M yelinating Schwann cells regulate the localization of ion channels on the surface of the axons they ensheath. This function depends on adhesion complexes that are positioned at specific membrane domains along the myelin unit. Here we show that the precise localization of internodal proteins depends on the expression of the cytoskeletal adapter protein 4.1G in Schwann cells. Deletion of 4.1G in mice resulted in aberrant distribution of both glial adhesion molecules and axonal proteins that were present along the internodes. In wild-type nerves, juxtaparanodal proteins (i.e., Kv1 channels, Caspr2, and TAG-1) were concentrated throughout the internodes in a double strand that flanked paranodal junction components (i.e., Caspr, contactin, and NF155), and opposes the inner mesaxon of the myelin sheath. In contrast, in 4.1G−/− mice, these proteins “piled up” at the juxtaparanodal region or aggregated along the internodes. These findings suggest that protein 4.1G contributes to the organization of the internodal axolemma by targeting and/or maintaining glial transmembrane proteins along the axoglial interface.

Introduction

Fast and efficient conduction along myelinated axons requires the precise positioning of ion channels and other proteins at distinct domains along the axolemma. These domains include the node of Ranvier, the paranodal junction (PNJ), the juxtaparanode (JXP), and the internodal region (Poliak and Peles, 2003; Hedstrom and Rasband, 2006; Salzer et al., 2008). The local differentiation of the axolemma depends on the function of cell adhesion molecules (CAMs) that are uniquely present at each domain. For example, clustering of Na+ channels at the node is regulated by binding of the axonodal CAM neurofascin 186 (NF186) to gliomedin present at the Schwann cell microvilli (Eshed et al., 2005, 2007), as well as by the bordering PNJ, which maintains these channels at the nodal gap (Eshed et al., 2007; Feinberg et al., 2010). The PNJ forms between the paranodal loops of myelinating glia and the axon, and its formation is mediated by an adhesion complex consisting of Caspr, contactin, and the glial 155-kD isoform of neurofascin (NF155; Bhat et al., 2001; Boyle et al., 2001; Gollan et al., 2003; Sherman et al., 2005). The JXP separates nodal Na+ channels from Kv1 K+ channels present at the JXP axolemma (Wang et al., 1993; Rhodes et al., 1997; Rasband et al., 1999; Vabnick et al., 1999). The accumulation of Kv1 channels at the JXP requires the presence of a Caspr-dependent membrane barrier at the PNJ (Bhat et al., 2001; Boyle et al., 2001; Gollan et al., 2003; Sherman et al., 2005), as well as a juxtaparanodal adhesion scaffold consisting of Caspr2 and TAG-1 (Poliak et al., 2003; Traka et al., 2003).

Throughout the internode, Kv1.2 is located in a double strand that apposes the inner mesaxon of the myelin sheath, and also forms a circumferential ring just below the Schmidt-Lanterman incisures (SLI; termed the juxtamesaxon; Arroyo et al., 1999). The juxtamesaxonal strands of Kv1.2 flank a single line of Caspr, which is reminiscent of the domain organization.
where it is present at the periaxonal and mesaxonal membranes (Ohno et al., 2006). Here we report that protein 4.1G plays an essential role in the molecular organization of myelinated axons.

Results and discussion

Protein 4.1G is located at the axoglial interface in peripheral myelinated nerves

To examine whether protein 4.1G participates in the organization of the axoglial interface in myelinated nerves, we first compared its distribution with that of MAG and Necl4, two glial CAMs that are located at the periaxonal membrane and inner mesaxon. Caspr and Necl proteins bind to cytoskeletal adapters of the protein 4.1 family (Peles et al., 1997; Poliak et al., 1999; Gollan et al., 2002; Denisenko-Nehrbass et al., 2003; Zhou et al., 2005; Hoy et al., 2009). These adapters play an important role in membrane organization by linking various transmembrane components, including ion channels (Li et al., 2007; Baines et al., 2009) and receptors (Binda et al., 2002; Lin et al., 2009), to the underlying actin/spectrin cytoskeleton (Bennett and Baines, 2001). For example, it was previously shown that the interaction between 4.1B and Caspr proteins is required to stabilize Caspr at the PNJ and to maintain Kv1 channels at the JXP (Gollan et al., 2002; Horresh et al., 2010; Buttermore et al., 2011; Cifuentes-Diaz et al., 2011). Protein 4.1 family consists of four members, of which three (4.1B, 4.1N, and 4.1R) are expressed by sensory neurons in the peripheral nervous system (PNS; Ohara et al., 2000; Poliak et al., 2001; Arroyo et al., 2004; Ogawa et al., 2006), whereas the fourth (4.1G) is expressed by myelinating Schwann cells (Horresh et al., 2010), where it is present at the periaxonal and mesaxonal membranes (Ohno et al., 2006). Here we report that protein 4.1G plays an essential role in the molecular organization of myelinated axons.

