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Early events in node of Ranvier formation during myelination and remyelination in the PNS

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Abstract

Action potential conduction velocity increases dramatically during early development as axons become myelinated. Integral to this process is the clustering of voltage-gated Na⁺ (Nav) channels at regularly spaced gaps in the myelin sheath called nodes of Ranvier. We show here that some aspects of peripheral node of Ranvier formation are distinct from node formation in the CNS. For example, at CNS nodes, Nav1.2 channels are detected first, but are then replaced by Nav1.6. Similarly, during remyelination in the CNS, Nav1.2 channels are detected at newly forming nodes. By contrast, the earliest Nav-channel clusters detected during developmental myelination in the PNS have Nav1.6. Further, during PNS remyelination, Nav1.6 is detected at new nodes. Finally, we show that accumulation of the cell adhesion molecule neurofascin always precedes Nav channel clustering in the PNS. In most cases axonal neurofascin (NF-186) accumulates first, but occasionally paranodal neurofascin is detected first. We suggest there is heterogeneity in the events leading to Nav channel clustering, indicating that multiple mechanisms might contribute to node of Ranvier formation in the PNS.

Keywords

Myelin; demyelination; Na⁺ channel; paranode; axon–glia interaction

INTRODUCTION

The development of nodes of Ranvier provides an outstanding example to illustrate how neuron–glia interactions shape the form and function of the nervous system. It is clear that the localization and retention of voltage-gated Na⁺ (Nav) channels at nodes of Ranvier, and Kv1 channels at juxtaparanodes, requires extrinsic factors derived from myelinating glia (for recent reviews see: Poliak and Peles, 2003; Salzer, 2003). For example, an axonal heterodimer comprised of the cell adhesion molecules caspr2 and TAG-1 interacts with Kv1 channels to restrict their localization to juxtaparanodal domains through *trans* interactions with glial TAG-1 (Poliak *et al.*, 2003; Traka *et al.*, 2003). Furthermore, the Kv1 channels of mutant mice with aberrant paranodal structure are not restricted to juxtaparanodes (Dupree *et al.*, 1999; Bhat *et al.*, 2001; Boyle *et al.*, 2001), which indicates that the paranode restricts Kv1-channel localization. Thus, two kinds of contact-dependent neuron–glia interactions restrict Kv1 channels to juxtaparanodal domains of myelinated axons.

In contrast to Kv1 channels, how Nav channels cluster at nodes of Ranvier is much more controversial. Although several studies indicate that some form of paranodal contact is required for channel clustering (Rasband *et al.*, 1999; Boiko *et al.*, 2001), others suggest that a soluble, oligodendrocyte derived factor initiates channel clustering (Kaplan *et al.*, 1997). Despite these differences in mechanism, one general conclusion is agreed: myelinating glia are crucial determinants of ion-channel localization in axons. The recent identification of gliomedin as a crucial contact-dependent mediator of channel clustering in the PNS has helped to resolve some of the confusion (Eshed *et al.*, 2005). However, gliomedin does not appear to be involved in CNS node formation.

The notion that myelinating glia actively regulate neuronal properties is supported further by experiments examining the subtypes of Nav channels found in myelinated and demyelinated axons (Boiko *et al.*, 2001; Kaplan *et al.*, 2001). For example, Boiko *et al.* (2001) show that during early development, Nav1.2 channels are detected at newly forming nodes of Ranvier in both the PNS and CNS. Surprisingly, as myelination progresses, Nav1.2 channels are gradually replaced by Nav1.6. However, this late onset of Nav1.6 localization at nodes of Ranvier was only tested in the CNS. Subsequent studies show that after demyelination in the CNS, Nav1.6 channel clusters are lost and there is a dramatic increase in Nav1.2 protein along the axon, which indicates that myelin actively regulates the localization and kinds of channels that occur in axons (Craner *et al.*, 2003; Rasband *et al.*, 2003a). Furthermore, during remyelination in the CNS Nav1.2 channels cluster at newly forming nodes of Ranvier (Dupree *et al.*, 2004).

In addition to Nav channels, other ion channels, cytoskeletal and scaffolding proteins, and cell adhesion molecules are also found at nodes and might be important contributors to node of Ranvier formation (Poliak and Peles, 2003; Salzer, 2003). Among these proteins the cell adhesion molecules neurofascin and NrCAM are good candidates for involvement in node formation and neuron–glia interactions because they are thought to be the first axonal proteins to cluster at nascent nodes (Lambert *et al.*, 1997). These proteins might act as pioneer molecules and nucleation sites for the recruitment of other nodal proteins such as ankyrinG (Lambert *et al.*, 1997).

