Nodal, paranodal and juxtaparanodal axonal proteins during demyelination and remyelination in multiple sclerosis

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Saltatory conduction in myelinated fibres depends on the specific molecular organization of highly specialized axonal domains at the node of Ranvier, the paranodal and the juxtaparanodal regions. Voltage-gated sodium channels (Nav) have been shown to be deployed along the naked demyelinated axon in experimental models of CNS demyelination and in multiple sclerosis lesions. Little is known about aggregation of nodal, paranodal and juxtaparanodal constituents during the repair process. We analysed by immunohistochemistry on free-floating sections from multiple sclerosis brains the expression and distribution of nodal (Nav channels), paranodal (paranodin/Caspr) and juxtaparanodal (Kv channels and Caspr2) molecules in demyelinated and remyelinated lesions. Whereas in demyelinated lesions, paranodal and juxtaparanodal proteins are diffusely distributed on denuded axons, the distribution of Na channels is heterogeneous, with a diffuse immunoreactivity but also few broad Na channel aggregates in all demyelinated lesions. In contrast to the demyelinated plaques, all remyelinated lesions are characterized by the detection of aggregates of Na channels, paranodin/Caspr, K channels and Caspr2. Our data suggest that these aggregates precede remyelination, and that Na channel aggregation is the initial event, followed by aggregation of paranodal and then juxtaparanodal axonal proteins. Remyelination takes place in multiple sclerosis tissue but myelin repair is often incomplete, and the reasons for this remyelination deficit are many. We suggest that a defect of Na channel aggregation might be involved in the remyelination failure in demyelinated lesions with spared axons and oligodendrogial cells.

Keywords: multiple sclerosis; demyelination; remyelination; sodium channels; potassium channels

Abbreviations: ALS = amyotrophic lateral sclerosis; MBP = myelin basic protein; Na = voltage-gated sodium channels; PBS = phosphate-buffered saline; PLP = proteolipid protein


Introduction

The molecular organization of the nodes of Ranvier in myelinated fibres allows a rapid saltatory conduction. The nodes of Ranvier are separated from the internode by two distinct domains of the axolemma, the paranodal axo–glial junction and the juxtaparanodal region, which are characterized by the presence of specific protein complexes (see review by Peles and Salzer, 2000). Voltage gated sodium (Na) channels, ankyrin G, NrCAM and 186 kDa neurofascin are highly enriched at the node (Bennett and Lambert, 1999; Jenkins and Bennett, 2001). At the paranodes, myelin loops are anchored to axons through septate-like junctions characterized by the enrichment of paranodin/Caspr and GPI-anchored cell adhesion molecule (CAM) contactin (Einhbeber et al., 1997; Menegoz et al., 1997; Rios et al., 2000). The juxtaparanodal region, just beyond the innermost paranodal junction (Wang et al., 1993), is enriched in Shaker-type potassium (K) channels, in association with Caspr 2, a second member of the Caspr family and the cell adhesion protein TAG-1 (Poliak et al., 1999; Traka et al., 2003).

A diffuse distribution of Na channels along the naked demyelinated axon has been reported in experimental models of demyelination and in multiple sclerosis lesions...
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(Rosenbluth et al., 1985; Moll et al., 1991; Noebels et al., 1991; Arroyo et al., 2002; Craner et al., 2004a, b). In addition to this diffuse distribution, these studies demonstrated a reversion of the Na\textsubscript{v} channel expression from a mature (Na\textsubscript{v}1.6) to an immature (Na\textsubscript{v}1.2) isoform, and this may limit axonal injury (Craner et al., 2004a, b).

In spite of producing a thinner myelin sheath with shorter internodes, remyelination not only restores efficient nerve conduction (Smith et al., 1979) but also protects axons from secondary degeneration (Kornek et al., 2000). Mechanisms involved in this myelin repair are many, but axo–glial interactions and the intrinsic properties of oligodendrocytes and axons are known to be important (see review by Franklin, 2002; Lubetzki et al., 2005). Little is known about the chronology and influence of the aggregation of nodal and perinodal axonal molecules in the process of remyelination.

