Novel clustering of sodium channel Na\textsubscript{v}1.1 with ankyrin-G and neurofascin at discrete sites in the inner plexiform layer of the retina

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Received 8 June 2004; revised 6 October 2004; accepted 18 November 2004

Voltage-gated sodium channels cluster at sites of action potential generation and propagation by interacting with partner proteins such as neurofascin, an adhesion molecule in the L1 family, and ankyrin-G, a spectrin-binding protein required for sodium channel accumulation at axon initial segments. Here, we describe in the inner plexiform layer of the retina a novel site of high-density sodium channel clustering, marked by ankyrin-G and neurofascin. The sodium channel isoform at this site is Na\textsubscript{v}1.1, instead of the Na\textsubscript{v}1.6 channels more commonly found in association with the clustering machinery. During development, Na\textsubscript{v}1.2 channels first associate with ankyrin-G in the inner plexiform layer but are later replaced by Na\textsubscript{v}1.1, similar to the switch from Na\textsubscript{v}1.2 to Na\textsubscript{v}1.6 at nodes of Ranvier and initial segments. This represents the first instance of high-density clustering of Na\textsubscript{v}1.1 channels, which may contribute to synaptic interactions among retinal neurons in the inner plexiform layer.

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Introduction

The polarized localization of voltage-gated sodium (Na\textsubscript{v}) channels in membrane domains is critical for proper signal conduction in excitable cells. Because Na\textsubscript{v} channels initiate the action potential, they are enriched in subcellular regions involved in generation and propagation of action potentials, such as axon initial segments (IS), nodes of Ranvier, and muscle endplates. At these sites, a high density of pore-forming Na\textsubscript{v} channel α subunits is maintained by cytoskeletal and extracellular interactions, mediated by proteins that are also enriched at these regions, including ankyrin-G, βIV spectrin, Na\textsubscript{v} channel β subunits, neurofascin 186, and NrCAM (reviewed in Poliak and Peles, 2003; Salzer, 2003). Ankyrin-G is believed to link Na\textsubscript{v} channel α and β subunits to the spectrin-based cytoskeleton (Lemaill et al., 2003; Malhotra et al., 2000) and is necessary for Na\textsubscript{v} channel accumulation at IS (Jenkins and Bennett, 2001; Zhou et al., 1998).

Ten genes encoding Na\textsubscript{v} channel α subunits have been identified in mammals, four of which are highly expressed in the CNS (Na\textsubscript{v}1.1, 1.2, 1.3, and 1.6). Ankyrin-G has been shown to bind to a 9-amino acid motif in the II–III cytoplasmic linker of Na\textsubscript{v}1.2, which is necessary and sufficient for compartmentalization to the IS (Garrido et al., 2003; Lemaill et al., 2003). Although this site is conserved among all Na\textsubscript{v}1 isoforms, only Na\textsubscript{v}1.2, Na\textsubscript{v}1.6, and Na\textsubscript{v}1.8 have been reported to cluster at ankyrin-G-enriched sites in the CNS (see Arroyo et al., 2002; Trimmer and Rhodes, 2004). Na\textsubscript{v}1.2 channels are predominantly localized diffusely along unmyelinated axons (Gong et al., 1999; Westenbroek et al., 1989), but they also cluster at IS and nodes of Ranvier early in development (Boiko et al., 2001, 2003; Kaplan et al., 2001), before being replaced at these sites by Na\textsubscript{v}1.6 channels. Apparently, it is important not only that a high density of Na\textsubscript{v} channels is maintained at nodes and IS, but also that a particular Na\textsubscript{v} subtype is expressed at those locations.

Here, we report that Na\textsubscript{v} channels, ankyrin-G, and neurofascin are also clustered in a unique subset of processes in the inner plexiform layer (IPL) of the mammalian retina. Unlike adult nodes and IS, where Na\textsubscript{v}1.6 channels are found, the clusters of Na\textsubscript{v} channels in the adult IPL fibers consist of Na\textsubscript{v}1.1 channels, which are usually located diffusely in neuronal somata and dendrites in the CNS (Gong et al., 1999; Schaller and Caldwell, 2003; Westenbroek et al., 1989; Whitaker et al., 2001). This represents the first instance of high-density accumulation of Na\textsubscript{v}1.1 channels at sites where the clustering machinery of nodes and IS is found. Early in development, Na\textsubscript{v}1.2 channels colocalize with ankyrin-G in the IPL processes, but Na\textsubscript{v}1.1 replaces Na\textsubscript{v}1.2 approximately in parallel with the similar switch from Na\textsubscript{v}1.2 to Na\textsubscript{v}1.6 at nodes of Ranvier (Boiko et al., 2001) and axon IS (Boiko et al., 2003). The functional significance of the specialized Na\textsubscript{v}1.1-rich domains in the IPL is not yet clear, but Na\textsubscript{v}1.1 channels may help to amplify electrical signals or integrate synaptic input in a subset of IPL neurites.
Results

