Axo-glial Interactions Regulate the Localization of Axonal Paranodal Proteins

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Abstract. Mice incapable of synthesizing the abundant galactolipids of myelin exhibit disrupted paranodal axo-glial interactions in the central and peripheral nervous systems. Using these mutants, we have analyzed the role that axo-glial interactions play in the establishment of axonal protein distribution in the region of the node of Ranvier. Whereas the clustering of the nodal proteins, sodium channels, ankyrinG, and neurofascin was only slightly affected, the distribution of potassium channels and paranodin, proteins that are normally concentrated in the regions juxtaposed to the node, was dramatically altered. The potassium channels, which are normally concentrated in the paranode/juxtaparanode, were not restricted to this region but were detected throughout the internode in the galactolipid-deficient mice. Paranodin/contactin-associated protein (Caspr), a paranodal protein that is a potential neuronal mediator of axon-myelin binding, was not concentrated in the paranodal regions but was diffusely distributed along the internodal regions. Collectively, these findings suggest that the myelin galactolipids are essential for the proper formation of axo-glial interactions and demonstrate that a disruption in these interactions results in profound abnormalities in the molecular organization of the paranodal axolemma.

Key words: paranodin • potassium channels • sodium channels • galactolipids • axo-glial interactions

Myelinated axons contain regularly spaced unmyelinated gaps known as nodes of Ranvier, which are critical for the proper function of the central nervous system (CNS)1 and the peripheral nervous system (PNS) (M orell et al., 1994). Both the structure and molecular organization of the nodal region are dependent on the formation of the appropriate axo-glial interactions. Structurally, these interactions establish the spacing of adjacent myelin segments and ultimately determine the position and length of the nodal gaps. In addition, septate-like junctions are formed at the interface between the myelin sheath and axon in the paranode (for review see Salzer, 1997). Based on the location of these axo-glial junctions, Rosenbluth (1990) proposed that they function in the establishment and maintenance of axolemmal protein domains of the nodal and paranodal regions.

The most recognized domains of the nodal/paranodal region are those of the sodium and potassium channels. The positioning and segregation of these channels along the axonal membrane of myelinated fibers is crucial for the rapid conduction of nerve impulses in both the CNS and PNS (Bl ack et al., 1990). Voltage-gated sodium channels, which are concentrated at the node of Ranvier are mainly responsible for the axonal depolarization that is required for action potential induction (for review see Ritchie, 1995). In the paranodal and adjacent juxtaparanodal regions, Shaker-type Kv1.1 potassium channels cluster (W ang et al., 1993). The function of these channels is not completely understood, but they appear to prevent aberrant neuronal firing during development (V abnick et al., 1999) and may modulate action potential duration and frequency (H ille, 1992; Smart et al., 1998).

In addition to the sodium and potassium channels, other axolemmal proteins are also clustered in the region of the node of Ranvier of mature, myelinated axons (for review see Scherer, 1996). Several of these proteins are initially distributed along the entire axon of unmyelinated fibers. Although these proteins become relocated to the nodal region, the mechanisms that mediate these events are poorly understood. Nodal clustering of neurofascin, an L1-associated protein that is tightly coupled to the cytoskeleton...
(Tuvia et al., 1997), precedes the elaboration of compact myelin and may play an important role in defining the site of node formation (Lambert et al., 1997). In contrast, the clustering of ankyrin\(\text{G}\), a nodal protein that is associated with sodium channel distribution (Kordeli et al., 1990), appears to be dependent on the presence of the myelin sheath (Lambert et al., 1997). Similarly, paranodin, a 180–190-kD neuronal glycoprotein that is a potential cell adhesion molecule, accumulates in the paranodal axolemma after myelination (Eiheler et al., 1997; Menegoz et al., 1997) and is a component of the paranodal septate-like junctions that may be involved in establishment and maintenance of the protein domains of the nodal and paranodal axolemma (Rosenbluth, 1990). This protein was also identified as a partner of contactin, a glycosyl-phosphatidyl-inositol–anchored adhesion molecule, and termed contactin-associated protein (Caspr) (Peles et al., 1997). Nevertheless, the neuronal and/or glial molecules that are responsible for its localization in the paranodal junctions are not known.