Here we show, through immunohistochemical analysis of multiple sclerosis lesions, that in demyelinated plaques, nodal, paranodal and juxtaparanodal axonal molecules are diffusely distributed along the naked axons. However, in addition to this diffuse distribution of Na\textsubscript{v} channels, loose clusters of Na\textsubscript{v} channels persist on a percentage of denuded axons. By analysing remyelinated lesions, we show that during myelin repair the aggregation of nodal, paranodal and juxtaparanodal axonal molecules recapitulates that of development, with the initial step being Na\textsubscript{v} channel clustering.

Material and methods

Tissues
Post-mortem snap frozen human brain samples consisting of 2–3 cm\textsuperscript{3} blocks were obtained from the Salpêtrière brain bank and UK multiple sclerosis Tissue Bank. Multiple sclerosis tissue was obtained from eight multiple sclerosis patients. Disease evolution was secondary progressive in seven cases, and unknown in one case. Age range was 35–86 years, disease duration was 8–56 years and post-mortem delay was 11–24 h. The eight controls consisted of seven cases of amyotrophic lateral sclerosis (ALS) (55–82 years, post-mortem delay: 21–47 h) and one case with no neurological disease (92 years, post-mortem delay: 13 h). Blocks were frozen in isopentane, and stored at –80°C.

The frozen blocks were thawed and fixed during 2 hours in paraformaldehyde [4% in phosphate-buffered saline (PBS)], protected in 30% sucrose for 24 h, and then frozen on the stage of a sliding microtome. Sections (30-μm thick) were recovered in PBS for immunohistochemistry or collected and stored at –20°C in cryoprotectant tissue collecting solution (Burwell and Amaral, 1998).

Immunocytochemistry
Tissue sections were washed in PBS, microwaved (2 min at 600 W) in 10 mM (pH 6) citrate buffer, saturated for 2.5 h in PBS containing 5% Triton X-100, 10% goat serum, and 0.01% sodium azide, and then incubated for 5 days at 4°C, with primary antibodies diluted in 0.2% Triton X-100, 5% goat serum and 0.01% sodium azide in PBS. After washing, incubation with the secondary antibody was performed for 3 h followed by a streptavidin-Cy5 step for the biotinylated antibodies. Sections were then post-fixed in 4% paraformaldehyde and mounted in Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL, USA).

Primary antibodies
Anti-PLP (proteolipid protein) monoclonal rat Ig (clone AA3, diluted 1 : 10, provided by Kazuhiro Ikenaka, Okayama, Japan), anti-MBP (myelin basic protein) monoclonal rat Ig (diluted 1 : 50, Chemicon, Temecula, USA), anti-neurofilament M rabbit Ig (diluted 1 : 200, Chemicon, Temecula, USA), anti-neurofilament SM 31 and MI 32 mouse IgG1 (diluted 1 : 400, Sternberger Monoclonals Inc., Lutherville, USA), CD68 anti-human macrophage mouse IgG1 (diluted 1 : 50, Dako, Glostrup, Denmark), anti-paranodal and juxtaparanodal axonal molecules in the process of remyelination.

Secondary antibodies
Alexa 488 and Alexa 594 coupled goat anti-mouse IgG1, IgG2b, goat anti-rabbit IgG and goat anti-rat IgG (diluted 1 : 1000, Sigma, St. Louis, USA), anti-paranodin/Caspr and anti-Caspr2 polyclonal rabbit Ig (diluted 1 : 500; Denisenko-Nehrbass et al., 2003), anti-Ro, 12 mouse IgG2b (diluted 1 : 100, Upstate Lab, Charlottesville, USA).

Microscopic and confocal observation
A Leica DRMB fluorescent photomicroscope was used to analyse the Luxol myelin staining. Immunolabelling was analysed with a confocal microscope (Leica TCS 4D krypton/argon laser). Most images resulted from stacks (z-step of 0.20–0.26 μm) of 10–14 images of the same 100 × 100 μm field, superimposed with ImageJ (http://rsb.info.nih.gov/ij/).

Quantification
Na\textsubscript{v} channel aggregates
The length of Na\textsubscript{v} aggregates was analysed by double immunolabelling (Na\textsubscript{v}–paranodin/Caspr and Na\textsubscript{v}–MBP) in the demyelinated plaques, the periplaques, the remyelinated plaques and the control tissues. Only the aggregates fully included in the volumes analysed were measured. The axonal calibre was not quantified but was very similar between different regions.