**Na<sub>C</sub> channels cluster in a subset of processes in the inner plexiform layer of mammalian retina**

In retinal ganglion cells (RGCs), voltage-gated Na<sub>C</sub> channels accumulate at higher density in axon IS than in neighboring regions of the unmyelinated axon (Boiko et al., 2003). Like other channels, Na<sub>C</sub> channels are concentrated at nodes of Ranvier, the high density of Na<sub>C</sub> channels at IS is supported by the cytoskeletal anchoring protein ankyrin-G (Jenkins and Bennett, 2001). However, unlike Na<sub>C</sub> channels, which are present at appreciable density throughout the axon, ankyrin-G immunostaining is barely detectable in unmyelinated RGC axons outside the IS (Boiko et al., 2003). Ankyrin-G immunoreactivity therefore serves as a marker for RGC IS. In confocal optical sections focused in the RGC/nerve fiber layer at the vitreal surface of the retina, numerous ankyrin-G-positive IS were visible (see Fig. 1A), as expected. However, when we focused deeper into the retina, we also observed bright ankyrin-G immunoreactivity extending 10–25 μm along a subset of processes within the inner plexiform layer (IPL), as illustrated in Fig. 1B. The IPL is virtually devoid of cell bodies and consists of RGC dendrites, neurites of amacrine and bipolar cells, and glial processes. Therefore, these ankyrin-positive structures are unlikely to be RGC IS. Consistent with this interpretation, close inspection showed that the labeled structures often appeared to end in bulbous swellings (inset, Fig. 1B), which suggests they may be terminal endings of IPL processes. We will refer to these ankyrin-G-positive processes in the IPL as morphologically distinct processes (MDPs) to reflect their unusual appearance and distinctive terminal endings.

Because ankyrin-G is typically found in regions of high Na<sub>C</sub> channel density (Bennett and Lambert, 1999), we examined whether increased Na<sub>C</sub> channel immunoreactivity was associated with ankyrin-G in MDPs in the IPL. Immunostaining of rat retina with an antibody that recognizes all Na<sub>C</sub>1 isoforms (PAN) revealed intense Na<sub>C</sub> channel immunoreactivity associated with ankyrin-G staining, both at IS and in IPL processes (see Fig. 2A). Ankyrin-G may mediate clustering of Na<sub>C</sub> channels in the IPL as it does at other locations. In addition, dimmer, diffuse PAN staining was found in several strata of the IPL, as well as in some cell bodies in the INL, but a high density of Na<sub>C</sub> channels was seen in the IPL only in MDPs and only in association with ankyrin-G.

Another protein found at regions of high Na<sub>C</sub> channel density is the cell-adhesion molecule neurofascin (Davis et al., 1996). An antibody against neurofascin labels RGC IS (Boiko et al., 2003), and we found that neurofascin immunolabeling was also associated with Na<sub>C</sub> channel immunostaining in MDPs within the IPL. As shown in Fig. 2B, neurofascin immunostaining colocalized with regions of intense Na<sub>C</sub> channel immunoreactivity both in RGC IS and in the IPL. Therefore, two molecular components associated with a high density of Na<sub>C</sub> channels at IS and at nodes of Ranvier, ankyrin-G and neurofascin, are also present in MDPs of the IPL.

Although the location and the bulbous endings of MDPs are not consistent with their being IS of ganglion cells, the presence in MDPs of markers normally associated with the IS prompted us to seek further verification that MDPs are not ganglion-cell initial segments. For this purpose, retinal sections from mice expressing CFP under the Thy1 promoter were labeled with antibodies against Na<sub>C</sub> channels (red) and CFP (green) (Fig. 2C). These mice are reported to express CFP in all RGCs (and some amacrine cells), in which CFP labeling fills both dendrites and axons all the way to their terminals (Feng et al., 2000). As expected, PAN antibodies labeled RGC axon fascicles and cell bodies, but PAN-labeled MDPs did not colocalize with CFP-labeled RGC processes in the IPL. Thus, MDPs likely are not dendrites of RGCs, initial segments of displaced RGCs, or intraretinal RGC axon collaterals (Dacey, 1985).