In the present study, to determine if ion channel subtype localization is regulated in the PNS in a manner similar to that seen in the CNS, we have examined the localization of Nav1.2 and Nav1.6 channels in the PNS during myelination and remyelination. Additionally, we have determined the localization of glial and neuronal neurofascin splice variants during node and paranode formation. Together, our results show that some aspects of node of Ranvier formation in the PNS are distinct from those described in the CNS, and that neurofascin-dependent neuron–glia interactions precede the clustering of Nav channels.

OBJECTIVE

In this study we determine key events surrounding the development and maturation of peripheral nodes of Ranvier. In particular we determine the time course of Nav channel subtype localization at nodes of Ranvier during developmental myelination and during remyelination. Because previous studies indicate that the cell adhesion molecule neurofascin is a pioneer molecule for node of Ranvier formation, we also sought to distinguish the role of the neuronal NF-186 splice-variant from that of the glial NF-155 splice-variant in early peripheral node formation.

MATERIALS AND METHODS

Primary antibodies

The monoclonal anti-pan Nav channel (K58/35), anti-Nav1.2 (K69/3) and anti-pan neurofascin (L11A/41.6) antibodies have been described previously (Rasband *et al.*, 1999; Boiko *et al.*, 2001; Rasband *et al.*, 2003; Schafer *et al.*, 2004). The polyclonal anti-Nav1.2, anti-Nav1.6 and anti-caspr antibodies have been described previously (Rasband *et al.*, 1999; Boiko *et al.*, 2001; Rasband *et al.*, 2003) and were kindly provided by Dr. James Trimmer. The polyclonal anti-neurofascin-186 antibody was kindly provided by Dr. Alex Gow. This antibody has been tested by immunoblot (recognizes a band of the appropriate molecular weight) and stains only nodes of Ranvier and axon initial segments, which is consistent with previous reports of NF-186 localization (data not shown).

Immunohistochemistry

Sprague-Dawley rats (Charles River), aged ≥ 10 days, were sacrificed using halothane (Halocarbons Inc.). Neonatal rats aged ≤ 10 days were sacrificed by rapid decapitation. Sciatic nerves were dissected rapidly and immediately fixed in ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.2, for 30 minutes. After fixation, nerves were transferred to ice-cold 20% sucrose (w/v) in 0.1 M PB overnight. Nerves were then frozen in Tissue-Tek OCT mounting medium. Sections (10 μ m thick) were cut, placed in 0.1 M PB and spread on gelatin-coated glass coverslips. After sections were allowed to air dry, immunofluorescence was performed as described previously (Rasband *et al.*, 1999). Secondary antibodies used included Alexa-488 and Alexa-594 conjugated to goat anti-mouse and goat anti-rabbit IgG, respectively (Molecular Probes). Digital images were collected on a Zeiss (Thornwood) Axioskop 2 fluorescence microscope fitted with a Hamamatsu (Bridgewater) ORCA-ER camera. For some data, a z-stack of images was collected at 0.2 μ m intervals. These stacks were subsequently deconvolved by iterative restoration using Volocity software (Improvision). All images were taken using a 63 $\times$, 1.4 NA oil-immersion objective.

Demyelination

Lyssolecithin-induced acute demyelination was performed as described (Dugandzija-Novakovic *et al.*, 1995). All animal procedures were approved by the Institutional Animal Care Committee at the University of Connecticut Health Center and conform to the United States Public Health Service Policy on Human Care and Use of Laboratory Animals. Briefly, female Lewis rats were anesthetized. The sciatic nerve was exposed and injected with ~ 2 μ l of 1% lyssolecithin diluted in sterile Locke's solution (154 mM NaCl, 5.6 mM KCl, 2 mM CaCl₂, and 10 mM HEPES, pH 7.4). Following surgery, the incision was sutured and animals were allowed to recover for 4–11 days.