Para- and juxtaparanodal aggregates
The length of paranodin/Caspr, K\textsubscript{v} and Caspr2 aggregates was measured in the same way, in control tissue, and periplaque and remyelinated lesions, using double immunolabelling with the corresponding antibody and a myelin marker (anti-MBP or anti-PLP antibody).

Statistical analysis
The length of aggregates in the different types of tissue (ALS, control, periplaque, demyelinated plaque, partially remyelinated plaque, and axons are known to be important (see review by Franklin, 2002; Lubetzki et al., 2005). Little is known about the chronology and influence of the aggregation of nodal and perinodal axonal molecules in the process of remyelination.

Here we show, through immunohistochemical analysis of multiple sclerosis lesions, that in demyelinated plaques, nodal, paranodal and juxtaparanodal axonal molecules are diffusely distributed along the naked axons. However, in addition to this diffuse distribution of Na\textsubscript{v} channels, loose clusters of Na\textsubscript{v} channels persist on a percentage of denuded axons. By analysing remyelinated lesions, we show that during myelin repair the aggregation of nodal, paranodal and juxtaparanodal axonal molecules recapitulates that of development, with the initial step being Na\textsubscript{v} channel clustering.
and shadow plaque) was compared by ANOVA (analysis of variance) using Bonferroni’s least significant difference method. In addition, the percentage of isolated Na\textsubscript{v} or paranodin/Caspr aggregates and of aggregates consisting of both Na\textsubscript{v} and paranodin/Caspr was compared in periplaque regions and partially remyelinated plaques using a Chi-squared test.

**Results**

**Pattern of demyelinated and remyelinated lesions**

Multiple sclerosis demyelinated plaques (n = 8) were characterized as areas with complete myelin loss, as evidenced by Luxol staining and PLP immunoreactivity, and exhibited a typical ‘sharp edge’ separating the myelinated and demyelinated region (Supplementary Fig. 1A). The periplaque region was defined as a rim of normal appearing white matter in the vicinity of a demyelinated plaque (Supplementary Fig. 1A). Remyelinated lesions were identified as shadow plaque (n = 1) and partially remyelinated plaques (n = 11).

Shadow plaques were characterized by homogeneous myelin pallor with Luxol staining (Supplementary Fig. 1B) and the absence of denuded axonal segments with PLP immunolabelling, all the fibres being surrounded by PLP-positive myelin. Partially remyelinated plaques were detected either at the border or within a demyelinated plaque, and characterized by a more heterogeneous myelin pallor on Luxol staining (Supplementary Fig. 1C), with PLP-positive myelin sheaths coexisting with denuded axons. Remyelination was considered if the following criteria were present (Prineas and Connell, 1979; Lassmann, 1998): undisrupted myelin sheath; short internodes (mean internodal length, measured between two paranodin/Caspr-positive paranodes on 50 remyelinated fibres was 13 ± 7 μm); no myelin debris in the vicinity of the remyelinated segments; if macrophages were detected (using anti-CD68 antibody), they showed no immunoreactivity to myelin proteins (Supplementary Fig. 2A). These remyelinated internodes were clearly different from myelin sheaths in the process of disintegration, which were disrupted and often in close contact with macrophages containing MBP-positive myelin debris (Supplementary Fig. 2B).

**Na\textsubscript{v} channels are heterogeneously distributed along axons in demyelinated plaques**

Na\textsubscript{v} channel immunoreactivity was analysed in the plaque and in the periplaque regions. As in normal or ALS brain (Fig. 1A and B), well-delimited, dense Na\textsubscript{v} channel aggregates were detected in the periplaque (Fig. 1C and D). The mean length of these Na\textsubscript{v} channel clusters was 0.94 ± 0.26 μm (range: 0.32–1.6 μm), thus similar to the length of Na\textsubscript{v} channel clusters from controls (non-neurological and ALS cases) (Fig. 1K). In the demyelinated plaque, Na\textsubscript{v} channel immunoreactivity was diffusely distributed along most naked axons (Fig. 1E–H).

In addition to this diffuse distribution, a few broad Na\textsubscript{v} channel aggregates were detected on demyelinated axons in all lesions (Fig. 1F–J). The mean length of these aggregates was 3.3 ± 1.4 μm (range: 1.3–11 μm), thus 3-fold larger than Na\textsubscript{v} channel aggregates in the periplaque or normal tissue (Fig. 1K). These aggregates, which we have named ‘loose’ aggregates, were often associated with a diffuse Na\textsubscript{v} channel distribution further down the same axon (Fig. 1F–H).

