelination (Rasband et al., 1999a) and are believed to occur when two clusters approach and fuse to generate the node. Remyelinating lesions immunolabelled with anti-Nfasc186 and anti-MOG contained a number of Nfasc186^+ nodes separated by short myelinated internodes varying in length from 30–4 μm, as well as a pair of Nfasc186^+ clusters separated by a 2 μm un-myelinated gap that may represent two nodes about to fuse (Fig. 6D–F).

The majority of Nfasc155^+ structures within fully remyelinated shadow plaques appeared as discrete paranodes that

![Fig. 6](http://brain.oxfordjournals.org/). White matter plaques with areas of remyelination contain an assortment of normal and disrupted nodal profiles. (A) Short NFil^+ (red) internodes separated Nfasc155^+ (green) paranodes (arrows). (B) Remyelinating lesions contained triple-paranodal Nfasc155^+ structures (arrows) that associated with MOG^+ myelin and two distinctive unstained nodal gaps. (C) An area of remyelination labelled for Nfasc155 and pan-Na\textsubscript{v} channels (red) identified a triple-Nfasc155^+ structure separated by a binary nodal cluster of pan-Na\textsubscript{v} channels (arrows). (D) Discrete nodal Nfasc186^+ clusters (green) were separated by short MOG^+ myelinated internodes. (F) Binary Nfasc186^+ nodes (arrows) separated by a 2 μm un-myelinated gap. (G) A fully remyelinated shadow plaque from case MS179 had normal appearing Nfasc155^+ paranodes, with Kv1.2^+ channels located at the juxtaparanode adjacent to paranodal Nfasc155 (H and I). (J) A Nfasc186^+ node and paranodal MOG, demonstrating restored nodal domains in completely remyelinated areas. Scale bar; 5 μm, except (G).
associated with adjacent- but non-overlapping juxtaparanodal K$_{1.2}$ channels (Fig. 6H and I). Nfasc186 associated with discrete clusters with MOG$^+$ myelin sheaths indicating the restoration of nodal domains similar to those seen in non-pathological areas (Fig. 6) and K).

**Discussion**

Axonal Nfasc186 is a key component in the assembly of nodes of Ranvier that localizes the high density of voltage gated Na$^+$ channels to the node, which is vital to successful fast nerve transmission in central and peripheral nerves. Oligodendroglial-specific Nfasc155 is equally required for the formation of the paranodal septate junctions that separate the Na$^+$ channels at the node from the K$^+$ channels at the juxtaparanode (Sherman et al., 2005), thus permitting efficient nerve conduction. Disruption of this structure results in severely reduced conduction velocities, even in the absence of demyelination (Sherman et al., 2005). We report for the first time the expression pattern of Nfasc186 and Nfasc155 in human subcortical white matter and in multiple sclerosis lesions using optimally prepared human post-mortem material. Of particular note are our findings that paranodal Nfasc155 expression was dramatically altered at and near active demyelinating lesions and accompanied by the movement of juxtaparanodal components to nearer the node. This appeared to preclude changes in the expression of nodal components Nfasc186 and Na$_v$1 channels following demyelination. Partially remyelinated plaques contained short myelinated internodes, restored nodes of Ranvier and a number of binary nodes separated by atypical Nfasc155$^+$ structures, which may have important implications for the restoration of saltatory conduction in multiple sclerosis lesions.

**Early disruption to oligodendrocyte Nfasc155 expression in active multiple sclerosis lesions**

Nfasc155 is expressed as discrete paired structures either side of the node of Ranvier where it is believed to contact and stabilize a complex of axonal adhesion molecules comprising Caspr-1 and contactin (Tait et al., 2000; Charles, et al., 2002; Brophy 2003; Sherman et al., 2005). Nfasc155 has been demonstrated to be an excellent marker of the paranodal assembly as Nfasc155 and Caspr-1 protein are co-localized at ectopic sites along axons near lesions in experimental models of demyelination and in the Shiverer mutant (Tait et al., 2000; Arroyo, et al., 2002, 2004). Caspr-1 and contactin knockout animals still express neurofascin at myelin end loops, although conversely Nfasc155 expression appears necessary for normal Caspr-1 accumulation at the axonal membrane, indicating that Nfasc155 expression is associated with correctly assembled paranodes as well as weakened or disrupted axo-glial paranodal contacts. In light of these observations, we believe changes equivalent to those we have reported for Nfasc155 would also occur in the expression of Caspr-1 and contactin. Disrupted Nfasc155$^+$ paranodes, often seen in areas of inflammation without obvious demyelination, took the form of an elongated paranodal expression with an initially conserved node and dissipation of Nfasc155$^+$ immunolabelling to the juxtaparanode (Figs 1, 2 and 4). A loosening of the innermost wraps of the myelin sheath in response to inflammation results in the displacement of Nfasc155 (and Caspr-1) to juxtaparanodal domains (Arroyo et al., 2004) and such alterations to the tight axo-glial junction noted in our study may contribute to the neurological deficits induced by inflammation but preceding demyelination in multiple sclerosis (Bitsch et al., 1999). However, it should be noted that subtle changes to the myelin, that were undetectable by epifluorescence microscopy, may later result in demyelination. Thus our results may reflect subtle early changes to the myelin.

