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K^+ channel distribution and clustering in developing and hypomyelinated axons of the optic nerve

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Summary

The localization of *Shaker*-type K^+ channels in specialized domains of myelinated central nervous system axons was studied during development of the optic nerve. In adult rats Kv1.1, Kv1.2, Kv1.6, and the cytoplasmic β-subunit Kvβ2 were colocalized in juxtaparanodal zones. During development, clustering of K^+ channels lagged behind that for nodal Na^+ channels by about 5 days. In contrast to the PNS, K^+ channels were initially expressed fully segregated from nodes and paranodes, the latter identified by immunofluorescence of Caspr, a component of axoglial junctions. Clusters of K^+ channels were first detected at postnatal day 14 (P14) at a limited number of sites. Expression increased until all juxtaparanodes had immunoreactivity by P40. Developmental studies in hypomyelinating *Shiverer* mice revealed dramatically disrupted axoglial junctions, aberrant Na^+ channel clusters, and little or no detectable clustering of K^+ channels at all ages. These results suggest that in the optic nerve, compact myelin and normal axoglial junctions are essential for proper K^+ channel clustering and localization.

Introduction

At the mammalian node of Ranvier there is a sharp segregation of voltage-dependent ion channels. Na^+ channels are clustered at a very high density within the nodal gap, allowing for the rapid conduction velocities characteristic of myelinated axons (Huxley & Stampfli, 1949; Ritchie, 1982). Voltage-dependent K^+ channels, however, are located in juxtaparanodal regions beneath compact myelin. Repolarization of action potentials is thought to be mediated by other classes of slow or voltage-independent K^+ channels at the node (Chiu & Ritchie, 1980, 1981; Roper & Schwarz, 1989). The juxtaparanodal channels are believed to be electrically inactive in the adult PNS (Sherratt *et al.*, 1980; Rasband *et al.*, 1998), but experiments with blocking drugs and genetic deletion have shown that they may serve important functions at earlier developmental stages (Zhou *et al.*, 1998; Vabnick *et al.*, 1999), and during remyelination (Rasband *et al.*, 1998) and regeneration (Kocsis *et al.*, 1982). In the CNS there is some regional diversity since conduction in optic nerve fibers, but not the spinal dorsal column, is sensitive to K^+ channel block (Kocsis & Waxman, 1980; Foster *et al.*, 1982). Smart *et al.* (1998) have recently demonstrated hyperexcitability in the CNS after genetic deletion of Kv1.1. While axonal K^+ channel function and localization have been extensively studied during development in the PNS (Zhou *et al.*, 1998; Vabnick *et al.*, 1999), little is known in this regard in the CNS. Since these channels are targets for pharmacological compounds aimed at restoration of function in multiple sclerosis, knowledge of the events and mechanisms responsible for their segregation is of both basic and clinical interest.

There are currently two major hypotheses for the clustering of ion channels in myelinated axons. In one, nodal sites are specified by the axon, and a soluble factor secreted by glial cells is postulated to induce aggregation independently of cell-cell contact (Kaplan *et al.*, 1997). In the other, myelinating glia induce channel clustering through direct contact, and specify nodal spacing by the extent of their processes (Dugandzija-Novakovic *et al.*, 1995; Vabnick *et al.*, 1996; Rasband *et al.*, 1998). Although these ideas have been tested

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in numerous studies of the PNS, very little is known about the corresponding mechanisms for ion channel segregation in central neurons. A potential mediator of K^+ channel clustering is a family of neuronal proteins that contain PDZ domains, including PSD-95 (Kim *et al.*, 1995; Hsueh *et al.*, 1997). These proteins have been shown to induce clustering of *Shaker*-type K^+ channels in *in vitro* transfection systems (Kim & Sheng, 1996). Thus, PDZ domain-containing proteins or other as yet unidentified proteins may represent intrinsic (axonal) factors responsible for K^+ channel clustering. Alternatively, myelinating oligodendrocytes may present extrinsic (glial) interacting proteins that directly influence channel localization in a manner similar to that of Schwann cells in the PNS (Rasband *et al.*, 1998; Vabnick *et al.*, 1999). Indeed, the mechanism of clustering may consist of both extrinsic and intrinsic proteins that act serially or in parallel to facilitate juxtaparanodal K^+ channel clustering.

In this paper, we address the role of glial cells and myelination in *Shaker*-type K^+ channel localization in the optic nerve. We first analyze the expression and location of K^+ channels during normal optic nerve development. We then use the hypomyelinated *Shiverer* mutant mouse as an important preparation to test the conflicting hypotheses of ion channel localization.

Materials and Methods

PRIMARY ANTIBODIES

For Na^+ channel immunolocalization, rabbit polyclonal antibodies were raised against a highly conserved peptide sequence located between domains III and IV in the vertebrate Na^+ channel α-subunit and have been described previously (Dugandzija-Novakovic *et al.*, 1995). Polyclonal anti-Caspr was generated against a bacterial fusion protein containing the cytoplasmic domain (Peles *et al.*, 1997). The subtype-specific mouse monoclonal antibodies against Kv1.2, Kv1.4, Kv1.5, Kv1.6, Kv2.1, Kvβ1, and Kvβ2 have been described previously (Bekele-Arcuri *et al.*, 1996). The rabbit polyclonal antibodies Kv1.1C and Kv1.2C were described previously (Nakahira *et al.*, 1996).

IMMUNOFLUORESCENCE

Optic nerves from mice (*Shiverer* and littermate controls, C3HeB/FeJ-MBPshi; Jackson Laboratory, Bar Harbor, ME) or Lewis rats were dissected, fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.2, for 30 min, transferred to a 20–30% sucrose solution in 0.1 M PB for 3–24 hours, and frozen in OCT mounting medium (Miller). Tissue was cut in 10 um sections, placed in 0.1 M PB, spread on gelatin coated coverslips, and allowed to air-dry. Sections were permeabilized for 2 hours in 0.1 M PB, pH 7.4, containing 0.3% Triton X-100 and 10% goat serum (PBTGS). In all steps involving antibodies, the tissue was washed three times for 5 min each with PBTGS between succeeding steps. Primary polyclonal antibodies were diluted in PBTGS and incubated with the cryosectioned tissue overnight. For double-labeling, monoclonal antibodies were incubated with the tissue for a minimum of 2 hours. Secondary antibodies were goat anti-rabbit IgG conjugated to FITC (1 : 300; Sigma, St. Louis, MO) or goat anti-mouse antibodies, conjugated to TRITC (1 : 200; Sigma) or Cy-3 (1 : 2,000; Accurate Chemicals, Westbury, NY). In experiments where triple-labeling was performed using Caspr, Na^+ channel, and Kv1.2 primary antibodies in adult rat optic nerve, both the Na^+ channel and Caspr antibodies were polyclonal. In these experiments secondary goat anti-rabbit Fab-FITC (Accurate) and goat anti-rabbit Fab-Cy-3 (Accurate) were used in dilutions of 1 : 25 and 1 : 2,000, respectively. Further, the tissue was washed at least 6 times between incubations. The Kv1.2 labeling was performed using the mouse monoclonal antibody, followed by incubation with a secondary goat anti-mouse AMCA (1 : 100; Accurate). Labeled cryosections were rinsed consecutively in PBTGS, 0.1 M PB, and 0.05 M PB for 5 min each. The samples were air-dried and mounted on slides with an anti-fade mounting medium. The labeled tissue was examined on a Nikon Microphot fluorescence microscope fitted with a C4742-95 cooled CCD camera (Hamamatsu, Japan). Images were stored on a laboratory computer and later analyzed using Image Pro (Media Cybernetics).