**Paranodal and juxtaparanodal axonal proteins are diffusely distributed along naked axons**

In the periplaque, dense paranodin/Caspr aggregates were detected, and their mean length was 2.7 ± 0.72 μm (range: 1.3–5.4 μm), thus similar to the paranodin/Caspr aggregates analysed in control tissue (Fig. 2A–D). Kv channels and Caspr2 also formed dense juxtaparanodal aggregates (Fig. 2E–H and I–L). The mean length of Kv aggregates (counted on 47 and 60 aggregates in the control brain and periplaque regions, respectively) was slightly larger in the periplaque: 5.9 ± 3 μm (range: 1.8–14 μm), than in control tissue: mean length: 4.6 ± 1.4 μm (range: 2.6–8.2 μm). The mean length of Caspr2 aggregates (counted on 26 and 68 aggregates in the control brain and periplaque regions, respectively), was similar in the periplaque: 4.9 ± 1.2 μm (range: 3.0–7.2 μm) and in control tissue (mean length: 5.2 ± 1.8 μm; range: 3–11 μm). These observations demonstrate that the molecular organization of nodal, paranodal and juxtaparanodal axonal domains in the periplaque region is similar to their organization in normal adult brain tissue (Fig. 2M and N).

In contrast to the heterogeneous distribution of Na\textsubscript{v} channels, no paranodin/Caspr aggregates were detected in any demyelinated plaques. Instead, immunoreactivity of paranodin/Caspr, as well as the normally juxtaparanodal constituents Kv, channels and Caspr2, was always diffusely distributed along demyelinated axons (Fig. 2O–S).

**Remyelination is associated with the occurrence of aggregates of nodal and perinodal constituents**

In contrast to the demyelinated plaques, all remyelinated lesions (shadow plaque and partially remyelinated plaques) were characterized by the detection of aggregates of Na\textsubscript{v} channels, paranodin/Caspr (Fig. 3A and B), K\textsubscript{v} and Caspr2. However, these aggregates differed between the two types of remyelinated lesions. In the shadow plaque, the mean length of Na\textsubscript{v} channels and paranodin/Caspr aggregates was 1.1 ± 0.33 μm (range: 0.53–2.5 μm) and 2.8 ± 0.83 μm (range: 1.5–6.3 μm), respectively, thus similar to aggregates in the periplaque region and in the control
The same ‘normal’ aspect was detected for the juxtaparanodal molecules, Kv channels and Caspr2 (not shown). In contrast, in the partially remyelinated zones, the mean length of the Nav channels and paranodin/Caspr aggregates was significantly larger than in the periplaque and in the shadow plaque. In PLP-positive fibres, the mean length of Nav channel aggregates (1.6 ± 0.61 µm; range: 1.0–3.3 µm) was shorter than in the demyelinated zones, but larger than the clusters in the shadow plaques or periplaque (Fig. 3C). Similarly, paranodin/Caspr aggregates in PLP-positive fibres were larger (mean length: 3.8 ± 1.8 µm; range: 1.3–12 µm) than in the shadow plaque or periplaque (Fig. 3D). In PLP-negative fibres within these partially remyelinated lesions, Nav channel and paranodin/Caspr aggregates were also detected, with a length larger than in PLP-positive fibres (mean length: 2.5 ± 1.0 and 5.4 ± 3.4 µm, respectively) (Fig. 3C and D). These Nav channel aggregates in PLP-negative fibres were nevertheless