Figure 1. 4.1G colocalizes with Necl4 in myelinated peripheral nerves. (A and B) Immunofluorescence labeling of teased adult mouse sciatic nerves using antibodies to protein 4.1G, neurofilament, and MAG (A) or Necl4 (B). (C) Double labeling of teased sciatic nerves for protein 4.1G and Na⁺ channels. An arrowhead marks the location of the node. (D and E) Immunoelectron microscopy analysis of adult rat sciatic nerves using an antibody to protein 4.1G. Specific labeling is detected at the SLI (D), and along the adaxonal membrane (E). (F and G) Immunofluorescence labeling of sciatic nerve cross-sections using antibodies to protein 4.1G and dystroglycan (DG; F), or protein 4.1G and neurofilament (NF; G). The location of the adaxonal and abaxonal membranes is marked by arrows and arrowheads, respectively. Bars: (A–C) 10 µm; (D and E) 0.5 µm; (F and G) 5 µm.
4.1G is required for the localization of Necl4 at the axoglial internodal interface

In view of the remarkable similarity in the localization of Necl4 and 4.1G in myelinating Schwann cells, the fact that 4.1 proteins bind members of the Necl family (Yageta et al., 2002; Zhou et al., 2005; Hoy et al., 2009), and the function of 4.1 proteins in membrane organization (Baines et al., 2009), we examined whether the localization of Necl4 is altered in the absence of protein 4.1G. In wild-type (WT) nerves, Necl4 immunoreactivity was detected in the SLI, the periaxonal (adaxonal) membrane, and detected weakly at the paranodal loops (Fig. 2, A and B), as anticipated from previous studies (Maurel et al., 2007; Spiegel et al., 2007). In contrast, in sciatic nerves isolated from 4.1G-null mice, there was a striking reduction in the level of Necl4 in the periaxonal membrane and the SLI (Fig. 2 A), which was accompanied by an abnormal concentration of Necl4 at the paranodal loops (Fig. 2, B and G). Western blot analysis of sciatic nerve lysates prepared from WT and 4.1G−/− mice revealed that there was an ~90% reduction in the amount of Necl4 in the mutant (Fig. 2 C), whereas the expression of MAG, which colocalized with Necl4 in myelinating Schwann cells (Maurel et al., 2007; Spiegel et al., 2007), was unchanged (Fig. 2, C and E). The reduction of Necl4 did not result from an impaired formation of the SLI, as their morphology in the mutant was indistinguishable from that seen in WT nerves (Fig. 2 D). Furthermore, immunolabeling of teased sciatic nerves using antibodies to other SLI proteins such as MAG and E-cadherin showed that in contrast to Necl4, these proteins were normally localized in the absence of protein 4.1G (Fig. 2, E and F). The marked reduction in Necl4 immunoreactivity at the SLI was already apparent at P5, as was evident by immunolabeling of sciatic nerves isolated at different developmental days, using antibodies to Necl4 and MAG (Fig. 2 G). Nevertheless, analysis of ultrathin sections of sciatic nerves by electron microscopy revealed no significant differences in myelin thickness (G ratio: WT, 0.57 ± 0.01; 4.1G−/−, 0.52 ± 0.04), axonal caliber (WT, 4.40 ± 0.3 µm; 4.1G−/−, 3.88 ± 0.7 µm), width of the periaxonal space (WT, 23 ± 4.0 nm; 4.1G−/−, 22.6 ± 4.0 nm), or periodicity of compact myelin between 4.1G−/− and WT nerves (Fig. S1). Consistent with the observed normal morphology of the mutant, the levels of myelin proteins such as MBP, MAG, and P0 were not reduced in sciatic nerves of 4.1G−/− mice (Fig. S1 I).

The specific requirement of 4.1G for the targeting of Necl4 within myelinating Schwann cells is in line with previous observations demonstrating that: (a) 4.1–Necl complexes exist in different subcellular structures (Terada et al., 2010; Nagata et al., 2011), (b) 4.1 proteins bind the cytoplasmic...
domain of Necls (Yageta et al., 2002; Zhou et al., 2005; Hoy et al., 2009), (c) the interaction between Necl4 and 4.1G is required for targeting and localization of Necl4 in Sertoli cells (Yang et al., 2011), and (d) the targeting of the MPP6 (MAGUK p55 subfamily member 6), a scaffolding protein that binds both 4.1G (Terada et al., 2011) and Necl proteins (Shingai et al., 2003; Kakunaga et al., 2005) to the SLI, requires 4.1G (Terada et al., 2011).