ELECTROPHYSIOLOGY

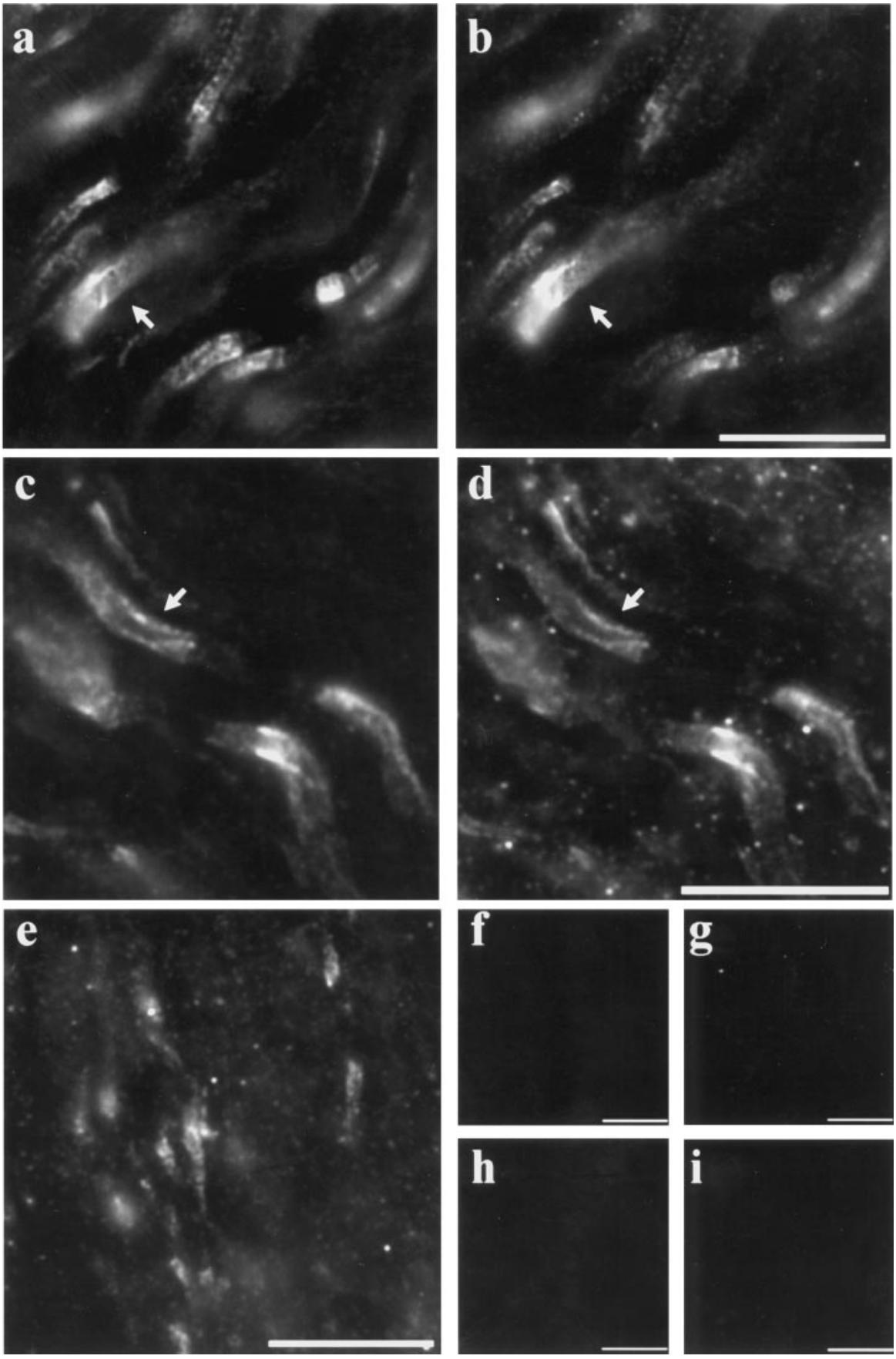
Rat optic nerves were dissected and placed in a recording chamber that was temperature-regulated, oxygenated, and continuously perfused with a standard Locke's solution containing (in mM): NaCl 154, KCl 5.6, $CaCl_2$ 2, D-glucose 5, and HEPES 10, pH 7.4. When present, 4-aminopyridine (4-AP) was used at a concentration of 1 mM. Each end of the nerve was drawn into a suction electrode for stimulation and recording of compound action potentials (Stys *et al.*, 1991). Stimuli were 50 μsec pulses with amplitudes adjusted to $\sim$10% above that required for a maximum response. Signals were amplified, digitized, and recorded on a laboratory computer for later analysis.

Results

MULTIPLE K^+ CHANNEL SUBTYPES ARE PRESENT IN MYELINATED ADULT OPTIC NERVE

Using subtype-specific antibodies, we have labeled adult rat optic nerves to determine the expression and location of K^+ channel α- and β-subunits. Kv1.1 and Kv1.2 immunofluorescence overlapped closely and was seen to occupy 2 zones flanking each node of Ranvier (Fig. 1a and b; arrows). The gap between these