Fig. 1 Heterogeneous redistribution of Nav channels in demyelinated plaques. (A–J) Free-floating 30-µm sections from control, ALS and multiple sclerosis brain tissue were immunolabelled with anti-pan Nav channel antibody (green), anti-neurofilament (red in G–H) and PLP antibody (red in D and J). Nav channels form dense nodal clusters in the periplaque (C, with myelin staining in D), similar to aggregates in controls (A) and ALS cases (B), whereas diffuse immunoreactivity is detected in the demyelinated plaque (E). In addition to this diffuse Nav channel distribution, loose aggregates of Nav channels are scattered along denuded axons (F–I, with higher magnification in inserts) in the demyelinated plaque where myelin loss is evidenced by the absence of PLP staining (I and J). Scale bar: 10 µm. (K) Quantification of the length of the Nav channel aggregates was performed as described in Material and methods, and expressed as mean ± standard deviation. The length of Nav channel aggregates was within a similar range in periplaque, shadow plaque, controls and ALS. Loose aggregates in demyelinated plaques are 3-fold larger than in the periplaque. N represents the number of aggregates counted for this length quantification. **p < 0.0001; C, control; PP, periplaque; D, demyelinated plaque.
of a shorter length than the loose aggregates in totally demyelinated lesions (Fig. 3C).

Caspr2 and Kᵥ channels aggregates were also detected in these partially remyelinated lesions, and their length, counted on 50 Caspr2-positive aggregates and 79 Kᵥ channel-positive aggregates, was not different from that in control tissue or the periplaque areas (data not shown).

In addition to this length heterogeneity, partially remyelinated lesions commonly contained abnormal nodal and paranodal architecture with heminodes, association of asymmetric nodal and paranodal clusters (dense nodal clusters and loose paranodal doublets, loose aggregates of Naᵥ channels with dense paranodin/Caspr doublets), or very short myelin sheaths (Fig. 3E, F and G).

### Aggregation of Naᵥ channels initiates remyelination

Although we used post-mortem tissue, the analysis of partially remyelinated lesions allowed us to study the chronology of the different steps of the remyelination process. In all remyelinated lesions analysed, myelin sheaths were always associated with either Naᵥ channel aggregates, paranodin/Caspr, Kᵥ channels or Caspr2 aggregates (Fig. 4A, C, E
and G), but these aggregates can also exist alone in the partially remyelinated zones (Fig. 4B, D, F and H), suggesting that remyelination follows the aggregation of these constituents. The chronology of Na\textsubscript{v} and paranodin/Caspr aggregation was inferred from the analysis of the percentage of isolated Na\textsubscript{v} channel clusters or paranodin/Caspr clusters, as compared with the clusters where the two molecules are associated (Fig. 5). Whereas isolated Na\textsubscript{v} channel aggregates represented 55% of the total number of Na\textsubscript{v} channel aggregates in the partially remyelinated lesions, isolated paranodin/Caspr aggregates represented only 15% of the total number of paranodin/Caspr aggregates, most of them (85%) being associated with Na\textsubscript{v} channels, as in the periplaque. These data strongly suggest that Na\textsubscript{v} channel aggregation precedes paranodin/Caspr reaggregation.

In addition, the shorter length of the Na\textsubscript{v} channel clusters associated with paranodin/Caspr (mean length: 1.4 ± 0.64 \(\mu\)m), compared with isolated Na\textsubscript{v} channel clusters (mean length: 2.5 ± 1.1 \(\mu\)m) suggests that Na\textsubscript{v} channel aggregates associated with paranodin/Caspr represent more mature nodal regions (data not shown).

Concerning the juxtaparanodal constituents, we observed that most of the myelin immunoreactivity was associated with K\textsubscript{v} channels and Caspr2 aggregates. However, some aggregates of Caspr2 on PLP-negative fibres were detected in partially remyelinated lesions, whereas K\textsubscript{v} channel aggregates on non-myelinated fibres were very rarely seen. This suggests that K\textsubscript{v} aggregation is a late event and just precedes or is contemporary to remyelination.

In summary, our data suggest that aggregation of nodal and perinodal axonal constituents precedes remyelination, and that Na\textsubscript{v} channel aggregation is the initial event, followed by aggregation of paranodal and then juxtaparanodal axonal proteins.