To compare the density of Na<sub>C</sub> channels at MDPs with other sites of known Na<sub>C</sub> channel clustering, images of optic nerve nodes, RGC IS, unmyelinated RGC axon fascicles, and MDPs were taken under identical conditions from a single representative retina/lamina cribrosa/optic nerve section stained with PAN, and the intensity of immunostaining was analyzed using NIH Image software (Fig. 3). The intensity of PAN staining at MDPs was similar to that at the IS, and labeling at nodes, IS, and MDPs was more intense than staining of the unmyelinated ganglion cell axon fascicles within the retina, where Na<sub>C</sub> channels are expressed but not clustered. Given the similarity in channel density at the MDPs and IS, Na<sub>C</sub> channels appear to be specifically targeted to the MDPs, just as they are to sites of action potential generation and propagation.

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**Fig. 1.** Ankyrin-G defines retinal ganglion cell axon IS in the ganglion cell layer (GCL), as well as a subset of morphologically distinct processes (MDPs) in the inner plexiform layer (IPL) of the flat mount adult rat retina. (A) A projection of optical sections spanning 3 μm from the nerve fiber layer to the ganglion cell layer, stained for ankyrin-G. Bright ankyrin-G immunofluorescence is found at ganglion cell IS, while diffuse staining is seen along axon fascicles. (B) A projection of optical sections spanning 3 μm of the inner plexiform layer, taken 4–7 μm directly ascending to the image in A. Intense ankyrin-G fluorescence can also be found at a subset of processes in the IPL. Inset shows an example of these processes at higher magnification. The projection of 3 μm contains the full length of all three processes, and was taken from a different field of view than B. Scale bars: A and B, 30 μm; inset, 10 μm. Fluorescence images were converted to grayscale and then inverted.
Nav1.1 accounts for Na\textsubscript{v} channel clustering in the IPL

Given the similarity in Na\textsubscript{v} channel density and molecular composition at MDPs in the IPL and at IS and nodes of Ranvier in RGC axons, the question arises of whether the same Na\textsubscript{v} channel isoform is expressed in all three regions. Boiko et al. (2001, 2003) showed that in mature retinal ganglion cell axons, Nav1.6 accounts for channel clusters at nodes of Ranvier and IS, while Nav1.2 is expressed uniformly throughout the unmyelinated axon. To identify the Na\textsubscript{v} channel isoform located at the MDPs, flat-mount adult rat retinas were double-labeled with PAN and isoform-specific antibodies for Nav1.1, Nav1.2, and Nav1.6 (see Fig. 4). Projections of confocal optical sections taken in the IPL showed that neither Nav1.2 (Fig. 4B) nor Nav1.6 (Fig. 4C) colocalized with brightly stained MDPs. Instead, Nav1.1 immunoreactivity coincided with pan-specific Na\textsubscript{v} channel staining in MDPs (Fig. 4A). The same staining pattern was seen in vertical retina sections (data not shown). Nav1.2 was occasionally observed in thinner, dimly PAN-stained processes in the IPL (e.g., Fig. 4B), which did not resemble the Na\textsubscript{v},1.1-positive MDPs. Nav1.6 immunoreactivity was not detectable in IPL processes. This is the first example of a high density of Na\textsubscript{v},1.1 channels at sites where the clustering machinery of the node and IS is found.

Developmental switch from Nav1.2 to Nav1.1 in IPL processes

In axons of RGCs, Na\textsubscript{v},1.2 channels are expressed at both nodes of Ranvier and IS early in development, but are replaced by Na\textsubscript{v},1.6 channels during maturation (Boiko et al., 2001, 2003). Because the Na\textsubscript{v} channel-enriched domains in the adult IPL express Na\textsubscript{v},1.1, as opposed to the Na\textsubscript{v},1.6 channels of adult nodes and IS, we asked whether a similar isoform switch occurs in MDPs during development. Na\textsubscript{v} channel-enriched IPL processes first appear around P8; however, at this time not all ankyrin-G-positive processes have detectable PAN staining (see Fig. 5). This suggests that ankyrin-G appears before Na\textsubscript{v} channels in developing IPL processes, which is similar to the developmental pattern at nodes of Ranvier and IS, where ankyrin-G is also detected prior to Na\textsubscript{v} channel clustering (Jenkins and Bennett, 2001; Rasband et al., 1999). It should be noted that by P2, >95% of RGCs already express Na\textsubscript{v} at their IS.
Boiko et al., 2003), further indicating that the IPL processes are not IS of displaced RGCs. At P8–P9, when Na_v channel-rich processes first appear in the IPL, Na_v1.1 immunostaining was not detectable in the retina (see Fig. 6). The first Na_v1.1 staining appeared at P10 in scattered processes throughout the IPL. The number of Na_v1.1-positive processes dramatically increased over the following 2 weeks, particularly between P10 and P14, and by P21 almost all PAN labeled processes were positive for Na_v1.1. At P21, MDPs were similar in morphology to those in the adults, with the majority of fibers terminating in bulbous swellings.