4.1G is required for the accurate localization of internodal adhesion complexes

We next examined whether the absence of protein 4.1G in Schwann cells, which results in the mislocalization of Necl4 along the internodal axoglial interface, also affects the molecular organization of the axon. Immunofluorescence analysis of teased sciatic nerves from WT and 4.1G-null mice demonstrated that ankyrin G, NrCAM, and Na+ channels were concentrated at the nodes of Ranvier in both genotypes (Fig. 3, A–C). Also, Caspr, contactin, and NF155 were present at both sides of the nodes (Fig. 3, A–C), which indicates that the PNJs are formed in the absence of protein 4.1G. This conclusion was further supported by transmission electron microscopic analysis of the mutant nerves, which showed the formation of the PNJ septa and a close (2–3 nm) association of the glial paranodal loops with the axolemma in mutant samples (Fig. 3, D–H). Nevertheless, although in WT axons Caspr, contactin, and NF155 were also present along the mesaxonal line that projects from the PNJ (arrowhead in Fig. 3 A), they were rarely detected at this location in the mutant nerve. In 4.1G−/−, but not in WT nerves, we often detected aberrant accumulation of Caspr and NF155 adjacent to the PNJ (arrows in Fig. 3, A and B). We also compared the distribution of NF155 and Caspr in sciatic nerves isolated from WT and 4.1G mutant at early postnatal days (i.e., P3, P5) and found that these two proteins are always found together in the mutant, making it impossible to determine whether glial components accumulation at paranodes preceded that of axonal ones (unpublished data).

Absence of 4.1G results in aberrant axonal clustering of Kv1

Immunofluorescence labeling of teased sciatic nerves isolated from WT and 4.1G−/− mice using antibodies to Caspr2, Kv1.2, and TAG-1, revealed that these proteins abnormally “piled up” at the mutant JXP (Fig. 4, A–C). In contrast to WT mice, where Kv1.2 was present along the internodes in a double strand that apposes the inner mesaxon of the myelin sheath (Fig. 4 D, top), in the absence of protein 4.1G these channels redistributed from the internodes toward the JXP and were occasionally detected in small aggregates along the internodes (Fig. 4 D). Western blot analysis of sciatic nerve lysates demonstrated that 4.1G−/− mutant nerves contained normal levels of Caspr and Kv1.2 channels, further supporting the conclusion that these proteins were relocated from the internodes toward the paranodes. Abnormal accumulation of Kv1 channels was already detected at P12 in 4.1G−/− nerves, which suggests that it represents an early developmental defect (Fig. 4 F). In agreement with previous developmental studies (Vabnick et al., 1999; Poliak et al., 2001), at earlier time points (P10), Kv1.2 was detected at the PNJ and JXP in both WT and 4.1G−/− nerves. Electrophysiological analysis of sciatic nerves isolated from adult WT and 4.1G−/− mice revealed that
4.1G−/− defines a novel internodal phenotype

The distribution of ion channels along myelinated axons is critically dependent on Schwann cells and represents a sensitive indicator of myelin abnormalities (Dupree et al., 1999; Poliak et al., 2001; Arroyo et al., 2004; Lonigro and Devaux, 2009). The precise positioning of these channels at the axolemma is regulated by their association with axoglial CAMs and cytoskeleton-linker proteins (Susuki and Rasband, 2008). In peripheral myelinated axons, Kv1 channels are present in the JXP and in the juxtamembranous lines that run through the internodes (Wang et al., 1993; Arroyo et al., 1999). At these sites, Kv1 channels are well positioned to act as active dampers of reentrant excitation, and help in maintaining the internodal resting potential (Chiu, 1980; Chiu and Ritchie, 1984; Zhou et al., 1998; Vabnick et al., 1999). Our results indicate that the mechanisms underlying the clustering of Kv1 channels at the JXP and the internodal axolemma are likely to be distinct (Fig. 5). Juxtaparanodal clustering of Kv1 channels depends on both the formation of a membrane barrier at the PNJ and the generation of a juxtaparanodal adhesion scaffolding complex by Caspr2 and TAG-1 (Poliak and Peles, 2003; Salzer et al., 2008).

Although Kv1 channels aberrantly accumulate immediately adjacent to the nodes in peripheral nerves of mutants lacking Caspr (Bhat et al., 2001; Gollan et al., 2003), contactin (Boyle et al., 2001), NF155 (Pillai et al., 2009), or ceramide galactosyltransferase (cgt; Dupree et al., 1999), they are still present at the internodal juxtamembranous channels in these mutants (Fig. 5 B). Similarly, in mutants with juxtaparanodal aberrations such as mice lacking Caspr2 (Poliak et al., 2003), TAG-1 (Poliak et al., 2003; Traka et al., 2003), or protein 4.1B (Horresh et al., 2010), Kv1 channels do not cluster at the JXP and mainly concentrate at the juxtamembranous lines (Fig. 5 C). In the present study, we found that the localization of Kv1 channels along the mesaxonal line depends on the expression of protein 4.1G in myelinating Schwann cells. In the absence of protein 4.1G, the overall level of Kv1 channels is unchanged, but they aberrantly “pile up” at the JXP and aggregate along the internodal axolemma (Fig. 5 D). Notably, in the absence of 4.1G, both Caspr and Caspr2 are not localized at the juxtamembranous line, indicating that the clustering of Kv1 channels