Fig. 1. K^+ channel organization and subtypes in adult rat optic nerve. (a, b) An optic nerve cryosection double-labeled for Kv1.1 (a) and Kv1.2 (b); (c, d) Kvβ2 (c) immunoreactivity colocalizes with Kv1.2 (d); (e) Kv1.6 labeling is present at juxtaparanodes; (f, g, h, i) Kv1.4 (f), Kv1.5 (g), Kv2.1 (h), and Kvβ1 (i) immunoreactivities were undetectable. Scale bars, 10 μm.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zones corresponds to paranodal and nodal regions (see below). The immunoreactivity was most intense adjacent to the gap, and decreased toward the internode. This pattern of staining was similar to that seen in PNS sciatic nerve preparations (Mi *et al.*, 1995; Rasband *et al.*, 1998), and in other regions of the CNS (Wang *et al.*, 1993; Rhodes *et al.*, 1997). Cytoplasmic β-subunits are found in association with each K^+ channel α-subunit and can affect both function and expression (Trimmer, 1998). Specifically, the Kvβ1 subunit has been shown to increase inactivation rates of Shaker-type K^+ channels (Rettig *et al.*, 1994), and the Kvβ2 subunit mediates efficient cell surface expression (Shi *et al.*, 1996). In optic nerves, Kvβ2 was found to be colocalized with Kv1.2 (Fig. 1c and d; arrows) and Kv1.1 (not shown), but Kvβ1 immunoreactivity was not detected (Fig. 1i). We also tested for the presence of Kv1.4, Kv1.5, Kv1.6, and Kv2.1 K^+ channel α-subunits. Of these, only Kv1.6 was detected (Fig. 1e). Immunofluorescence for the latter was faint and was seen predominantly in small axons. Since this antibody has been shown to label other regions of the CNS very strongly (Bekele-Arcuri *et al.*, 1996; Rhodes *et al.*, 1997), the weak signal in the optic nerve may reflect a relatively low density of this channel. When it could be detected clearly, Kv1.6 colocalized with Kv1.1 and Kv1.2 (data not shown). Immunoreactivity for Kv1.4 (Fig. 1f), Kv1.5 (Fig. 1g), and Kv2.1 (Fig. 1h) α-subunits was not found.

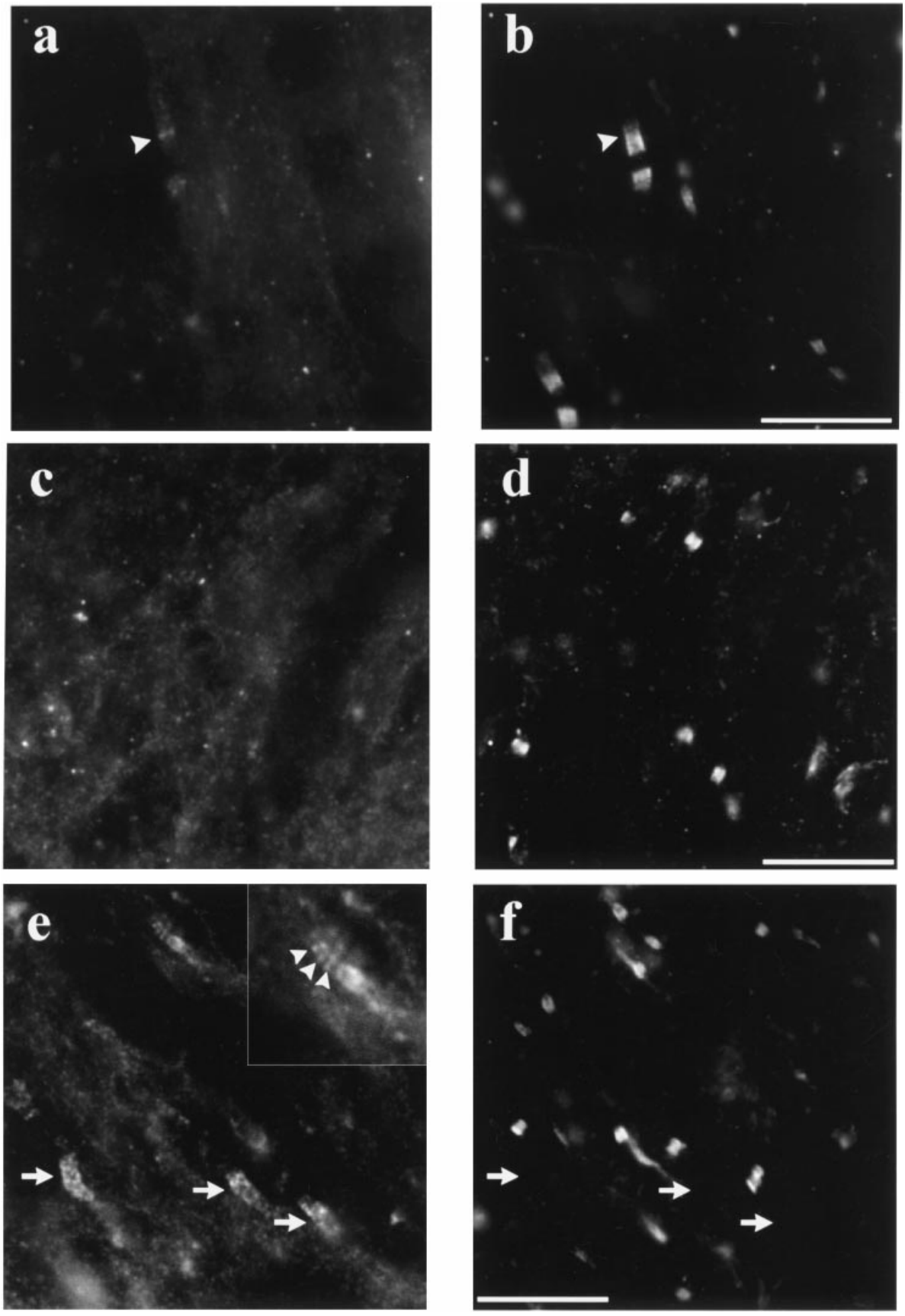
DURING DEVELOPMENT, K^+ CHANNELS ARE DETECTED FIRST AT JUXTAPARANODES

To examine the localization of K^+ channels to their specific subcellular domains during myelination and development, we analyzed Kv1.2 immunoreactivity in rat optic nerves beginning at postnatal day 8 (P8). Nodal regions were identified and delineated either by pan-specific Na^+ channel antibodies, which label the nodal gap, or with antibodies against Caspr, which identify paranodes, since this protein (also known as paranodin; Menegoz *et al.*, 1997) is a component of axoglial junctions (Einheber *et al.*, 1997). The earliest Caspr staining was detected at P7, at zones under the edges of myelinating oligodendrocyte processes. Na^+ channel clusters were seen by P9, in broad zones that were bordered by Caspr labeled paranodes (Rasband *et al.*, 1999). In contrast, Kv1.2 immunoreactivity was not observed until P14. Sites were counted as positively labeled for Kv1.2 if at least one side of the nodal region was stained. At this stage of development, Kv1.2 was present as a faint band of immunofluorescence that decreased in intensity toward the internode (Fig. 2a; arrowhead). It is important to note that the Kv1.2 immunoreactivity first appeared in the juxtaparanode, the region immediately adjacent to the Caspr-labeled paranode (Fig. 2b). This is in contrast to the PNS where during both development (Vabnick *et al.*, 1999) and remyelination (Rasband *et al.*, 1998), K^+ channels were detected initially, but transiently, at the node and paranode, before finally appearing in juxtaparanodal zones.