To further explore the role that axo-glial interactions play in the organization of axolemmal nodal proteins, we have analyzed the distribution of these proteins in mice that contain a disruption in the gene that encodes UDP-galactose–ceramide galactosyltransferase (CGT), an enzyme that is essential for the production of galactocerebroside (GalC) and sulfatide (Morell and Radin, 1969). The galactolipid-deficient mice display several CNS nodal and paranodal abnormalities, including increased heminode formation, altered node length, and terminal loops that frequently face away from the axon (Dupree et al., 1998; Popko, 2000). Moreover, the complete absence of CNS and PNS transverse bands, which are prominent components of the paranodal septate-like junctions, indicates that axo-glial interactions are severely disrupted in the galactolipid-deficient animals (Dupree et al., 1998; Du- pree and Popko, 1999). These studies described here should enhance our understanding of the role that axo-glial interactions play in the organization of axolemmal proteins.

Materials and Methods

**Immunocytochemical Analysis**

Spinal cords (C3) and sciatic nerves were harvested from 30-d-old galactolipid-deficient and wild-type mice, diced into small pieces, homogenized in 1% SDS in PB, and placed in a boiling water bath for 10 min. Insoluble material was removed by centrifugation at 5,000 \(g\) for 10 min. Protein concentrations were determined using a modified Lowry assay (Bio-Rad, Inc.). 100 \(\mu\)g of protein was separated using a precast 4–15% gradient polyacrylamide gel (Jule, Inc.), transferred to nitrocellulose, and stained with Ponceau S. The nitrocellulose was blocked for 1 h in 5% milk solids, 5% normal goat serum, and 0.1% Triton X-100 in PB, incubated in anti-paranodin (1:3,000) for 3 h at room temperature, rinsed in PB, blocked, incubated in goat anti-rabbit secondary antibody conjugated to HRP, rinsed in PB, and visualized using ECL™ according to the manufacturer’s instructions (A mersham Pharmacia Biotech).

**Results**

**Distribution of Ion Channels**

To better understand the consequences that altered axo-glial interactions of the CNS (Dupree et al., 1998) and PNS (Dupree and Popko, 1999) have on the establishment of ion channel domains, we analyzed the distribution of the nodally clustered voltage-gated sodium channels and the paranodal/juxtaparanodal \(\text{Kv1.1}\) potassium channels in the CGT-deficient mice. In both the mutant and wild-type mice, CNS and PNS sodium channels were concentrated in small regions that were presumptive nodes of Ranvier (Fig. 1). Similar results have been reported previously regarding sodium channel distribution in the PNS of galactolipid-deficient mice (Bosio et al., 1998). Since we reported previously that CNS nodal length is increased in the galactolipid-deficient mice (Dupree et al., 1998), we measured the length and the width of the sodium channel domain and report node length as a function of axon caliber. In the CNS, the length to width ratio was significantly greater in the mutant (1.17 ± 0.05; \(n = 3\) mice and 36 nodes; \(P < 0.02\) by \(t\) test) compared with littermate wild-type mice (1.05 ± 0.13; \(n = 3\) mice and 35 nodes) mice, indicating that the sodium channel domains, which likely corresponds to nodal length, were increased in the galactolipid-deficient animals.