both genotypes have similar nerve conduction velocities (WT, 39.6 ± 0.7 m/s; mutant, 40.4 ± 5 m/s), which is likely due to the fact that although these channels are abnormally clustered, they are still concealed under the compact myelin in the mutant (Vabnick et al., 1999; Poliak et al., 2003).
at this site requires the presence of at least one of the two Caspr proteins.

We thus propose that two membrane barriers that are present at both the PNJ and the juxtaaxonal line regulate the clustering of Kv1 channels in peripheral myelinated axons. Although the PNJ barrier controls the axial (longitudinal) distribution of Kv1 channels, the presence of this membrane barrier at the juxtaaxonal line affects the distribution of these channels around the axon circumference (radial). In the absence of the PNJ barrier in Caspr-deficient nerves, Kv1 channels are still trapped at the juxtaaxonal line by Caspr2, whereas in Caspr2-deficient axons, these channels are located at the juxtaaxonal line because of the presence of the Caspr-mediated membrane barrier at this site. In contrast, in 4.1G/−/− nerves, both the juxtaaxonal NF155/CaspR-dependent membrane barrier and the TAG-1/Caspr2-dependent scaffold are missing, resulting in the diffusion of Kv1 channels away from the mesaxonal line. In these axons, Kv1 channels are mainly accumulated by Caspr2 at the JXP, which is formed near a Caspr-dependent membrane barrier at the PNJ. Our findings suggest that protein 4.1G plays an important role in the polarized distribution of proteins in myelinating Schwann cells, which is required for the precise molecular organization of the underlying axonal membrane.

Materials and methods

Mice

Generation of 4.1G/−/− mice was achieved by replacing the first coding exon with a neomycin selection cassette as described previously (Wozny et al., 2009). Genotyping was done by PCR of genomic tail DNA using primers: 5′-TTTCCATACCTCACCACCGAGACTG-3′ and 5′-CAGTCAGGACGCAGAACCACCTCAG-3′. Specificity and cross-reactivity of these antibodies to 4.1G, human 4.1G (GenBank accession no. AF027299.1). Guinea pig anti-rabbits with a GST fusion protein containing amino acids 673–837 of 4.1G were generated by immunizing rabbits with a GST fusion protein containing amino acids 673–837 of 4.1G

Electron microscopy

Mice were perfused with 2.5% glutaraldehyde and 4% PFA in phosphate buffer as described previously (Möbius et al., 2010). Sciatic nerve specimens were embedded in epoxy resin (Serva GmbH). Ultrathin sections (50–70 nm) were cut on an ultramicrotome and collected on copper grids, stained with an aqueous solution of 4% uranyl acetate followed by lead citrate, or aqueous KMnO4, stained with an aqueous solution of 4% uranyl acetate followed by lead citrate, or aqueous KMnO4, followed by lead citrate. Cryo-immunoelectron microscopy sections were embedded in epon resin (Serva GmbH). Ultrathin sections (50–70 nm) were cut on an ultramicrotome and collected on copper grids, stained with an aqueous solution of 4% uranyl acetate followed by lead citrate, or aqueous KMnO4, followed by lead citrate. Cryo-immunoelectron microscopy sections were embedded in epon resin (Serva GmbH). Ultrathin sections (50–70 nm) were cut on an ultramicrotome and collected on copper grids, stained with an aqueous solution of 4% uranyl acetate followed by lead citrate, or aqueous KMnO4, followed by lead citrate. Cryo-immunoelectron microscopy sections were embedded in epon resin (Serva GmbH). Ultrathin sections (50–70 nm) were cut on an ultramicrotome and collected on copper grids, stained with an aqueous solution of 4% uranyl acetate followed by lead citrate, or aqueous KMnO4, followed by lead citrate. Cryo-immunoelectron microscopy sections were embedded in epon resin (Serva GmbH). Ultrathin sections (50–70 nm) were cut on an ultramicrotome and collected on copper grids, stained with an aqueous solution of 4% uranyl acetate followed by lead citrate, or aqueous KMnO4, followed by lead citrate. Cryo-immunoelectron microscopy sections were embedded in epon resin (Serva GmbH). Ultrathin sections (50–70 nm) were cut on an ultramicrotome and collected on copper grids, stained with an aqueous solution of 4% uranyl acetate followed by lead citrate, or aqueous KMnO4, followed by lead citrate.
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