The number of Na^+ channel clusters within a given field of view increased 7-fold from P12 to P20 (Rasband *et al.*, 1999). However, at P15, only 10% of Na^+ channel labeled nodes of Ranvier had associated juxtaparanodal Kv1.2 staining (Fig. 2c and d). By P19, Kv1.2 was more clearly detected, and was associated with 24% (89/369) of nodes (Fig. 2e and f). The nerve fibers that had juxtaparanodal channels at this stage appeared to be among the largest in diameter. At some sites there was an apparent asymmetry in Kv1.2 staining, with juxtaparanodes on one side of the node preferentially labeled (Fig. 2e; arrows). Vabnick *et al.* (1999) documented an asymmetric maturation of K^+ channels in the PNS, but concluded that while the lower total number of channels might be important, the asymmetry itself had no electrophysiological significance. At several stages of development, discrete bands of K^+ channel immunoreactivity were visible near the paranode (Fig. 2e; inset, arrowheads). This banding was seen to alternate with Caspr immunoreactivity (see below), and was reminiscent of the paranodal lakes of particles that have been described in freeze fracture studies (Rosenbluth, 1984; Fields *et al.*, 1986). These results suggest that K^+ channels are excluded from regions of close contact between axons and cytoplasmic loops of myelinating glial cells.

By P26, 78% of sites had detectable Kv1.2 staining in both large and small diameter fibers. In comparison, about 80% of optic nerve fibers are myelinated by this age (Hildebrand & Waxman, 1984). Figure 3a (Kv1.2) and b (Na^+ channels) show that the K^+ channel staining, like that seen at earlier stages, was characterized by intense immunofluorescence at juxtaparanodes that extended into internodes where it gradually diminished to background levels. Further, bands of immunofluorescence were seen at some sites (Fig. 3a). By P50, nearly all (347/353) nodal regions had Kv1.2 immunostaining (Fig. 3c and d). In adult rat optic nerves zones of Na^+ channels, Caspr, and Kv1.2 were always mutually exclusive, with little or no overlap. This can be seen most clearly in the triple-labeled preparation of Fig. 5a. This point is emphasized by the largest fiber in this figure,

Fig. 2. K^+ channel clustering during early myelination, P14-P19. (a, b) At P14, the earliest Kv1.2 (a, arrowhead) immunoreactivity was located in the juxtaparanode, immediately adjacent to Caspr (b) staining; (c, d) A P15 rat optic nerve double-labeled for Kv1.2 (c) and Na^+ channels (d); (e, f) A P19 optic nerve double-labeled for Kv1.2 (e) and Na^+ channels (f). The inset figure shows discrete banding in K^+ channel immunoreactivity (e, arrowheads). Scale bars, 10 μm.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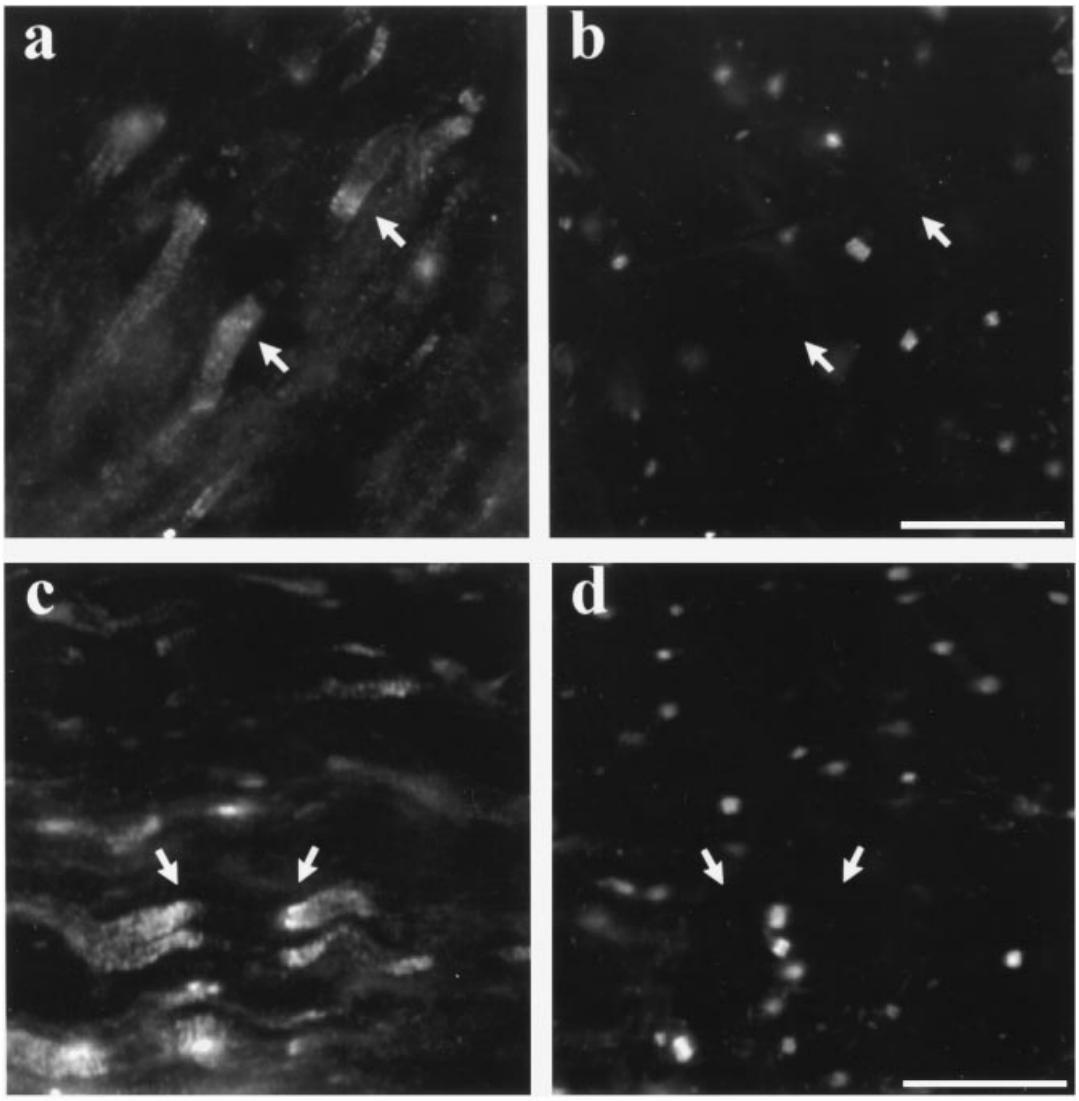
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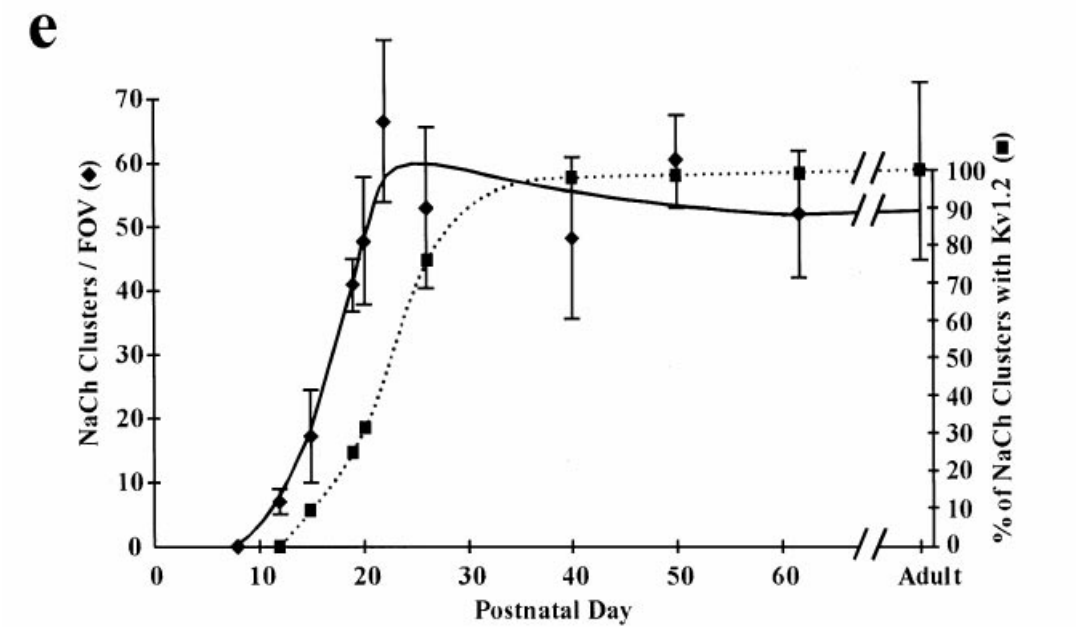
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NaCh Clusters / FOV (◆)
% of NaCh Clusters with Kv1.2 (■)
Postnatal Day
0
10
20
30
40
50
60
Adult
70
80
90
100