Since the first detectable Na_v1.1 staining appeared in the IPL 1–2 days after pan-specific Na_v staining, the question remains as to what isoform is found in early MDPs. At both the node of Ranvier and IS, the first Na_v channel clusters consist of Na_v1.2 channels, which remain at these domains until Na_v1.6 replaces them as the predominant channel isoform (Boiko et al., 2001, 2003). Similarly, in sections from P9 retina, PAN-labeled MDPs in the IPL were enriched in Na_v1.2 (Fig. 7). Like Na_v1.1 staining in adult, Na_v1.2 in young MDPs was segregated in the putative distal end of the process (colocalized with ankyrin-G and neurofascin; data not shown), and was not detected diffusely throughout the processes. By P14, however, Na_v1.2 intensity decreased and was no longer detectable in many Na_v channel-enriched processes (see Fig. 7). This corresponds to the period when Na_v1.1 appeared in MDPs (cf., Fig. 6). By P24, Na_v1.2 was detectable at only low levels in a small proportion of MDPs. Double labeling with anti-Na_v1.2 and anti-Na_v1.1 (Fig. 8) revealed the absence of Na_v1.1 staining at P9, but colocalization of Na_v1.2 with Na_v1.1 at some IPL processes at P14, which is consistent with an isoform switch during this period. The time course for the switch from Na_v1.2 to Na_v1.1 at MDPs is strikingly similar to the Na_v channel isoform switch occurring at nodes of Ranvier and IS, where Na_v1.6 replaces Na_v1.2 (Boiko et al., 2001, 2003).

Attempts to identify the cellular origin of MDPs

The morphology of MDPs is unusual. They are varicose processes extending multidirectionally in the IPL, oftentimes changing direction, but always terminating in lobular swellings averaging 2 μm diameter. Whereas most cell processes are confined to a particular stratum of the IPL, the MDPs appear throughout both ON and OFF sublaminae, occasionally reaching into the INL but most frequently seen near the INL/IPL border. Extensive double-labeling experiments were carried out to determine which cell class contains the cells of origin, but we were unable to find a marker that colocalized with MDPs. Selected examples of some of the obvious candidate markers are shown in Fig. 9. Bipolar cells often have distinctive bulbous processes which
terminate in the IPL, and cone bipolars are known to display sodium currents (Pan and Hu, 2000). However, in vertical sections of rat retina, PAN-labeled MDPs (red, Fig. 9A) do not colocalize with recoverin (green), a marker for On and Off subpopulations of cone bipolar cells, or the rod bipolar cell marker PKC (not shown). Another plausible candidate is the amacrine cell, whose estimated 30 subtypes (see Masland, 2001) contain markers for either glycine or GABA (Marc et al., 1995). We were unable to detect colocalization between PAN staining in the IPL and either the glycine transporter GlYT1, a marker for glycinergic neurons (green, Fig. 9B), GAD, a marker for GABAergic neurons (not shown), or the selective amacrine cell marker HPC-1 (Barnstable et al., 1985; not shown). Interestingly, MDPs often appeared to contact cell bodies and dendrites of glycinergic amacrines, as shown in Fig. 9B. By rotating 3D confocal reconstructions of such projections, we confirmed that GlyT1 immunoreactivity is adjacent to, but not present within the PAN-labeled MDP lobule. Among the cells that receive putative MDP contact are AII amacrines, labeled...
by parvalbumin (red) in Fig. 9C. In this projection taken near the IPL/INL border, PAN-labeled MDPs (green) appear to grasp the lobular appendages of AII amacrine cells, further suggesting that they are indeed process endings. Remarkably, the bulbous MDP terminals are devoid of synaptic vesicle markers, including synaptophysin (Fig. 9D) and synaptotagmin (not shown). Among further antibodies tested but not shown here were markers for polyaxonal amacrine axons (SMI-31; Volgyi and Bloomfield, 2002), interplexiform cells (TH), and glia (GFAP, vimentin, ox42). These studies indicate that MDPs are a unique domain in the IPL belonging to an unusual cell type, not labeled by markers for most major retinal cell types.