The absence of axonal Caspr-1 immunoreactivity in chronically demyelinated lesions, and the disruption of Caspr-1$^+$ paranodes at the borders of active and chronic lesions has been noted previously (Wolswijk and Balesar, 2003). Caspr-1$^+$ paranodes were also significantly reduced in density in demyelinated EAE rodent optic nerve (Craner et al., 2003). In the present study we noted that Nfasc155 expression was always absent from demyelinated fibres, demonstrating the absence of oligodendrocytes from the lesions. Nfasc155$^+$ paranode number (complete paranodes per mm$^2$) was greatly reduced at the borders and centres of active lesions, reflecting the reduction in myelin. In addition, we noted the early disruption to Nfasc155$^+$ structures within areas of inflammation and also the significant association of disrupted Nfasc155$^+$ profiles (length and diameter) with damaged or stressed axons containing dephosphorylated neurofilaments. These were clearly present in lesioned as well as non-pathological normal appearing white matter, indicating the continued spreading of damage in brain areas without obvious myelin disruption or loss.

The disruption of Nfasc155$^+$ profiles in inflammatory lesions was associated with changes in the location of juxtaparanodal and nodal ion channels that might be expected to lead to defective axonal conduction. In these areas, Nfasc155$^+$ paranodes associated with apparently normal nodes often had a displaced expression that overlapped with distinct and strong expression of K$_{1.2}$ channels, suggesting that Nfasc155 disruption allowed the dislocation of normally juxtaparanodal K$^+$ channels towards the node of Ranvier. Such changes are consistent with the proposed role of paranodal junctions as a ‘molecular fence’ to transmembrane protein diffusion (Pedraza et al., 2001). The overlapping expression of K$_{1.2}$ channels with Nfasc155 near demyelinating lesions is comparable to the reported unwinding of paranodal junctions and the intercalation of K$_{1.2}$ channels between regions of Caspr-1 noted in lysolecithin-induced demyelination in the rodent (Arroyo et al., 2004).

Structures with overlapping K$_{1.2}$ and Nfasc155 expression were accompanied by a regular nodal space, suggesting
that continued oligodendrocyte contact at the paranode blocked the full displacement of K\textsubscript{v}1.2 channels to the node, with the outermost myelin wraps being the last to be disrupted. Continuous K\textsubscript{v}1.2 profiles were noted in the absence of Nfasc155 expression within demyelinated lesion centres where K\textsubscript{v}1.2 immunoreactivity was reduced and overlapped with nodal Nfasc186, similar to another recent study of demyelinated multiple sclerosis lesions (Coman et al., 2006). K\textsubscript{v}1 channel displacement to paranodal and nodal domains is a reproducible feature of experimental and genetic demyelinating paradigms (Rasband et al., 1998, 1999a; Baba et al., 1999; Bhat et al., 2001; Mathis et al., 2001; Arroyo et al., 2004), where they are a substrate for the reduced excitability and altered conduction properties of the affected axons. Thus, changes to electrical conduction leading to neurological symptoms in MS might occur due to early disruption to the paranodes, in addition to the direct action of inflammatory mediators on the axon, in areas that are not yet demyelinated.

**Nfasc186 and Na\textsuperscript{+} channels at the node of Ranvier are disrupted following loss of the paranodal junctions**

Voltage gated Na\textsuperscript{+} channels are dispersed along the axon membrane in demyelinating conditions and do not cluster at developing nodes of Ranvier in animals lacking Nfasc186 (Moll et al., 1991; Waxman et al., 2004; Sherman et al., 2005). We have demonstrated an overlapping expression of Nfasc186 and Na\textsuperscript{+} at normal nodes and a near identical disruption of Nfasc186\textsuperscript{+} and Na\textsuperscript{+} nodal clusters along the axon length with demyelination or loss of Nfasc155\textsuperscript{+} paranodal expression, respectively, in multiple sclerosis lesions. These observations support an intimate association between Nfasc186 and Na\textsuperscript{+} channels at the node and demonstrate the important function of oligodendrocytes and preserved axo-glial junctions to the stability of the node of Ranvier. We also demonstrated a wide variation in the dimensions of Na\textsuperscript{+} nodal profiles that did not associate with Nfasc155\textsuperscript{+} paranodes, indicating a measured disruption of nodal clusters following the loss of axo-glial junctions in demyelinated lesions. The presence of nodal Na\textsuperscript{+} clusters has also recently been suggested to indicate nodal re-organization preceding remyelination (Coman et al., 2006). The preservation of clustered nodes has been noted in dysmyelinating and knockout mice shortly following the loss of paranodal junctions (Bhat et al., 2001; Boyle et al., 2001; Arroyo et al., 2002; Schaeren-Wiemers et al., 2004) that are noted to deteriorate following prolonged absence of stabilizing paranodal contacts (Mathis et al., 2001; Ishibashi et al., 2002; Rios et al., 2003). Within the established demyelinated multiple sclerosis lesion it would therefore be expected that a range of differently disrupted nodal profiles could co-exist reflecting the evolving damage (Figs 4 and 5).