where a single Kv1.2 band is present adjacent to the paranode (Fig. 5a; arrowhead), but separated from the majority of the K^+ channels by a strip of Caspr immunoreactivity.

The results for the time course of Kv1.2 channel clustering during development are summarized in figure 3e. The number of Na^+ channel clusters (♦) per FOV was counted at several ages. It was then determined whether Kv1.2 immunoreactivity was present near these nodes, and this result was plotted as a percentage (■). Clustering of Kv1.2 lagged behind that of Na^+ channels by about 5 days, but eventually was present at virtually all nodal regions. Kv1.6 was not detectable at P26 (Fig. 4a). By P34 faint immunofluorescence was usually diffuse all along axons, but could also be found in focal distributions at a few sites (Fig. 4b; arrowhead). These results suggest that the developmental expression of Shaker-type channels in myelinated optic nerve fibers may be differentially regulated.

The drug 4-aminopyridine (4-AP) blocks voltage-dependent Shaker-type K^+ channels by diffusing across the membrane and binding near the cytoplasmic pore (Stephens *et al.*, 1994). To determine the role of voltage-dependent K^+ currents during development in optic nerves, we compared compound action potentials in the presence and absence of 1 mM 4-AP (Fig. 4c). Application of this drug resulted in both increased amplitude and duration of CAPs. Thus, in contrast with the situation in the sciatic nerve, 4-AP sensitive K^+ channels contribute to the normal excitability of the optic nerve.

IS K^+ CHANNEL CLUSTERING DEPENDENT ON MYELIN?

K^+ channel localization was examined in the hypomyelinating mouse mutant *Shiverer (Shi)* to determine if myelin and axoglial junctions influence K^+ channel clustering in the optic nerve. In these animals the first 5 exons of the myelin basic protein (MBP) gene are deleted (Roach *et al.*, 1985). Oligodendrocytes may ensheathe axons only partly or with multiple lamellae of cytoplasmic processes, but fail to form compact myelin (Rosenbluth, 1980; Inoue *et al.*, 1981). Further, zones of axoglial junctions are very irregular (Rosenbluth, 1981). In optic nerves from littermate control mice, the localization of Kv1.2, Na^+ channels, and Caspr was identical to that observed in adult rats (Fig. 5a, b and d). In *Shi* mice, K^+ channels were diffusely distributed throughout axons, with only a few identifiable aggregates (Fig. 5c and e, arrowheads). K^+ channel expression appeared to be elevated overall, in agreement with the results of Wang *et al.* (1995). Na^+ channel organization was similarly disrupted, with very few normal clusters present (Fig. 5c). When node-like structures were seen, they occasionally had K^+ channel immunoreactivity immediately adjacent to or overlapping with Na^+ channels (Fig. 5c; arrowhead), a very different pattern from that seen in control littermates (Fig. 5b). In *Shi* optic nerves, there were abundant Caspr-labeled zones, but their shapes were highly irregular (Fig. 5e). Some K^+ channels were concentrated in regions close to more normal appearing Caspr sites (arrowhead), but there was usually no correlation in arrangement of the two.

K^+ channel localization in optic nerves from *Shi* mice was also investigated during development. Mice were examined at P16, P23, P26, and P38. Juxtaparanodal K^+ channels were not seen at any of these ages, and axons had only diffuse K^+ channel immunoreactivity. Results at P26 are shown in Fig. 6a and c. As in the adult, numerous abnormal Na^+ channel aggregates and Caspr-labeled sites were discernable (Fig. 6b and d), and relatively normal appearing regions of immunoreactivity were rare (Fig. 6d; arrowhead). In control mice, K^+ channel clustering was similar to that for the rat at P26 (data not shown; compare Fig. 3e). In contrast to the results of Wang *et al.* (1995) in *Shi* spinal cord, we find that in the *Shi* optic nerve not only the maintenance of K^+ channel distributions, but also the initial clustering is disrupted.