Discussion

Na\textsubscript{v} channel clustering machinery delimits a specialized initial segment-like domain in IPL of mammalian retina, where Na\textsubscript{v}1.1 is expressed

In this study, we found that in addition to demarcating nodes of Ranvier and IS of retinal ganglion cell axons, ankyrin-G immunostaining defines a new domain of Na\textsubscript{v} channel clustering in the retina, within a subset of processes in the inner plexiform layer. The density of Na\textsubscript{v} channels at these IPL processes is similar to that at RGC IS, where action potentials are believed to originate. Like sites that support action potential generation and propagation, the Na\textsubscript{v} channel-enriched IPL domains also contain the cell adhesion molecule neurofascin. Immunostaining for all three proteins was restricted to a limited region, 10–25 µm in length, at the apparent termination of the IPL process, which suggests that this terminal domain represents a functional specialization of the process.

In the adult CNS, the Na\textsubscript{v} channel subtype found at sites of high density, such as nodes and IS, is predominantly Na\textsubscript{v}1.6, although Na\textsubscript{v}1.2 is also found at these sites earlier in development (Boiko et al., 2001, 2003). By contrast, Na\textsubscript{v}1.1 channels, and not Na\textsubscript{v}1.2 or Na\textsubscript{v}1.6, are specifically targeted to the Na\textsubscript{v} channel-enriched IPL domains in the retina. This is the first evidence of Na\textsubscript{v}1.1 recruitment to sites where ankyrin-G and neurofascin concentrate. Previous reports of Na\textsubscript{v}1.1 protein localization place it in somata and dendrites of hippocampal pyramidal cells, cerebellar Purkinje neurons and granule cells, layer V cortical pyramidal cells, and spinal motor neurons (Gong et al., 1999; Westenbroek et al., 1989). In these cells and their processes, expression is diffuse, and no colocalization with Na\textsubscript{v} channel clustering machinery has been reported. Our finding that Na\textsubscript{v}1.1, ankyrin-G, and neurofascin colocalize at sites of high Na\textsubscript{v} channel accumulation indicates that Na\textsubscript{v}1.1 interacts with the same anchoring mechanisms as both Na\textsubscript{v}1.2 and Na\textsubscript{v}1.6.

How, then, are Na\textsubscript{v}1.6 and Na\textsubscript{v}1.1 differentially targeted in adults? The ankyrin-G binding domain of Na\textsubscript{v}1.2 is conserved among all Na\textsubscript{v}1 isoforms (Garrido et al., 2003; Lemaillet et al., 2003), and our results show that neurofascin clusters with Na\textsubscript{v}1.1, as well as with Na\textsubscript{v}1.2 and Na\textsubscript{v}1.6. Therefore, differential targeting of Na\textsubscript{v}1 isoforms is likely independent of interactions with ankyrin-G and neurofascin. This also implies that in order to prevent Na\textsubscript{v}1.1 clustering at the IS and nodes of neurons that express multiple Na\textsubscript{v} channel isoforms, such as cerebellar Purkinje neurons and retinal ganglion cells (Fjell et al., 1997), mechanisms must be in place to prevent Na\textsubscript{v}1.1 channels from entering the axon, to restrict Na\textsubscript{v}1.1 from accessing binding partners at nodes and IS, or to selectively retain Na\textsubscript{v}1.6 at axonal sites of Na\textsubscript{v} channel clustering. Interestingly, a sequence was also found on Na\textsubscript{v}1.2 that acts as an axonal determinant and as an endocytic signal in somatodendritic membranes, yet this domain is also conserved among the CNS isoforms (Garrido et al., 2001). Another less-conserved endocytic signal was recently found in the Na\textsubscript{v}1.2 II–III loop which is thought to remove Na\textsubscript{v}1.2 from somatodendritic, and possibly axonal, membranes if not tethered to the...
cytoskeleton at the IS (Fache et al., 2004). To date, however, no signals for selective retrieval of Nav1.2 from nodal membranes or for selective insertion of Nav1.6 or Nav1.1 have been identified. An alternative explanation for the differential isoform localization is that Nav1.1 expression is downregulated in most retinal ganglion or cerebellar Purkinje cells, limiting the number of channels available to compete with Nav1.6 for interactions with clustering proteins. Any such reduction in expression would have to be dramatic, however, since as little as 10–20% of the normal amount of Nav1.6 protein is sufficient to replace Nav1.2 at optic nerve nodes in medJ mutant mice, albeit with a developmental delay (Kearney et al., 2002).