Quantification

Nodal or node-associated clusters were counted from 10 fields of view ($>P1$) or entire nerves ($<P1$). These raw data were then summed. For comparing Nav channel subtypes to total pan-Nav positive clusters, sum totals of clusters positive for either Nav1.6 or Nav1.2 were calculated as a percentage of total clusters positive for pan-Nav channel. These percentages were averaged across three independent animals; results are mean $\pm$ SEM. Nerves were also double-labeled for Nav1.6 and Nav1.2. In these experiments, Nav1.6 and/or Nav1.2 positive clusters were summed and expressed as a percentage of total immunoreactive clusters (Nav1.6 positive, Nav1.2 positive, and Nav1.2/1.6 positive). These percentages were averaged across two independent animals. Immunostaining for Nav1.6, Nav1.2 and caspr, with pan-NF was performed in 2–3 independent animals. Clusters of Nav1.6, Nav1.2 and caspr were counted and expressed as percentages of total clusters positive for pan-NF. Similarly, clusters of NF-186

were counted and expressed as a percentage of clusters positive for pan-NF $\pm$ SEM of three experiments. Nodes that were NF-186-positive and NF-155-negative were defined by complete colocalization of NF-186 and pan-NF antibodies. Clusters defined as NF-186- and NF-155-positive had colocalization of pan-NF and NF-186 with some flanking pan-NF immunoreactivity. Clusters were defined as NF-155-positive if they were immunoreactive for pan-NF and negative for NF-186.

RESULTS

Nav Channel subtypes at developing nodes of Ranvier in the PNS

In the developing CNS, Nav1.2 is initially expressed at all newly forming nodes of Ranvier. Upon maturation, nodal Nav1.2 is downregulated and replaced by Nav1.6 (Boiko *et al.*, 2001). Boiko *et al.* (2001) also detected Nav1.2 during PNS-node formation, which suggested that switching of Nav-channel subtypes is a general phenomenon in both the CNS and PNS. However, in these studies Nav1.6 was not examined directly in PNS nerves. Therefore, to determine if Nav channel-subtype switching at nodes is a generalized phenomenon, we analyzed Nav1.2 and Nav1.6 subtype localization by immunofluorescence microscopy in the developing rat sciatic nerve at postnatal days 0–4 (P0–P4). To identify developing nodes of Ranvier in the PNS, we used a pan-Nav channel monoclonal antibody (which recognizes all neuronal Nav channel subtypes) together with rabbit polyclonal antibodies specific for either Nav1.2 or Nav1.6. As in the CNS and consistent with previous reports (Boiko *et al.*, 2001), Nav1.2 is detected at nearly every Nav channel-positive cluster during early myelination (Fig. 1a). However, unlike the CNS, Nav1.6 is also localized to nearly every pan-Nav channel cluster during early myelination (84–90% at P0–P1; Fig. 1c). As myelination progresses, nodal Nav1.2 immunoreactivity decreases and nodal Nav1.6 localization is retained and increased. By P3–P4, <25% of pan-Nav channel-labeled clusters also had Nav1.2 immunoreactivity (Fig. 1b), but nearly every node had Nav1.6 channels (Fig. 1d,e). In adults, nodal Nav1.2 immunoreactivity is not detected and every node is labeled for Nav1.6 (data not shown).

The results described above indicate that a substantial fraction of newly forming nodes contained both Nav1.2 and Nav1.6. To examine this directly, we double-labeled nodes using polyclonal antibodies specific for Nav1.6 and a mouse monoclonal antibody specific for Nav1.2. At each age, nodes were characterized as immunoreactive for Nav1.2, Nav1.6, or both Nav1.2 and Nav1.6. As the data above indicate, we found nodes with both Nav1.2 and Nav1.6 immunoreactivity (Fig. 2a,c). Nodes that were positive for only Nav1.2 (Fig. 2b, arrow) are also detected at the earliest time points of myelination (Fig. 2c), but are uncommon as myelination progressed. Interestingly, even at these early time points of PNS node formation, Nav1.6 as the only subtype was detected at a small percentage of nodes (22% at P0). This percentage increases rapidly with age and is virtually 100% by P4 (Fig. 2c). The number of nodes positive for both Nav1.2 and Nav1.6 (Fig. 2a) increases from P0 to P1, but begins to decrease after P2, presumably because the Nav1.2 channels are removed from nodes (Fig. 2c).

To confirm that early clusters of Nav1.2 and Nav1.6 channels are destined to become nodes of Ranvier, we immunostained developing nerve with antibodies against caspr, a component of the paranodal axoglial junction. Early clusters of Nav1.2 and Nav1.6 form adjacent to caspr-labeled paranodes. For example, we observed Nav1.2 and Nav1.6 channels at heminodes (Fig. 3a,b), in binary clusters (Fig. 3c,d) and in focal clusters (Fig. 3e,f). These patterns of immunoreactivity are consistent with previous descriptions of Nav channel clustering in the developing PNS (Vabnick *et al.*, 1996). Thus, in contrast to the CNS, Nav1.6 is detected at many newly forming nodes of Ranvier in the PNS.