Discussion

K^+ CHANNELS IN THE OPTIC NERVE

The upstroke of the action potential in mammalian myelinated axons depends on a voltage-dependent increase in inward current that is provided by a high density of nodal Na^+ channels. Repolarization requires an outward current, but its source is less well defined since voltage-dependent K^+ channels are absent from the nodal gap (Chiu *et al.*, 1979). Indeed, immunocytochemical studies have shown that axonal heteromultimeric Kv1.1 and Kv1.2 channels are found in juxtaparanodal regions beneath myelin in both CNS and PNS tissues (Wang *et al.*, 1993; Mi *et al.*, 1995). The function of these voltage-dependent channels has been tested pharmacologically, most often with 4-AP. Conduction in adult peripheral nerves and in myelinated rat dorsal columns is insensitive to K^+ channel blockade (Kocsis

Fig. 3. K^+ channels during late myelination. (a, b) P26 optic nerve double-labeled for Kv1.2 (a) and Na^+ channels (b); (c, d) A P50 optic nerve double-labeled for Kv1.2 (c) and Na^+ channel immunoreactivity (d); (e) The number of Na^+ channel clusters per field of view (♦; from Rasband *et al.*, 1999), and the percentage of Na^+ channel clusters with juxtaparanodal Kv1.2 immunostaining (■). Curves were drawn by eye to indicate trends. Error bars ± SD. Scale bars, 10 μm.

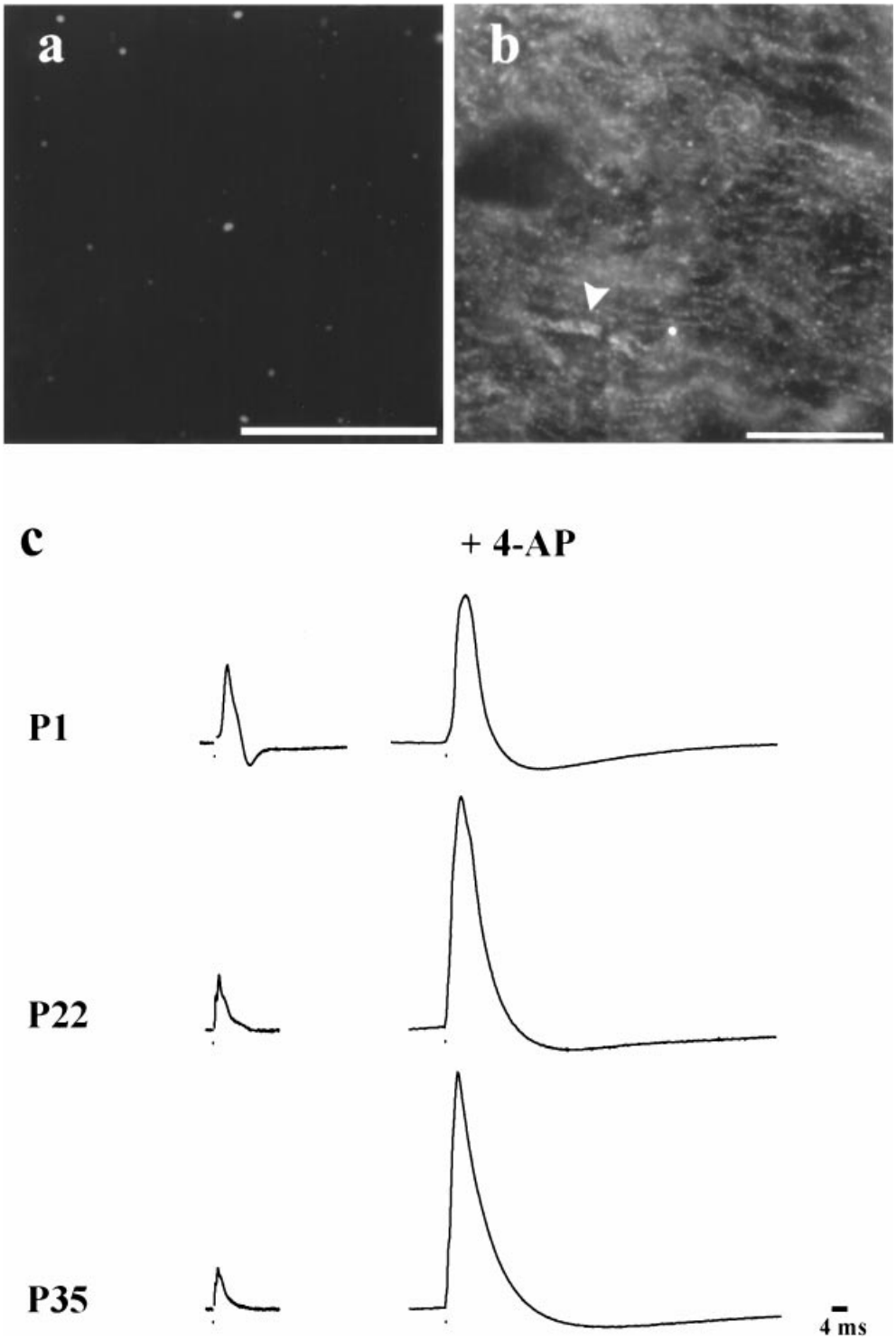


Fig. 4. Kv1.6 immunoreactivity and electrical signals during development. (a, b) Anti-Kv1.6 immunolabeling at P26 (a) and P34 (b). Scale bars, 10 μm. c, Compound action potentials recorded before and after exposure to 1 mM 4-AP at P1, P22, and P35.

& Waxman, 1980; Sherratt *et al.*, 1980; Bostock *et al.*, 1981). Since mammalian Shaker-type K^+ channels are inhibited by 4-AP (Stuhmer *et al.*, 1989; Grissmer *et al.*, 1994; Stephens *et al.*, 1994), the sensitivity of conduction to this drug is dependent on the physiological contribution of these channels to action potential repolarization. Juxtaparanodal channels, isolated by tight axoglial junctions, may be electrically silent, conferring insensitivity to 4-AP on many axons. Therefore, it was of interest that inhibition of voltage-dependent K^+ currents in the adult optic nerve results in significantly broadened action potentials, as shown both here and in earlier

work (Gordon *et al.*, 1988, 1989). K^+ channel localization had not been described in this preparation, and as part of our investigation we sought an explanation for this sensitivity.

We tested for the presence of Kv2.1, Kvβ1, Kvβ2, and 5 *Shaker*-type α-subunits. Of these, Kv1.1, Kv1.2, Kv1.6, and Kvβ2 were found in the optic nerve, although Kv1.6 was not detected until later stages of development and staining for this channel subunit was very weak. Identifying paranodes with immunohistochemistry indicated that all of these K^+ channel subunits are found exclusively in juxtaparanodal and internodal zones, and do not extend into the node or paranode. Thus, we found no evidence from these studies for an obvious candidate channel to explain the pharmacological sensitivity. As an alternative hypothesis, we considered the possibility that axoglial junctions in the optic nerve might be leaky and allow for juxtaparanodal K^+ currents. However, we have shown that Caspr labeling of paranodes in the optic nerve is similar to that in the PNS, and ultrastructural studies confirm the conclusion that axoglial junctions in these axons have a normal morphology (Wiggins *et al.*, 1988). Thus, while we can rule out some plausible reasons, the unusual sensitivity of adult optic nerve axons to K^+ channel block remains unexplained. It is, of course possible that an as yet untested channel is present within the nodal gap. The absence of voltage-dependent K^+ channels at the mammalian node was measured electrophysiologically only in sciatic fibers (Chiu *et al.*, 1979). Finally, we note that the expression of Kv1.6 in these axons, colocalized with Kv1.1 and Kv1.2, broadens the potential range of heteromultimers that may form and the possible extent of function and regulation (Isacoff *et al.*, 1990; Hopkins *et al.*, 1994).