It is also possible that Nav channel auxiliary β subunits may be involved in channel targeting. In addition to modulating physiological properties of Nav channels, β subunits assist in their cell surface expression and provide interactions at the nodal complex (Malhotra et al., 2000; McEwen et al., 2004; Ratcliffe et al., 2001; Srinivasan et al., 1998). While neither β1 nor β2 is necessary for...
localization of Na\textsubscript{\textalpha}1.6 to optic nerve nodes (Chen et al., 2002, 2004), a subset of hippocampal neurons in \beta1-null mice undergo a selective decrease in Na\textsubscript{\textalpha}1.1 protein expression, and substitution by Na\textsubscript{\textalpha}1.3 not seen in other brain areas (Chen et al., 2004). During development, \beta2 is present in the rat retina at P1, whereas \beta1 is not detected until P14 (Wollner et al., 1988), around the time we begin to see prominent Na\textsubscript{\textalpha}1.1 staining in retinal processes. The role that \beta subunits play in Na\textsubscript{\textalpha} channel localization is not yet clear, but it is conceivable that \beta1 may also be important for surface expression or targeting of Na\textsubscript{\textalpha}1.1 in these retinal processes.

Developmental switch from Na\textsubscript{\textalpha}1.2 to Na\textsubscript{\textalpha}1.1 in ankyrin-G positive IPL processes

As with nodes of Ranvier and axon IS, the molecular composition of the morphologically distinct IPL processes is also developmentally regulated. Na\textsubscript{\textalpha} channel clusters are first detectable in the IPL around P8–P9, about the same time as the first appearance of nodes in the optic nerve (Rasband et al., 1999). At this age, many of the ankyrin-G-labeled MDPs are not positive for Na\textsubscript{\textalpha} channels, suggesting that ankyrin-G precedes Na\textsubscript{\textalpha} channels at morphologically distinct IPL processes, as it does at nodes of
Ranvier (Rasband et al., 1999) and axon IS (Jenkins and Bennett, 2001). Like their axonal counterparts, early MDPs contain Nav1.2 channels, which are transiently expressed and then replaced by Nav1.1 as MDPs mature. The timing of this transition matches the appearance of Nav1.6 at optic nerve nodes and at RGC IS, as well as the general increase of Na+,1.1 expression in the CNS (see Goldin, 2001). Nav1.1 colocalizes with dim Nav1.2 staining at some IPL processes during the switch in isoform expression, suggesting that Nav1.1 and Nav1.2 are indeed targeted to the same region during the developmental transition. The switch is likely rapid, however, since the proportion of processes expressing both isoforms at any given developmental point is small. Although numerous developmental transitions in ion channel isoform expression have been observed in the nervous system, both in normal development and in cases of disease or nerve injury, this is the first reported transition involving a switch to Nav1.1.

The functional significance of the developmental switch from Nav1.2 to Nav1.1 in IPL processes is not yet clear. When expressed in *Xenopus* oocytes, Na+,1.6 inactivated more quickly than Na+,1.1 or Na+,1.2, but similar voltage dependence and inactivation kinetics were found when these isoforms were coexpressed with β1 and β2 subunits (Smith et al., 1998). Although differences between heterologously expressed Na+,1.1 and Na+,1.2 are subtle, with or without coexpression of β subunits, the functional distinction may be magnified in a cell-type-specific manner by interactions with other proteins or by posttranslational modifications (Goldin, 2003; Grieco et al., 2002; Maurice et al., 2001). Identification of the cells of origin of the Na+,1.1-expressing IPL processes may help in understanding the apparently specific targeting of Na+,1.1 channels to the high-density domain.

The IPL contains an intricate plexus of processes belonging to bipolar (input), amacrine, glial, and ganglion (output) cells. Together these processes form a multilayered signaling network responsible for shaping, sharpening, and decoding visual signals. Sodium current, Na, channel mRNA, and/or Na, channel immunoreactivity have been reported in subsets of each of these cell classes (Chao et al., 1993; Fjell et al., 1997; Gustincich et al., 1997; Pan and Hu, 2000; Velte and Masland, 1999; Zenisek et al., 2001). Although we do observe low to moderate levels of Na, channel immunoreactivity in some of these candidate cells, Na, channel staining at the MDPs does not colocalize with a variety of cell type-specific markers in double-label immunohistochemical studies. Nevertheless, the morphology, number, and projection pattern of the Na, channel-enriched processes suggest they may be dendrites of a small subset of ganglion or amacrine cells, for which a selective marker has not yet been identified. Enhanced local sodium current provided by a concentration of Na+,1.1 or Na+,1.2 at these unusual domains may lower the threshold for generation of action potentials, or aid in signal amplification and synaptic integration.