In contrast, potassium channel distribution was dramatically altered in the CNS of the CGT-deficient mice. In the CNS and PNS of wild-type mice, intense labeling was commonly observed in the juxtaparanodal region (Fig. 1, a and c; Table I). The juxtaparanode was easily distinguished from the paranodal and nodal regions, since its diameter was conspicuously larger. Potassium channel antibody reactivity was occasionally observed in the paranodal region; however, the labeling intensity was greatly reduced. In the
CNS of the mutant animals, fewer juxtaparanodal regions were immunolabeled with the Kv1.1 antibody (Fig. 1 b), whereas diffuse labeling over long stretches of axons was occasionally observed (see Fig. 3 b). When paranodal/juxtaparanodal potassium channel accumulations were present, the width of the labeling pattern did not change, indicating that the diameter of these axons does not change at the interface between the paranode and juxtaparanode in the galactolipid-deficient mice. In the PNS, paranodal/juxtaparanodal regions were typically labeled, although the labeling was frequently less intense and more diffuse as compared with the wild-type sciatic nerve fibers (Fig. 1, c and d).

To verify the location of the sodium and potassium channel accumulations, we double-labeled CNS and PNS tissues with antibodies directed against the ion channels (Fig. 1). The findings from these double-labeling experiments supported the single immunolabeling experimental observations that the sodium channel distribution was not altered in the mutants, since these channels maintained a nodal localization in both the CNS and PNS. Using the sodium channel clustering as an indicator of node position, we have quantitatively demonstrated that the potassium channels are concentrated in the CNS and PNS juxtaparanodal regions with a minority of axons exhibiting paranodal distribution. In the galactolipid-deficient mice, the channels were rarely limited to the juxtaparanode. Instead, the potassium channels in the CNS were frequently clustered in the paranodal region, diffusely distributed along the internode, or were not detected, whereas in the PNS they were primarily observed only in the paranodes. In addition to facilitating the potassium channel quantitative analysis, the double-labeling approach also revealed that the prominent separation between the ion channel domains in the

Table I. Quantitation of Potassium Channel Distribution

|                | Percent of total |
|----------------|------------------|
|                | ND   | Internodal | Paranodal | Juxtaparanodal |
| CNS            |      |            |           |                |
| +/+            | 10.3 | 4.8        | 13.3      | 71.6           |
| −/−            | 25.8 | 35.0       | 37.5      | 1.7            |
| PNS            |      |            |           |                |
| +/+            | 0.0  | 7.7        | 0.0       | 92.3           |
| −/−            | 11.5 | 7.7        | 80.8      | 0.0            |

The quantitation of Kv1.1 potassium channel localization demonstrates that the channels are concentrated in the CNS and PNS juxtaparanodal regions in the wild-type (+/+), but not in the galactolipid-deficient (−/−) mice. In the CNS of the −/− animals, potassium channels are often not detected (ND), diffusely distributed throughout the internode, or restricted to the paranode but rarely limited to the juxtaparanodal region. In the PNS, the channels were predominately concentrated in the paranodal region. For the CNS, 128 nodes from five wild-type mice and 120 nodes from four mutant mice were analyzed. For the PNS, 26 nodes from three mice were analyzed for both groups.
wild-type tissue is frequently absent in the mutant. In the mutant mice the potassium and sodium channel domains occasionally overlapped (Fig. 1).

**Distribution of Cell Adhesion and Cell Adhesion–associated Molecules**

The structural abnormalities at the node of the galactolipid-deficient mice appear to be related to compromised axo-glial interactions, such that we have analyzed the distribution of two potential neuronal adhesion molecules: paranodin and neurofascin. In addition, we have determined the distribution of the cytoskeleton-associated molecule ankyrin<sub>G</sub>. Using a combination of immunocytochemical techniques and confocal microscopy, we demonstrated the complete absence of paranodin accumulation in the paranodal regions of the myelinated fibers of the spinal cord in the CGT<sup>−/−</sup> mouse (Fig. 2, a and b). In the galactolipid mutants, paranodin appeared to be diffusely distributed along the axon (Fig. 2, c and d), resembling the expression pattern of unmyelinated fibers (Einheber et al., 1997). In the sciatic nerve, paranodin was localized to the paranodal region; however, the staining intensity was reduced and the border between the paranode and the juxtaparanode was not as clearly defined as in the wild-type sciatic nerve (Fig. 2, c and d). Paranodin was not detected in the paranode of any of the CNS fibers examined and reduced accumulations of paranodin were always observed in the paranode of the PNS fibers observed. Western blot analysis revealed no difference in the level of paranodin expression between the galactolipid mutant and wild-type animals for either the spinal cord or the sciatic nerve (Fig. 3), indicating that the diminished immunoreactivity was a result of abnormal paranodal accumulations. In contrast, the distribution of the nodal proteins neurofascin (Fig. 4) and ankyrin<sub>G</sub> (data not shown) did not appear altered in either the spinal cord or the sciatic nerve of the mutant.