Nav channel subtypes at nodes of Ranvier during remyelination in the PNS

Recent experiments have shown that CNS demyelination results in the loss of Nav1.6 channel clusters and a dramatic upregulation of Nav1.2 in axons (Rasband *et al.*, 2003a; Craner *et al.*, 2004a). These data indicate that oligodendrocytes actively regulate the complement and localization of Nav channels expressed in CNS axons. Consistent with this idea, Dupree *et al.* (2004) reported, using the cuprizone model of CNS demyelination and remyelination, that demyelinated CNS axons have clusters of Nav1.2 channels, and that nodes in the remyelinated corpus callosum eventually have only Nav1.6 channels. Thus, clustering of Nav channels during CNS remyelination might recapitulate developmental myelination.

To compare node formation during remyelination in the PNS to events during PNS developmental myelination, we used an intraneural injection of lyssolecithin to induce an acute, focal demyelinated lesion in the sciatic nerve. In this model, PNS myelin in the area of injection is lost within 1 week. Thereafter, Schwann cells proliferate and begin to remyelinate denuded axons. Previous studies have shown that clusters of Nav channels accumulate and form new nodes of Ranvier during remyelination in the second week after surgery (Dugandzija-Novakovic *et al.*, 1995). When we double-labeled remyelinating axons with antibodies against Nav1.2 and Nav1.6, we were unable to detect Nav1.2 clusters. This is in contrast to newly forming nodes during PNS developmental myelination (Fig. 4a,b). We labeled demyelinated axons with antibodies against Nav1.6 and found focal clusters of Nav1.6 (Fig. 4c) and heminodes (Fig. 4d, arrow) with smaller clusters of Nav1.6 channels that extend into the demyelinated zone (Fig. 4d, arrowheads); this immunoreactivity might represent Nav channels that are dispersing throughout the axolemma as a consequence of demyelination (Arroyo *et al.*, 2004). During remyelination (11 dpi), all newly forming nodes (identified by pan-Nav staining (not shown) or pan-neurofascin staining) contain Nav1.6 channels (Fig. 4e,f). Thus, in contrast to the CNS (Dupree *et al.*, 2004), new nodes of Ranvier that form during PNS remyelination contain Nav1.6.

Neurofascin localization in the developing PNS

Neurofascin is reported to be the first protein to accumulate at newly forming nodes of Ranvier, and to provide the nucleation site for attachment of ankyrinG, Nav channels and other nodal proteins (Lambert *et al.*, 1997). The recent identification of the Schwann cell microvilli protein gliomedin as the likely glial binding partner of axonal neurofascin provides additional evidence for the importance of this protein in recruiting Nav channels to nodes of Ranvier (Eshed *et al.*, 2005). Therefore, we investigated the connection between the cell adhesion molecule neurofascin and Nav channel clustering. We used a pan-neurofascin antibody together with either Nav1.2 or Nav1.6 specific antibodies for this analysis. At P0, we found that 69% of neurofascin labeled sites lacked Nav1.6 immunoreactivity and 62% lacked Nav1.2 immunoreactivity (Fig. 5a,b,e). We never observed Nav channel clusters without neurofascin staining. As myelination progressed, the number of neurofascin labeled sites with both Nav1.2 and/or Nav1.6 immunoreactivity increased (Fig. 5e). Consistent with the results described above (Figs 1 and 2), we found that by P4, only 23% of neurofascin labeled sites had Nav1.2 immunoreactivity. In contrast, Nav1.6 co-immunostaining steadily increased to 100% of neurofascin-labeled clusters. Careful examination of the staining patterns for neurofascin and Nav channels revealed sites where neurofascin clustered before Nav channels (Fig. 5a,b). As myelination progressed, Nav1.6 immunoreactivity overlapped with neurofascin immunoreactivity, but the neurofascin immunoreactivity always appeared to extend beyond the Nav1.6 staining either on one side (Fig. 5c, heminode, arrowhead) or both sides (Fig. 5d, a mature node, arrowheads). Our results are consistent with those of others (Lambert *et al.*, 1997; Eshed *et al.*, 2005) and indicate that neurofascin accumulates before Nav channels and is likely to have crucial roles in the earliest events associated with node of Ranvier formation.