**Experimental methods**

**Tissue preparation**

Animal use followed guidelines established by the NIH and the Institutional Animal Care and Use Committee. Sprague–Dawley rats were sacrificed using CO₂ at ages >P14, and animals of postnatal ages P2–P14 were sacrificed by rapid decapitation. Mice...
expressing CFP under the Thy1 promoter (strain name: B6.Cg-Tg(Thy1-CFP)23Jrs; Feng et al., 2000) were purchased from the Jackson Laboratory (Bar Harbor, Maine) and sacrificed using CO2. Immediately after death, eyes were dissected out, hemisected, and immersion-fixed for 2 h on ice in freshly prepared 4% paraformaldehyde. For retinal sections including the lamina cribrosa and optic nerve, eyes together with the proximal portion of the optic nerve were dissected out and immersion-fixed for 2 h at 4°C in 4% paraformaldehyde. For flat-mount preparation (Voigt and Wässle, 1987), retinas were processed free-floating. For sections, retinas were hemisected, cryoprotected overnight at 4°C in a sucrose gradient, then frozen in M1 medium (Shandon Lipshaw, Pittsburgh, PA), and cryosectioned at 12 or 30 μm in a plane perpendicular to the surface of the retina.

Fig. 9. MDPs do not colocalize with many neuronal and synaptic markers. (A) In vertical sections of rat retina, no colocalization is detected between PAN-labeled MDPs (red) and processes of recoverin-labeled ON and OFF cone bipolar cells (green). Image spans 7 μm. (B) PAN staining at MDPs (red) does not colocalize with the glycinergic cell marker GlyT1 (green) in sections from rat retina. MDP swellings are frequently found adjacent to GlyT1-labeled processes near the IPL/INL border. Image spans 2 μm. (C) A projection taken through a flat mount rat retina stained for PAN (green) and parvalbumin (Parv; red) indicates that AII amacrine cells are among the glycinergic cells which receive putative contacts from MDPs. Although parvalbumin staining does not coincide with PAN-labeled MDPs, PAN-labeled processes often reach toward the IPL/INL border and extend their endings around AII cell bodies and lobular appendages. Image spans 5 μm. (D) In rat retina sections, PAN-labeling at MDPs (green) does not colocalize with synaptic vesicle marker synaptophysin (Syp; red, D), indicating the MDPs are devoid of synaptic vesicles. Image spans 3.6 μm. Ticks indicate IPL/INL border. Scale bar, 20 μm (A), 10 μm (B–D).
An anti-Na$_{1.1}$ mouse monoclonal antibody (K74/71) was generated against a fusion protein consisting of the C-terminus of rat Na$_{1.1}$ (amino acids 1929–2009) and GST carrier. In immunoblots of rat brain membranes, the antibody labeled a single band at the same position as the band labeled with a pan-specific anti-Na$_{1.1}$ antibody (see Supplemental Fig. S1). K74/71 was positive by ELISA against the GST-Na$_{1.1}$ fusion protein, positive by immunohistochemistry in sections of rat brain, and positive by immunofluorescence in COS cells expressing full-length human Na$_{1.1}$ (see Supplemental Fig. S2). No cross reactivity was detected by ELISA against GST-Na$_{1.2}$ (amino acids 1882–2005) and GST-Na$_{1.6}$ (amino acids 1904–1976). In further tests of specificity, K74/71 was also negative in immunofluorescence staining of COS cells expressing full-length Na$_{1.2}$ or Na$_{1.6}$ (see Supplemental Fig. S2), with expression being confirmed by immunostaining with pan-specific anti-Na$_{1.1}$ antibody. In addition, no immunofluorescence with K74/71 was detected in nodes of Ranvier in the adult rat optic nerve (see Supplemental Fig. S3C), where Na$_{1.6}$ channels are found at high density (Boiko et al., 2001). To test staining specificity in retinal tissue, adjacent rat retina cryosections were incubated with K74/71 alone, with K74/71 preincubated with 10× molar excess of Na$_{1.2}$ C-terminus GST fusion protein as a non-specific blocker, or with K74/71 preincubated with 10× molar excess of the Na$_{1.1}$ fusion protein used for its generation. Staining was blocked by preincubation with GST-Na$_{1.1}$ fusion protein but not by incubation with GST-Na$_{1.2}$ (see Supplemental Fig. S3A,B). No staining above background was detectable in sections incubated with the secondary antibody alone.