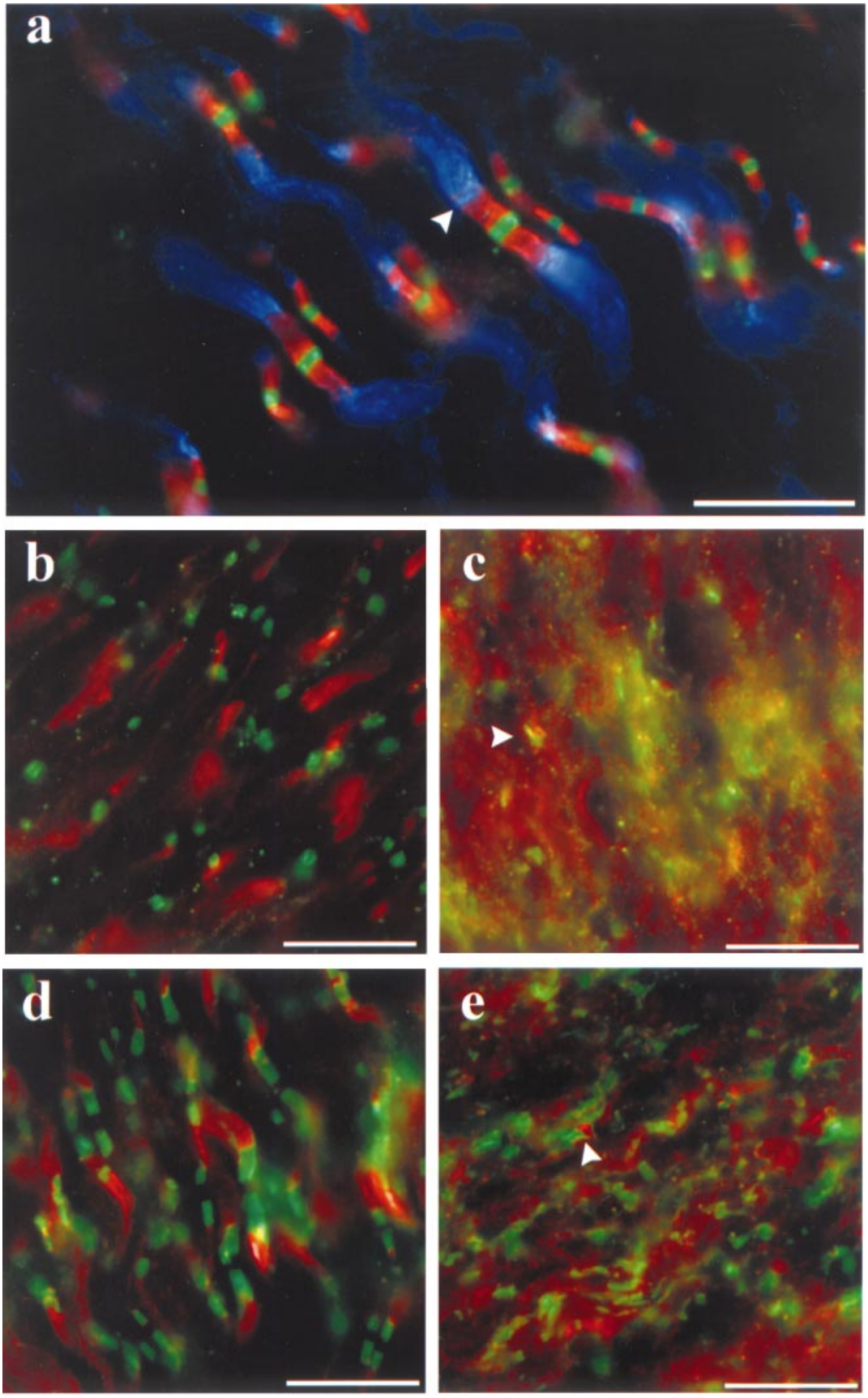
JUXTAPARANODAL CLUSTERING OF K^+ CHANNELS

Since K^+ channels in PNS axons appear initially in nodal and paranodal zones during both development and remyelination, and only later cluster under compact myelin (Rasband *et al.*, 1998; Vabnick *et al.*, 1999), we anticipated a similar sequence of events in the optic nerve. In contrast, however, in the optic nerve the first clusters of *Shaker*-type channels were detected at juxtaparanodes, adjacent to the Caspr-labeled paranode. Consequently, there seems to be little channel reorganization in these fibers. These clusters of K^+ channels were not uniform, and generally were of higher density adjacent to the paranode. Further, at the paranode/juxtaparanode border, bands of K^+ channels alternated with those of Caspr-labeled axoglial junctions, in both young animals and in adults. Similar bands or spirals of K^+ channels have been described by Vabnick *et al.* (1999) during PNS development. Earlier freeze-fracture studies of myelinated nerve fibers revealed zones of intramembranous particles separated by regions with axoglial junctions with a geometry that was similar to the immunocytochemically defined structures described above (Rosenbluth, 1984; Fields *et al.*, 1986). The graded density and mutually restricted zones, combined with the fact that Caspr is detected prior to K^+ channels suggest a clustering mechanism in which K^+ channels are excluded from regions of close contact between axons and glial cells. While this process may be involved, it probably does not represent the sole mechanism since the exclusion is one-way and K^+ channels are not seen, even transiently, within the nodal gap. Further, Wang *et al.* (1995) showed that despite the hypomyelination in *Shi* mice the initial localization of K^+ channels in the spinal cord remained juxtaparanodal.

Factors other than myelin that may be responsible for determining the sites of Kv1.1 and Kv1.2 aggregation in the CNS may include PSD-95 or other PDZ domain containing proteins that have been shown to induce clustering of *Shaker*-like K^+ channels in transfected cells *in vitro* (Kim *et al.*, 1995, 1996; Kim & Sheng, 1996) and at *Drosophila* synapses *in vivo* (Tejedor *et al.*, 1997). However, the only evidence for colocalization of *Shaker*-type channels and PSD-95 in the mammalian CNS is at basket cells, where there is robust labeling of terminals (Laube *et al.*, 1996). Future experiments will be necessary to address the role of PDZ domain containing and other K^+ channel interacting proteins in localization of K^+ channels at juxtaparanodes in myelinated nerve fibers.