**Discussion**

We reported previously that mice incapable of synthesizing the myelin galactolipids GalC and sulfatide exhibit structural abnormalities of the nodal and paranodal regions that are likely due to compromised axo-glial interactions (Dupree et al., 1998; Dupree and Popko, 1999). In this study, we demonstrate that in the CNS, the distribution of the Shaker-type K<sub>v1.1</sub> potassium channels is altered, whereas the clustering of the voltage-gated sodium channels is only mildly affected. Furthermore, we show a complete disruption in the axolemmal organization of the potential axo-glial adhesion molecule paranodin. In the PNS, we demonstrate similar trends with regard to ion channel organization and paranodin distribution; however, the abnormalities are less dramatic. The regional differences correlate well with the structural data, since the morphological abnormalities in the PNS are less severe.

**Figure 2.** In the wild-type mice paranodin (green) is highly concentrated in the paranodal regions of spinal cord (a) and sciatic nerve (c) axons. In contrast, the galactolipid-deficient mice exhibit a more diffuse labeling pattern. In the mutant spinal cord (b) paranodin is evenly distributed in the axolemma throughout the internode. In the sciatic nerve (d) of these mice, paranodin is concentrated in the paranode but the interface between the paranode and the juxtaparanode is not clearly demarcated. a and b, eight images 0.26 μm apart; double-labeled for paranodin in green and phosphorylated neurofilament in red. c and d, eight images 0.4 μm apart; double-labeled for paranodin in green and myelin basic protein in red. Bar, 5 μm.
Taken together, we propose that the disruption in the distribution of paranodin is further evidence that axo-glial interactions are disrupted in the galactolipid-deficient mice, and that these aberrant interactions impair appropriate ion channel segregation.

Compromised Axo-glial Interactions Result in Abnormal Ion Channel Distribution

The pattern of potassium channel distribution that we report for the galactolipid-deficient mice is consistent with a previous report of shiverer mice (Wang et al., 1995), which also display compromised axo-glial interactions in the CNS (Rosenbluth, 1980a). In both shiverer and galactolipid-deficient mice, potassium channels frequently do not cluster in the paranodal/juxtaparanodal region but are diffusely distributed throughout the internodal regions. Furthermore, the sciatic nerve of shiverer mice display elongated paranodal/juxtaparanodal potassium channel labeling (Wang et al., 1995). This alteration in ion channel distribution correlates well with the mild disruption in paranodal Schwann cell–axon interactions (Rosenbluth, 1980b). Likewise, potassium channel distribution is altered in the PNS of the CGT<sup>−/−</sup> mice coinciding with compromised axo-glial interactions and reduced paranodal accumulation of paranodin.