**Discussion**

**Nodal and perinodal axonal proteins behave differently during demyelination**

Diffuse distribution of Na\textsubscript{v} channels on denuded axons in multiple sclerosis plaques has been previously detected by autoradiography and immunolabelling (Moll \textit{et al}., 1991; Craner \textit{et al}., 2004\textit{a}, \textit{b}). It remains unknown whether this change is due to a Na\textsubscript{v} channel synthesis or to a

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**Fig. 3** Remyelination is associated with nodal and paranodal reaggregation. Free-floating 30-\(\mu\)m sections from multiple sclerosis plaques were immunolabelled with anti-pan Na\textsubscript{v} channel antibody (green) and anti-paranodin/Caspr antibody (red) in A, B, E, F, or anti-MBP (red) and anti-paranodin/Caspr antibody (green) in G. Nodal and perinodal aggregates are similar in periplaques A and shadow plaque (B). In the partially remyelinated plaques, abnormal architecture is seen, such as heminodes (E), loose nodal aggregates (F), abnormally short myelinated internodes (arrows in G). The node is marked by a star, the myelin sheaths by arrows and the paranodal aggregates of paranodin/Caspr by arrowheads (G). The quantification of the length of the Na\textsubscript{v} channels (C) and paranodin/Caspr aggregates (D) was performed as described in Material and methods, and expressed as the mean ± standard deviation. Na\textsubscript{v} channels and paranodin/Caspr form dense clusters in the shadow plaque. In the partially remyelinated plaques, the Na\textsubscript{v} channels (C) and paranodin/Caspr (D) aggregates are larger on PLP-negative fibres than on PLP-positive fibres. Na\textsubscript{v} channel aggregates on PLP-negative fibres are nevertheless shorter than on denuded axons. Aggregates on PLP-positive fibres within the partially remyelinated plaques are larger than in the shadow plaque. **P < 0.0001. PP, periplaque; D, demyelinated plaque; Sh.P., shadow plaque (fully remyelinated); R-PLP+, remyelinated fibre within a partially remyelinated plaque; R-PLP-, non-remyelinated fibre within a partially remyelinated plaque. Scale bar: 10 \(\mu\)m.
perinodal clusters (myelin sheaths were always associated with nodal and denuded MBP-negative fibres (represented 55% of the Nav channel aggregates. In contrast, isolated Nav channel or paranodin/Caspr aggregates, and aggregates are more frequent than paranodin/Caspr aggregates. Remyelinated lesions, isolated Nav channel aggregates in partially remyelinated plaques were immunolabelled with anti-MBP antibody (red) and either anti-pan Na⁺ channel antibody (green) (A and B), or paranodin/Caspr antibody (green) (C and D), or anti-K⁺ channel antibody (green) (E and F) or anti-Caspr2 antibody (green) (G and H). Whereas aggregates were detected on some denuded MBP-negative fibres (B, D, F, H), MBP-positive myelin sheaths were always associated with nodal and perinodal clusters (A, C, E, G). Scale bar: 10 μm.

Fig. 4 Reaggregation of nodal and perinodal proteins precedes remyelination. Free-floating 30-μm sections from partially remyelinated plaques were immunolabelled with anti-MBP antibody (red) and either anti-pan Na⁺ channel antibody (green) (A and B), or paranodin/Caspr antibody (green) (C and D), or anti-K⁺ channel antibody (green) (E and F) or anti-Caspr2 antibody (green) (G and H). Whereas aggregates were detected on some denuded MBP-negative fibres (B, D, F, H), MBP-positive myelin sheaths were always associated with nodal and perinodal clusters (A, C, E, G). Scale bar: 10 μm.

Fig. 5 In partially remyelinated plaques, isolated Na⁺ channel aggregates are more frequent than paranodin/Caspr aggregates. Isolated Na⁺ channels or paranodin/Caspr aggregates, and Na⁺-paranodin/Caspr-associated aggregates were counted in remyelinated lesions and in the periplaque. In partially remyelinated lesions, isolated Na⁺ channel aggregates represented 55% of the Na⁺ channel aggregates. In contrast, only 15% of paranodin/Caspr aggregates were isolated aggregates, and this percentage is not significantly different than in the periplaque.

Re-distribution of Na⁺ channels from nearby nodes into the demyelinated, previously internodal membrane. Our data confirm this diffuse distribution, but also provide evidence that loose aggregates of Na⁺ channels are present on denuded fibres. These aggregates may represent remaining nodes, anchored by ankyrin G, or alternatively, they may correspond to attempts to remyelinate the axons. In contrast to this heterogeneous distribution of Na⁺ channels, paranodal and juxtaparanodal proteins only exhibit a diffuse distribution on demyelinated axons. Previous work (Wolsiwik and Balesar, 2003) has reported a diffuse distribution of paranodin/Caspr in multiple sclerosis tissue, but only at the border of the plaque, whereas no paranodin/Caspr immunoreactivity was detected in the plaque. Although diffuse distribution of paranodin/Caspr at the edge of the plaque was often detected in our samples, our data clearly show diffuse paranodin/Caspr immunoreactivity along naked axons in the plaque. This discrepancy is most probably linked to technical points, with improved antibody access using the free-floating technique used in our study. To our knowledge, this is the first report describing the expression of the juxtaparanodal proteins Kᵥ channel and Caspr2 in multiple sclerosis lesions.