We have described evidence that channel clustering was a consequence of axon-glial contact, and paranode formation in particular. Our previous work has shown that K^+ channels are disrupted during demyelination and that during remyelination in the PNS, Schwann cells may directly influence clustering and channel localization (Rasband *et al.*, 1998). To test further the role of myelin in these processes in the CNS, we analyzed *Shi* mouse optic nerves. Studies during development showed that normal clusters of K^+ channels were not detected at any age, indicating that their initial aggregation is dependent on the integrity of myelination. In contrast to these results in the optic nerve, Wang *et al.* (1995) found that in *Shi* spinal cord, early K^+ channel clustering still occurred, but these aggregates were unstable and were later lost. Since these authors did not ascertain the state of myelination in their experiments, it will be important to determine if levels of myelin and axoglial junction formation in the *Shi* spinal cord, at the earliest stages of ensheathment and channel clustering, are different from those in the optic nerve. Should this be the case, disrupted K^+ channel arrangement in *Shi* spinal cord may be seen only at later times, possibly after progressive loss of axoglial junctions.

Ion channels are involved in numerous ways in demyelinating disease or injury. In multiple sclerosis, 4-AP has been used for restoration of neurologic deficits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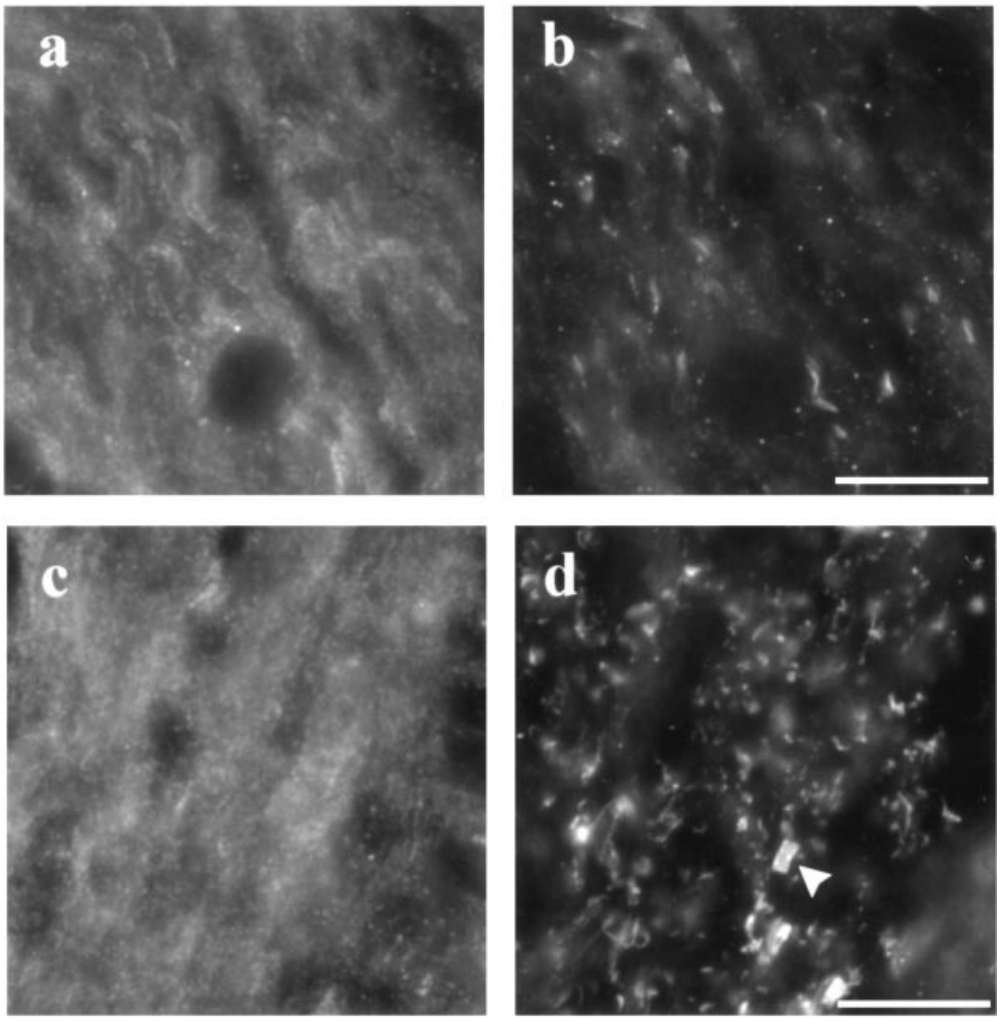

Fig. 6. The initial K^+ channel clustering at juxtaparanodes is disrupted in *Shi* mutant mice. P26 *Shi* optic nerves double-labeled for Kv1.2 (a, c) and Na+ channel (b) or Caspr (d) immunoreactivity. Scale bars, 10 μm.

(Bever *et al.*, 1994), since most axons are unaffected unless they are demyelinated (Kocsis & Waxman, 1980; Sherratt *et al.*, 1980; Bostock *et al.*, 1981; Rasband *et al.*, 1998). It was initially assumed that 4-AP sensitivity resulted from an exposure of juxtaparanodal K^+ channels following myelin damage. We later showed that in the PNS during remyelination, K^+ channels appear first in the gap of new nodes of Ranvier, a site that allowed them to contribute voltage-dependent K^+ currents (Rasband *et al.*, 1998). While inhibition of K^+ channels can restore conduction in demyelinated axons, it may also induce re-excitation and repetitive firing (Chiu & Ritchie, 1981; Kocsis *et al.*, 1982). Since seizures can be a serious side effect of 4-AP use, we need better information on K^+ channel properties in CNS axons. Further, Vabnick *et al.* (1999) have demonstrated an important role of *Shaker*-type channels in stabilizing conduction in PNS axons during development. Clearly, an understanding of the mechanisms of expression and localization of K^+ channels in CNS axons should help in the design of better therapeutic approaches to restore and stabilize function in disease.

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Fig. 5. Kv1.2, Na^+ channel, and Caspr distributions in adult rat, control littermate, and *Shi* optic nerves. (a) Adult rat optic nerve triple-labeled for Kv1.2 (blue), Caspr (red), and Na^+ channels (green); (b, c) Optic nerves from littermate control (b) and *Shi* (c) mice, double-labeled for Na^+ channel (green) and Kv1.2 K^+ channel (red) immunoreactivity; (d, e) Optic nerve cryosections from control (d) and *Shi* (e) mice, double-labeled for Caspr (green) and Kv1.2 (red). Scale bars, 10 μm.

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