Following demyelination and disruption of saltatory conduction, the axon redistributes and upregulates the expression of Na\textsuperscript{+} channels along continuous stretches of the denuded membrane in an attempt to maintain signal conduction (Waxman, 1998). Such dramatic changes allow conduction through the demyelinated area but also predispose the axon to further damage, due in part to the increased influx of Na\textsuperscript{+} ions (Waxman et al., 2004; Bechtold and Smith, 2005). Alterations in the excitability of the axonal membrane contribute to a worsening of symptoms for the sufferer (Compston and Coles, 2002; Devor, 2006). We have demonstrated for the first time the displacement of Na\textsubscript{v}1 channels at nodes not associated with Nfasc155\textsuperscript{+} paranodes, together with a comparable displacement of Nfasc186 in demyelinated axons. The equivalent disruption in the localization of Nfasc186 and Na\textsubscript{v} channels is in agreement with their close affinity during nodal formation and supports an integral role for neurofascins in the mature human nervous system (Sherman et al., 2005). In Fig. 7 we present a schematic summarizing the changes in paranodal and nodal constituents with demyelination that is informed from this and other studies.

**Remyelination is associated with restructuring of neurofascin domains and unusual binary nodal clusters separated by axo-glial contacts**

For efficient and effective remyelination there needs to be a rapid and appropriate re-assembly of nodal and paranodal domains. As part of our analysis of neurofascin expression in remyelinating lesions, we observed some Nfasc155\textsuperscript{+} structures organized as three closely associated domains demarcating a pair of nodes. The presence of such unusual triple-Nfasc155\textsuperscript{+} profiles, and their association with binary Na\textsubscript{v}1 nodal clusters of regular dimensions, suggest that they were newly generated structures. Double immunolabelling of remyelinating lesions for MOG and Nfasc186 revealed a number of discrete Nfasc186\textsuperscript{+} nodal clusters separated by short myelinated internodes of as little as 4 \textmu m in length. In addition, a pair of Nfasc186\textsuperscript{+} clusters separated by a \textasciitilde 2 \textmu m section of naked axon was observed that may represent two nodes that were about to fuse. Binary Na\textsubscript{v} nodes, nodal clusters that fuse to form a single cluster of ion channels at the node are a feature of CNS developmental myelination (Rasband et al., 1999b). Fusion of nodes has been proposed to occur during peripheral nerve remyelination and regeneration (Hildebrand et al., 1986; Dugandzija-Novakovic et al., 1995; Vabnick et al., 1996; Custer et al., 2003) but has yet to be demonstrated within remyelinating lesions in multiple sclerosis.

Atypical Nfasc155\textsuperscript{+} profiles seen in remyelinating lesions may be temporary oligodendrocyte contacts during the ongoing process of internodal remodelling of myelin repair (see Fig. 7 for a schematic representation). In support of this possibility is the previous demonstration that very short Schwann cell internodes exist in peripheral nerve injury that cover as little as 1–10 \textmu m of the axon. These internodes were
postulated to be temporary contacts that retracted during nodal and internodal remodelling by glial competition for longitudinal axonal space (Hildebrand et al., 1986). Such internodes might originally express paranodal components at either end of the myelinated segment and an enforced retraction from the axonal membrane may temporarily display a single point of attachment before complete uncoupling of the glial process from the axon. Likewise, single paranodal contacts may precede internode expansion and the generation of new paranodal junctions. Alternatively, it is possible that such unusual structures represent a failure of appropriate remyelination and it would seem unlikely that they would support rapid saltatory conduction. In support of this suggestion was their presence in lesions that were not fully remyelinated. However, due to the limiting nature of human post-mortem tissue it is not possible to determine how triple-Nfas155 paranodes contribute to the ongoing process of remyelination, but due to their compact appearance and association with essential nodal components they could be important features of repair that are worthy of further investigation.

**Pioneering role for neurofascins in demyelination and repair**

Our studies implicate Nfas155 as an early and sensitive marker of inflammation and myelin damage that associates with an early index of axonal pathology in multiple sclerosis. Consequent to paranodal disruption, the incursion of K₅,1.2 channels with Nfas155 and the eventual diffusion of nodal Naᵥ channels along fully demyelinated axons will predispose the axon to further damage (Waxman et al., 2004). The loss of paranodal components Nfas155 or Caspr-1 in experimental models results in aberrant paranodal junctions and severely disrupted conduction velocities along myelinated axons, demonstrating how small changes within nodal domains can manifest as significant phenotypic abnormalities (Bhat et al., 2001; Sherman et al., 2005). We believe that Nfas155 also plays an important role in remyelination, being associated with clustered Naᵥ channels at heminodes and at triple-Nfas155 structures separating very close nodes. This work points to a pivotal role for neurofascin in the disruption and restoration of nodal architecture in demyelinating and remyelinating multiple sclerosis lesions.

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Neurofascin localization in multiple sclerosis


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