Consistent with abnormal potassium channel distribution, particularly in conjunction with aberrant paranode structure, action potential duration is increased in the CNS of the galactolipid-deficient mice (Coetzee et al., 1996). In addition, action potential amplitude is decreased in the CNS (Coetzee et al., 1996), and to a lesser degree in the PNS (Dupree et al., 1998). Furthermore, the addition of 4-aminopyridine, an inhibitor of potassium channels, results in little or no change in PNS amplitude, whereas CNS amplitude increased 25%. This difference likely reflects...
the greater alteration of potassium channel distribution in the CNS compared with the PNS in these mutants. A xo-gial interactions do not only influence potassium channel distribution. Clustering of sodium channels in the PNS also appears dependent upon the appropriate association of the Schwann cell with the axon (Dugandzija-Novakovic et al., 1995). In the CNS, Kaplan et al. (1997) reported that oligodendrocyte contact is not required for initial sodium channel clustering in vitro, but a recent report demonstrates that axo-gial contact, as indicated by paranodin and myelin-associated glycoprotein labeling, is required for the sodium channel accumulation in vivo (Rasband et al., 1999). In the galactolipid-deficient mice, sodium channels are concentrated in nodal regions. This finding demonstrates that normal paranodal axo-gial contacts are not essential for nodal clustering of sodium channels. Although gross clustering of sodium channels to the nodal gap is not dependent upon the formation of the paranodal septate-like junctions, these axo-gial junctions may be important in establishing and maintaining the interface between the sodium and potassium domains, since these domains occasionally overlap in both the CNS and the PNS of the galactolipid-deficient mutant.

**Galactolipids Are Essential for Proper Formation of Axo-gial Interactions**

Using ultrastructural analysis, we have shown previously that axo-gial interactions are disrupted in the galactolipid-deficient mice (Dupree et al., 1998; Dupree and Popko, 1999). Here we provide evidence that these interactions are also disrupted at the molecular level, since paranodin, a known component of the septate-like junctions that form between the myelin sheath and the axolemma (Einheber et al., 1997), does not appropriately accumulate in the paranodal region. Presently, the mechanism responsible for proper axolemal distribution of paranodin is not known. Since paranodin has multiple potential cell adhesion domains including an extracellular lectin-binding domain (Menegez et al., 1997; Peles et al., 1997), an attractive model is that GalC and/or sulfatide directly bind paranodin and facilitate its accumulation in the paranodal axolemma. Nevertheless, there is no evidence to suggest that the galactolipids accumulate in the paranodal region. A another possibility centers on the role that the galactolipids play in detergent-insoluble-complex (DIGs) formation and trafficking. In oligodendrocytes, DIGs, which are raft-like microdomains composed of the myelin galactolipids and proteins, are thought to be responsible for the molecular organization of the myelin sheath (Kramer et al., 1997, 1999). Therefore, in mice that lack GalC and sulfatide, an as yet unidentified paranodin ligand may be abnormally distributed in the oligodendrocyte of CGT<sup>−/−</sup> mice, resulting in the disruption of paranodal intracellular targeting.

The rearrangement of axolemmal proteins in the CGT<sup>−/−</sup> mice appears to be specific to the paranodal region, since the membrane arrangement of ankyrin<sub>G</sub> and neurofascin is not grossly affected. The clustering of neurofascin precedes myelination (Lambert et al., 1997), therefore it is not surprising that its distribution is not affected by a myelin gene mutation. In contrast, the clustering of ankyrin<sub>G</sub> and voltage-dependent sodium channels is temporally associated with the elaboration of myelin-associated glycoprotein-positive myelin-forming processes (Lambert et al., 1997). Therefore, if the distribution of sodium channels and ankyrin<sub>G</sub> is dependent on myelin, the mechanism by which these proteins are spatially organized apparently does not require the myelin galactolipids and is distinct from the process that regulates potassium channel and paranodin distribution.

In summary, mice that are incapable of producing the myelin galactolipids have compromised axo-gial interactions as evidenced by CNS and PNS structural abnormalities (Dupree et al., 1998; Dupree and Popko, in press). Furthermore, the distribution of paranodin, a prominent component of the paranodal septate-like junctions (Menegez et al., 1997; Einheber et al., 1997), is dramatically altered with no paranodal accumulation in the CNS. The disruption in axo-gial interactions leads to the abnormal distribution of the Shaker-type K<sub>V</sub>1.1 potassium channels in both the CNS and the PNS. Therefore, our data indicate that axo-gial interactions are essential not only for the proper myelin formation but also axolemmal organization.

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