The differential distribution of nodal and perinodal constituents during demyelination is in agreement with experimental observations

During recent years, several groups have analysed the expression of nodal, paranodal and juxtaparanodal proteins in dysmyelinating mutants. In the jimpy or md murine mutant, characterized by a PLP mutation with premature post-natal death of oligodendrocytes, or in the ceramide galactosyltransferase (CGT)-deficient mice, characterized by a deficit of specific myelin galactolipids, it was shown that, even in the absence of axo-glial junctions, Na⁺ channel clusters are detected, but with frequent abnormal distribution along the axons (Dupree et al., 1999; Mathis et al., 2001; Arroyo et al., 2002). The same type of observation was reported with Caspr- and contactin-deficient mutants (Rios et al., 2000; Boyle et al., 2001). In the transgenic mice containing extra copies of the PLP gene and characterized by a normal myelin formation followed by the occurrence of chronic demyelination, Na⁺ channels persist, but their density decreases gradually with demyelination, and their distribution is often abnormal (Ishibashi et al., 2003). These findings suggest that intact axo-glial junctions are not necessary for the maintenance of Na⁺ channel aggregation, and are in agreement with our results showing that, despite the total absence of myelin and thus of axo-glial junctions, loose Na⁺ channel clusters are detected. However, as shown recently using Caspr and PAPS sulfotransferase-deficient mutants (Rios et al., 2003; Suzuki et al., 2004), interaction of paranodal loops with the axon promotes the transition between Na⁺ channel subtypes in the CNS, an observation which is in accordance with results on experimental demyelination and in multiple sclerosis (Crane et al., 2004a, b). In contrast, and in agreement with our observations, paranodin/Caspr has been shown to be diffusely distributed in these dysmyelinating mutants (Dupree et al., 1999; Mathis et al., 2001; Arroyo et al., 2002), suggesting that axo-glial junctions and...
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Glia contact are necessary for the maintenance of paranodin/Caspr aggregation.

For juxtaparanodal molecules, Kᵥ channel mislocalization or diffuse distribution has also been reported (Dupree et al., 1999; Boyle et al., 2001; Mathis et al., 2001). We detected such mislocalization at the edge of the multiple sclerosis demyelinated lesions, with transitional forms (data not shown), but only diffuse distribution within the demyelinated plaque. In the jimpy mutant, mislocalized aggregates of Kᵥ channels were detected at P15, but with a similarly diffuse distribution at a later age, suggesting that diffuse distribution may be associated with long-lasting demyelination. Mislocalization of Caspr2 has been reported in the CGT-deficient mutant, which lacks proper paranodal junctions (Poliak et al., 2001).

Thus, these experimental data showing the different distribution of nodal and perinodal constituents when myelination is impaired are in accordance with our results in multiple sclerosis tissue. Persisting aggregates of nodal Naᵥ channels are most probably related to the persisting presence of their anchoring partners at the node, and the lack of axo–glial interactions may result in the diffuse distribution of the perinodal molecules.

**Naᵥ channel clustering is the initial event in the remyelination process**

Aggregation of nodal and perinodal constituents during the repair process has been studied in experimental models. In the PNS, after lyssolecithin demyelination, it has been shown that Naᵥ channel immunoreactivity is an early event, followed by detection of Kᵥ immunoreactivity (Dugandzija-Novakovic et al., 1995; Novakovic et al., 1996; Rasband et al., 1998). In the CNS, Naᵥ channel reclustering has been reported together with evidence of remyelination in the corpus callosum after cuprizone treatment (Dupree et al., 2004), and restoration of normal Naᵥ and Kᵥ channels following ethidium bromide intraspinal injection (Black et al., 2006). These experimental results are in agreement with our data.