Interpretation of the data described above and that of Lambert *et al.* (1997) is limited because the antibodies used in both studies do not distinguish between the two main splice variants of neurofascin in the nervous system: the 155 kD glial splice variant (NF-155) found at paranodes (Tait *et al.*, 2000), and the 186 kD axonal splice variant (NF-186) found at nodes and axon initial segments (Davis *et al.*, 1996). Consequently, we have been unable to determine whether the initial clusters of neurofascin consist of NF-155 at developing paranodes and/ or NF-186 at nascent nodes. In the CNS, paranodal proteins, including neurofascin, appear to cluster before localization of nodal proteins (Rasband *et al.*, 1999; Schafer *et al.*, 2004). Therefore, we sought to determine which splice variant of neurofascin is the first to appear at newly forming nodes in the PNS.

At P1–P2 ~25% of all pan-NF immunoreactive sites had precisely colocalized NF-186 immunoreactivity (Fig. 6a). The remaining pan-NF labeled sites had immunoreactivity that extended beyond NF-186 (indicating both NF-186 and adjacent glial NF-155; Fig. 6b,c). Surprisingly, we also detected sites that were labeled only with the pan-NF antibody (data not shown). It is possible that these sites represent NF-155 at forming paranodes (see below). Alternatively, the pan-NF antibody might detect lower concentrations of NF-186 than the NF-186 specific antibody. By P4, all pan-NF immunostaining extended beyond NF-186 immunoreactivity (Fig. 6c). A summary of the relative frequency for each of these patterns is shown in Fig. 6d. Taken together, these results indicate that NF-186 is the first splice variant of neurofascin to be clustered at nascent nodes of Ranvier. However, our data also indicate that, in some cases, glial NF-155 might accumulate before NF-186 (see below).

To better determine whether clustering of nodal NF-186 precedes accumulation of NF-155 at paranodes, we double-labeled developing nerve fibers with the pan-NF antibody and an antibody against caspr, an axonal component and marker of the paranodal axoglial junction (Einheber *et al.*, 1997). In early PNS myelination we found clusters of NF without caspr immunoreactivity (Fig. 7a), but we never observed caspr immunoreactivity without colocalized neurofascin immunoreactivity. These sites might represent clustered NF-186 without any adjacent components of paranodes, like that seen in Fig. 6a. We also found paranodal junctions defined by precisely colocalized caspr and pan-NF immunoreactivity (Fig. 7b). Because caspr is a definitive marker of paranodal junctions in myelinated nerve and is a putative binding partner for NF-155 (Charles *et al.*, 2002), these sites presumably represent paranodal junctions that have formed before the accumulation of adjacent NF-186. In addition to these patterns of immunoreactivity, most caspr and pan-NF labeled sites had a region of colocalized immunoreactivity corresponding to the paranode (NF-155 and caspr) (Fig. 7c, arrowhead, 7f), and an adjacent region of only pan-NF immunoreactivity corresponding to the node (NF-186; Fig. 7c, arrow, 7d,e). As nodes matured, every pan-NF labeled site had caspr immunoreactivity at the paranodes (Fig. 7e). Together, these results indicate that clustering of NF-186 usually precedes formation of paranodal junctions and the attendant clustering of NF-155. However, there are examples (Fig. 7b) where paranodal junctions form prior to accumulation of NF-186. Thus, multiple mechanisms may exist and work synergistically to facilitate clustering of Nav channels at nodes of Ranvier.

CONCLUSIONS

- In contrast to the CNS, Nav1.6 is localized to all newly forming nodes of Ranvier in the PNS.
- Similar to the CNS, Nav1.2 is initially localized to forming nodes but is lost subsequently.
- In the lysolécithin model of PNS demyelination/ remyelination Nav1.6, but not Nav1.2, is detected at newly forming nodes of Ranvier.

- Neurofascin clusters appear at the very earliest stages of node formation and can be detected before the clustering of Nav channels.
- There is heterogeneity among early nodes of Ranvier in the splice variants of neurofascin that accumulate first at nascent nodes and paranodes: the majority of nodes have both NF-186 and NF-155, but others have only NF-186 or only NF-155.