All other immunocytochemistry was performed using previously characterized antibodies. Pan-specific polyclonal (Dugandzija-Novakovic et al., 1995) and monoclonal (K58/35, Rasband et al., 1999) Na$_{1}$ channel antibodies (PAN) were generated against a conserved sequence present in all vertebrate Na$_{1}$ isoforms. Anti-peptide rabbit polyclonal antibodies against the Na$_{1.2}$ isoform were developed against a unique sequence in the Na$_{1.2}$ C-terminus (Gong et al., 1999). Anti-peptide rabbit polyclonal antibodies against Na$_{1.6}$ were generated against a synthetic peptide corresponding to a unique sequence in the large intracellular domain I–II loop of Na$_{1.6}$ (Caldwell et al., 2000). Monoclonal anti-neurofascin antibody was generated against a neurofascin-GST fusion protein and detects both neurofascin 155 and neurofascin 186 (Schaf er et al., 2004). Rabbit polyclonal anti-Caspr antibodies (Boiko et al., 2001) were made against the same fusion protein as previously characterized anti-Caspr rabbit polyclonal antibodies (Peles et al., 1997). Rabbit polyclonal anti-recoverin (Lambrecht and Koch, 1991) was provided by Dr. Stephen Yazulla. The following antibodies were purchased: mouse anti-ankyrin-G antibody (clone 4G3F8; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-parvalbumin (PARV-19) and mouse synaptophysin (SVP-38; Sigma-Aldrich, St. Louis, MO), goat anti-GlyT1 (Chemicon, Temecula, CA), and mouse anti-synaptotagmin-I (Synaptic Systems, Göttingen, Germany). CFP-expressing neurons were labeled with rabbit anti-GFP, which also recognizes CFP, purchased from BD Biosciences Clontech (Palo Alto, CA). Alexa 488-conjugated secondary antibodies (Molecular Probes, Eugene, OR) were used to detect rabbit and goat polyclonal antibodies, and Cy-3-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) were used for visualization of the mouse monoclonal antibodies.

Staining was carried out using a protocol similar to that used for the cryosections with the following exceptions. Whole retinas were incubated free-floating in solutions inside sealed plastic containers (1.5 ml centrifuge tubes or 2 ml plastic autoanalyzer cups). Primary incubations were done at RT for 3–5 days on a nutator in the presence of 3 mM sodium azide, and then washed 3 × 10 min in PBS. Secondary incubations were done for 1 h, followed by 1 × 15 min PBST and 2 × 10 min PBS washes. Several cuts were then made at the edges of the retina towards the optic disc, and after being dipped in deionized water to eliminate excess salt, retinas were spread flat and mounted, RGC side down, onto a c overslip which was covered with a slide bearing a drop of Vectashield (Vector Laboratories).

Images were acquired using a laser-scanning confocal microscope [LSM 510 (Zeiss, Thornwood, NY) or FV-300 (Olympus Optical, Tokyo, Japan)], initially processed using Zeiss LSM or Olympus FluoView software, and later exported into Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA) for final processing. Images comparing peptide-blocked and unblocked antibody labeling were acquired and digitally processed identically. No staining above background was detectable in sections incubated with secondary antibody alone or with primary antibody preincubated with blocking peptide (data not shown). For imaging of retinal flat mounts, where a series of confocal optical sections was taken, nominal confocal section thickness was 0.3 μm, and successive sections were separated by 0.5 μm. As indicated in the captions, figures show either planar projections of a series of successive confocal images, or representative, individual confocal sections.

Confocal images of retina and optic nerve were taken from the same sections labeled with mouse monoclonal PAN antibody.
Individual optical sections of the nodes, IS, and MDPs were acquired under identical conditions within the linear range of the photomultiplier. The fluorescence intensity of PAN staining per unit of total volume was measured in examples from each site and analyzed using the NIH Image software. Data were submitted to a t test to determine the significance of intensity differences between each site.

Acknowledgments

The first two authors contributed equally to this work. We thank Dr. Gail Mandel (SUNY Stony Brook) for access to her confocal microscope, and Dr. S.R. Levinson (University of Colorado Medical School) for generously providing the polyclonal pan-specific Na+, channel and anti-Na+,1,6 antibodies. We thank Ted Cummins (Indiana University School of Medicine) for providing rat Na+,1,6 cDNA, Rob Dunn (McGill University) for rat Na+,1,2 cDNA, and Al George (Vanderbilt University) for human Na+,1,1 cDNA. Excellent technical assistance in monoclonal antibody production, screening, and characterization was provided by Lynn Buchwalder, Kaori Misono, and Diane Henry, Supported by NIH grants EY03821 (G.M.), NS34383 (J.S.T.), and NRSA Fellowship F31 NS048762 (A.V.).

Appendix A. Supplementary data


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