Analysis of partially remyelinated and shadow plaques allowed us to detect Naᵥ channel and paranodin/Caspr aggregates in PLP-negative fibres, suggesting that aggregation of Naᵥ channels and paranodin/Caspr precedes remyelination. In addition, by comparing the percentage of isolated aggregates of either Naᵥ channels or paranodin/Caspr in partially remyelinated lesions, we conclude that Naᵥ channel clustering is likely to precede paranodin/Caspr aggregation in remyelination, as it does during development in normal myelination.

Alternatively, these non-uniformly scattered Naᵥ channel aggregates in PLP-negative fibres may instead represent persisting aggregates. Although we cannot rule out this possibility, the shorter length of these aggregates in partially remyelinated lesions compared with totally demyelinated lesions is not in favour of this hypothesis. Alternatively, these aggregates could represent phi-nodes, which are scattered aggregations of Naᵥ channels that have been shown to be established several days before nodes appear and correspond to precursors of nodes (Smith et al., 1982). The large length of these channels (2-fold the normal length) is similar to that described (Smith et al., 1982; Rosenbluth and Blakemore, 1984). Therefore, these Naᵥ channel aggregates on PLP-negative fibres within a partially remyelinated lesion may correspond to attempts to remyelinate in an area where both axons and oligodendrocyte precursors are spared, enabling the repair process to take place, although incompletely. However, it should be noted that broad aggregates of Naᵥ channels have been observed in mice in which oligodendrocytes had been destroyed before myelination (Mathis et al., 2001). In vitro culture studies have suggested that an oligodendroglial factor, present in the culture supernatant, induces Naᵥ channel clustering in retinal ganglion cell axons, in a contact-independent mechanism (Kaplan et al., 1997, 2001). In the PNS, gliome-<ref>den</ref>, expressed by Schwann cells, has been shown to trigger nodal-like clusters of Naᵥ channels and could represent a glial cue for the formation of nodes of Ranvier (Eshed et al., 2005). Here, we show that clusters of Naᵥ channels on PLP-negative fibres are more dense in an environment favourable for remyelination (the partially remyelinated lesion) compared with an unfavourable environment (the demyelinated plaque). This suggests that an oligodendroglial factor may be involved in this attempt to remyelinate, either by inducing reaggregation of Naᵥ channels from pre-existing nodes or through synthesis of new channels. We detected two Naᵥ channel aggregates on the same axon in very few cases, but in each case the length of the aggregate was similar, suggesting that the rate of aggregation is similar along a given axon in remyelination.

**Remyelination recapitulates development**

Controversial data have been reported on the temporal relationship of domain formation during development. It was reported in the developing murine optic nerve that Caspr clustering slightly precedes and is immediately adjacent to Naᵥ channel clusters (Rasband et al., 1999). In contrast, in the corpus callosum of 1-week-old mice, there are many nodal clusters of Naᵥ channels and ankyrin G, independent of paranodin/Caspr aggregates (Mathis et al., 2001). Comparable results have been obtained in the PNS (Vabnick et al., 1996; Rasband et al., 1998; Rios et al., 2000; Custer et al., 2003). Our data suggest that in human brain tissue Naᵥ channel clustering is an initial event in the repair process, preceding paranodin/Caspr aggregation. For juxtaparanodal domain formation, observations during development have shown that aggregation of Caspr2 and Kᵥ channels lags behind that of Naᵥ channels by several days (Baba et al., 1999; Rasband et al., 1999; Rasband and Shrager, 2000; Poliak et al., 2001). Our data cannot directly define the respective chronology of paranodal and juxtaparanodal...
aggregates. However, as isolated K, channel aggregates on naked axons were very rarely detected, and the length of K, channel aggregates in remyelinated lesions is similar to aggregates in control tissue, K, channel aggregation is probably a late event in the repair process, coincident to or just preceding remyelination. Therefore, our data suggest that remyelination in multiple sclerosis recapitulates the developmental sequence of domain formation.

Reorganization of nodal and perinodal axonal constituents preceding remyelination, and NaN, channel aggregation being the first step of the repair process, it could be hypothesized, although this is purely speculative, that a defect of NaN, channel aggregation might play a role in the remyelination failure in demyelinated lesions with spared axons and oligodendroglial cells.

Supplementary material
Supplementary material is available at Brain online.

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References

Nodal, paranodal and juxtaparanodal proteins in MS


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