DISCUSSION

In the present study we describe early events in node of Ranvier formation including the temporal localization of the Nav channels Nav1.2 and Nav1.6 to PNS nodes of Ranvier, and of two splice variants of neurofascin; NF-155 to paranodes and NF-186 to nodes. The analysis of the localization of Nav channels and their differential detection at nodes of Ranvier has received much attention in recent years because regulation of channel localization and expression provides an example of how myelinating glia actively control neuronal properties. Furthermore, some data indicate that dysregulation of these different types of channels during demyelinating diseases might lead to axonal pathology (Craner *et al.*, 2004b).

Role of myelination in Nav channel subtype switching

The first analyses of the clustering of Nav channels at PNS nodes of Ranvier during development and remyelination were performed by Dugandzija-Novakovic *et al.* (1995) and Vabnick *et al.* (1996), respectively. In each study, Nav channels were found to accumulate at the tips of (re)myelinating Schwann cells. Importantly, when myelination was blocked, Nav channels failed to cluster, pointing to the importance of Schwann cells and myelin in the clustering process. The spatial localization of Nav channels described in the present study using antibodies against Nav1.2 and Nav1.6 are consistent with their results.

Analysis of the localization of Nav1.2 and Nav1.6 subunits in the CNS has shown that Nav1.2 occurs mainly on unmyelinated axons and Nav1.6 is located at nodes of Ranvier and axon initial segments (Gong *et al.*, 1999; Caldwell *et al.*, 2000). Thus, a simple model might be that myelination regulates the kind of Nav channels that are expressed by axons. Consistent with this idea, Boiko *et al.* (2001) showed that during early myelination in the optic nerve Nav1.2 is expressed at nodes and in nonmyelinated regions (proximal to the *lamina cribrosa*). However, shortly after Nav1.2 channels cluster and form new nodes, they are replaced by Nav1.6 channels. These results indicate that myelin actively excludes Nav1.2 (or suppresses expression) from myelinated axons and promotes the localization of Nav1.6 to nodes of Ranvier. The molecular mechanisms regulating these processes are unknown. The results presented here show that, as in the CNS, Nav1.2 is removed from newly formed PNS nodes. However, our results differ in that most newly forming nodes already have Nav1.6. Thus, in contrast to CNS optic nerve axons, Nav1.6 appears to be expressed before myelination in the PNS. The reasons for this difference might be that the early expression of Nav1.6 in DRG and spinal motor neurons does not depend on myelination. Previous studies have shown that DRG neurons and motoneurons both express Nav1.2 and Nav1.6 channel mRNAs at the onset of myelination (Felts *et al.*, 1997; Alessandri-Haber *et al.*, 2002).

With respect to demyelination and remyelination, experiments in several different mutant mouse lines have also revealed the importance of CNS myelination in regulating Nav channel expression and localization. For example, Shiverer mice have a mutation in the gene that encodes myelin basic protein that results in a severe CNS hypomyelinating phenotype (Rosenbluth, 1980; Rosenbluth, 1981). Transgenic mice that overexpress the proteolipid protein (Plp) develop adult onset chronic demyelination (Kagawa *et al.*, 1994; Inoue *et al.*, 1996). In both model animals there is a dramatic upregulation of axonal Nav1.2 (Westenbroek *et al.*, 1992; Boiko *et al.*, 2001; Rasband *et al.*, 2003a). The increase in Nav1.2 is mirrored by

a loss of clustered Nav1.6 from nodes (Rasband *et al.*, 1999; Boiko *et al.*, 2001). The phenomenon of reversion from Nav1.6 as the main Nav channel subunit in axons to Nav1.2 as the main channel subunit is not restricted to mutant mouse models, but also occurs after demyelination in multiple sclerosis (Craner *et al.*, 2004b). Interestingly, Dupree *et al.* (2004) detected Nav1.2 clusters during remyelination in the CNS (in the corpus callosum), similar to those seen during developmental myelination.

In contrast to the results described above, mice with peripheral dysmyelinating or demyelinating phenotypes (e.g. Trembler-J and P0-null) retain nodal Nav1.6 localization, fail to upregulate Nav1.2 at nodes, but do show an increase in Nav1.8 localization to the node (Ulzheimer *et al.*, 2004; Devaux and Scherer 2005). One potential complication when interpreting these studies is that in both these models bouts of peripheral demyelination and remyelination occur over months, often within the same nerve fiber. In our demyelination/remyelination experiments we only checked for Nav1.2 and Nav1.6, and only detected Nav1.6 at new nodes. One potential explanation for the failure to upregulate Nav1.2 in both our lyssolecithin-demyelinated rats and in the mutant Trembler-J and P0-null mice might be that the demyelination/dysmyelination is segmental, and the remaining myelinated regions of the axon are sufficient to continue to suppress Nav1.2. Alternatively, whereas demyelination in the CNS results in upregulation of Nav1.2, demyelination in the PNS might result in upregulation of Nav1.8 or another Nav channel. For example, Wallace *et al.* (2003) showed that lyssolecithin-mediated demyelination upregulates Nav1.3 channel expression in DRG neurons. It is not known whether this Nav1.3 is transported into axons and/or localizes to specific membrane domains (e.g. nodes and/or nerve terminals). Similarly, Devaux and Scherer (2005) showed an increase in Nav1.8 in a model of peripheral demyelination, and found Nav1.8 clustered at newly formed nodes of Ranvier.

Role of paranodes in PNS node of Ranvier formation

Lambert *et al.* (1997) report that neurofascin is the first molecule that clusters at nascent nodes of Ranvier and precedes accumulation of both Nav channels and ankyrinG. However, later studies revealed that another splice variant of neuro-fascin, NF-155, is a crucial component of paranodal junctions (Tait *et al.*, 2000). The experiments of Lambert *et al.* (1997) could not distinguish between the neurofascin splice variants. So which neurofascin comes first, paranodal NF-155 or nodal NF-186? In the CNS, paranodal neurofascin precedes detection of nodal neurofascin (Schafer *et al.*, 2004). The importance of paranodes and axoglial contact in regulating channel localization was suggested first by Rosenbluth (1985). The use of paranodal markers such as caspr also argues in favor of a model in which paranodal contact occurs before node formation and facilitates ion-channel clustering (Rasband *et al.*, 1999). These conclusions have been questioned because paranodal mutant mice lacking transverse bands (e.g. caspr-null, contactin-null and CGT-null) develop normal Nav channel clusters in the PNS (Dupree *et al.*, 1999; Bhat *et al.*, 2001; Boyle *et al.*, 2001). However, nodes are much larger in the CNS of these paranodal mutant mice (Rasband *et al.*, 2003; Rios *et al.*, 2003). These results indicate that paranodes do not determine Nav channel clustering in the PNS. However, it is important to note that, in contrast to the CNS of these mutant mice where many paranodal loops are everted, most paranodal loops in the PNS retain some form of axoglial association because all ultrastructural features can be identified with the exception of transverse bands. Thus, strictly speaking, transverse bands are not required for channel clustering. However, the general conclusion that paranodal interactions are not important for regulating Nav channel localization and/or expression is an oversimplification.

In support of the idea that paranodes are important for regulation of Nav channels, Rios *et al.* (2003) showed that caspr-null mice have many CNS nodes that retain Nav1.2 channels. However, in other paranodal mutants Nav1.2 was not detected at PNS nodes of Ranvier

(Rasband *et al.*, 2003b). Thus, the function of paranodes in the PNS might not extend to regulation of expression of Nav channel subtypes.

A major advance, which is consistent with the idea that paranodes are not the central mediator of channel clustering in the PNS, comes from the work of Eshed *et al.* (2005), who show that gliomedin is the glial binding partner for NF-186. Gliomedin is restricted to the Schwann cell microvilli, where it is ideally positioned to interact with nodal NF-186. In culture models, loss of gliomedin by RNA interference or blocking the interaction between gliomedin and with NF-186 inhibits clustering of Nav channels, which indicates that this molecule provides a local, contact dependent cue for organizing nodes of Ranvier. Furthermore, addition of soluble gliomedin to neurons in culture is sufficient to induce clustering of channels along axons. Importantly, gliomedin only appears to be present at nodes of Ranvier in the PNS; the mechanisms that regulate the clustering of Nav channels in the CNS remain unknown.

Our results on the differential localization of neurofascin splice variants are consistent with the idea that gliomedin–NF-186 interactions are the main determinants of Nav channel clustering in the PNS. However, we also found NF-155 labeled paranodes without any adjacent NF-186 immunoreactivity. This indicates that, in some cases, paranodes might form before nodal clustering of Nav channels. We speculate that, as with Kv1 channels, the localization of Nav channel subtypes also has multiple levels of regulation including neuron–glia interactions at the node and the paranode (Rasband, 2004). Future *in vivo* experiments utilizing gliomedin-null, gliomedin-null/ caspr-null, and neurofascin-null mice should help to clarify the role of paranodes in the formation and maintenance of nodes of Ranvier in the PNS.

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Fig. 1. Nav 1.6 and Nav1.2 are present at the earliest stages of node formation

(a,b) P1 and P4 sciatic nerve double-labeled with Nav1.2 (green) and Pan-Nav (red) antibodies. (c,d) P1 and P4 sciatic nerve double labeled with anti-Nav1.6 (red) and anti-Pan Nav (green) antibodies. Insets show Nav1.6 staining alone. (e) Percentage of pan-Nav channel immunoreactive clusters positive for either Nav1.6 or Nav1.2. Data are mean $\pm$ SEM of 3–4 experiments. n, total number of nodes counted at each age. Scale bars: a, 5 μ m; b–d, 2 μ m.

Fig. 2. Nav1.6 and Nav1.2 colocalize at many nodes of Ranvier during early myelination
 (a,b) P1–P2 sciatic nerve double-labeled for Nav1.2 (green) and Nav1.6 (red). The arrow points to a node that is labeled by Nav1.2 only. Insets: anti-Nav1.6. (c) Percentage of total clusters positive for Nav1.6 versus Nav1.2. Data are mean $\pm$ SEM of 3–4 experiments. n, total number of nodes counted at each age. Scale bars: 2 μ m.

Fig. 3. The earliest detectable Nav channel clusters contain Nav1.2 and/or Nav1.6 and show three distinct patterns in relation to paranodal caspr immunostaining
 Developing rat sciatic nerve (P1–P4) were double-labeled with either (a,c,e) anti-Nav1.2 (red) and caspr (green) or (b,d,f) anti-Nav1.6 (red) and caspr (green). Patterns of node formation include: (a,b) heminodes, (c,d) binary clusters and (e,f) fusing/focal clusters. Scale bars: 2 μ m.

Fig. 4. Localization of Nav channel subtypes in remyelinating adult rat sciatic nerves

(a) In developing sciatic nerve (P1) Nav1.2 immunoreactivity (green) colocalizes with pan Nav immunoreactivity (red). However, in the remyelinating sciatic nerve at 11 days post-injection (11 dpi), Nav1.2 is not detected at newly forming nodes of Ranvier. (b) Quantification of Nav1.2 immunoreactivity at pan Nav clusters at P1 and P10–11. Data are mean $\pm$ SEM. (c–f) Nav1.6 localization at various stages of demyelination and remyelination in the lysocleithin model. (c,d) At peak demyelination (7 dpi), Nav channel clusters contain Nav1.6 (arrow), but these clusters begin to disperse throughout the axon as puncta of immunoreactivity (arrowheads). (e,f) During remyelination (11 dpi), examples of nodal reorganization and newly forming nodes include: (e) binary clusters and (f) heminodes. Reforming and new nodes are labeled with the pan-NF antibody (red), which recognizes both paranodal NF-155 and nodal NF-186, and by anti-Nav1.6 antibodies (green). Scale bars: a, 3 μ m; c,d, 10 μ m; e,f, 5 μ m.

Fig. 5. Neurofascin (NF) clustering precedes Nav1.2 and Nav1.6 clustering
(a–d) Sciatic nerve (P0–P4) was double labeled with anti-pan-NF (green) and either anti-Nav1.6 (red, b–d) or anti-Nav1.2 (red, a). (e) Quantification of immunostaining from 2–3 independent experiments. Boxes represent the range (maximum and minimum percentage values), with the mean shown as the horizontal line in the box. n indicates the total number of nodes counted at each age. Scale bars: 5 μ m.

Fig. 6. Clustering of neurofascin (NF) splice variants in the developing rat sciatic nerve (a–c) Double-labeling of developing nodes of Ranvier using anti-NF-186 antibodies (red) and pan-NF antibodies (green). (d) Quantification of immunostaining. Data are mean $\pm$ SEM of four experiments. n indicates the total number of nodes counted at each age. Scale bars: 5 μ m.

Fig. 7. Clustering of neurofascin (NF) precedes detection of caspr in the developing rat sciatic nerve (a–e) Sciatic nerve (P0–P4) was double-labeled with anti-pan-NF (red) and anti-caspr (green). (f) Quantification of pan-NF and caspr immunostaining (P0–P4). Error bars indicate SD of two separate experiments. n indicates the total number of nodes counted at each age. Scale bars: 5 μ m.