Polarized Distribution of Ion Channels within Microdomains of the Axon Initial Segment

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ABSTRACT
Voltage-gated sodium (Nav) channels accumulate at the axon initial segment (IS), where their high density supports spike initiation. Maintenance of this high density of Na\textsubscript{v} channels involves a macromolecular complex that includes the cytoskeletal linker protein ankyrin-G, the only protein known to bind Na\textsubscript{v} channels and localize them at the IS. We found previously that Na\textsubscript{v,1.6} is the predominant Na\textsubscript{v} channel isoform at IS of adult rodent retinal ganglion cells. However, here we report that Na\textsubscript{v,1.6} immunostaining is consistently reduced or absent in short regions of the IS proximal to the soma, although both ankyrin-G and pan-Na\textsubscript{v} antibodies stain this region. We show that this proximal IS subregion is a unique axonal microdomain, containing an accumulation of Na\textsubscript{v,1.1} channels that are spatially segregated from the Na\textsubscript{v,1.6} channels of the distal IS. Additionally, we find that axonal K\textsubscript{v,1.2} potassium channels are present within the distal IS, but are also excluded from the Na\textsubscript{v,1.1}-enriched proximal IS microdomain. Because ankyrin-G was prominent in both proximal and distal subcompartments of the IS, where it colocalized with either Na\textsubscript{v,1.1} or Na\textsubscript{v,1.6}, respectively, mechanisms other than association with ankyrin-G must mediate differential targeting of Na\textsubscript{v} channel subtypes to achieve the spatial precision observed within the IS. This precise arrangement of ion channels within the axon initial segment is likely an important determinant of the firing properties of ganglion cells and other mammalian neurons. J. Comp. Neurol. 500:339 –352, 2007. © 2006 Wiley-Liss, Inc.

Indexing terms: sodium channels; Na\textsubscript{v,1.1}; Na\textsubscript{v,1.6}; ankyrin-G; K\textsubscript{v,1.2}; channel targeting

The initial segment (IS) is a functionally specialized region of the axon where action potentials are generated. Like nodes of Ranvier, which are required for the saltatory conduction of action potentials, the adult IS contains a high density of a specific isoform of voltage-gated sodium (Na\textsubscript{v}) channel, Na\textsubscript{v,1.6} (Caldwell et al., 2000; Jenkins and Bennett, 2001). Targeting and restriction of Na\textsubscript{v} channels to these sites involves a macromolecular complex of interacting proteins, including the cytoskeletal proteins ankyrin-G and \( \beta \)IV spectrin, the cell adhesion molecules neurofascin-186 and NrCAM, and Na\textsubscript{v} auxiliary \( \beta \) subunits (Poliak and Peles, 2003; Salzer, 2003). Of particular importance is ankyrin-G, which is necessary to localize Na\textsubscript{v} channels and neurofascin to the IS and to maintain normal firing capabilities (Zhou et al., 1998; Bennett and Lambert, 1999; Jenkins and Bennett, 2001). Therefore, the precise distribution and targeting of ion channels at the initial segment is critical for determining firing properties.

Because of the importance of the IS in neuronal firing, we examined the distribution of ion channels at this site in more detail, with special attention to isoform-specific targeting of Na\textsubscript{v} channels. In retinal ganglion cells (GC), immunostaining for Na\textsubscript{v,1.6} channels largely colocalized with ankyrin-G and pan-Na\textsubscript{v}, channel immunoreactivity in...
the IS, as reported previously (Boiko et al., 2003). However, we found that Na,1.6 staining was reduced or absent in a short segment of the IS near the cell body, which did label with pan-Na, channel and anti-ankyrin-G antibodies. This led us to investigate whether other Na, channels might be present in the proximal IS microdomain. Surprisingly, the proximal IS exhibited strong staining for Na,1.1 channels, which are generally thought to have a somatodendritic distribution in the central nervous system (CNS). The Na,1.1-dense proximal and Na,1.6-dense distal microdomains both contain ankyrin-G, suggesting that association with ankyrin-G does not directly confer isoform specificity to the different zones within the IS, and that numerous Na, channel isoforms can likely interact with the same clustering/anchoring mechanisms in GC axons. Furthermore, the regolorizing delayed rectifier potassium channels of the K,1 subfamily exhibit a distinct pattern of subcellular localization in the GC axon, being segregated from the Na,1.1-containing subregion of the IS, but present in distal Na,1.6-containing IS, the unmyelinated GC axon, and at juxtaparanodes of nodes of Ranvier. Taken together, these results suggest that a complex molecular structure exists within the IS, producing unexpectedly precise and selective clustering patterns of Na, and K, channel isoforms. Such disparate channel microdomains at this crucial site for action potential initiation are likely to have important implications for neuronal firing patterns.

MATERIALS AND 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 (postnatal day), and animals of P2–14 were sacrificed by rapid decapitation. Immediately after death, eyes were removed, hemisected, and immersion-fixed for 2 hours on ice in freshly prepared 4% paraformaldehyde in phosphate-buffered saline (PBS). For flat-mount preparation (Voigt and Wässle, 1987), retinas were processed free-floating. For sections, retinas were hemisected, cryoprotected overnight at 4°C in 30% sucrose in PBS, then frozen in M1 medium (Shandon Lipshaw, Pittsburgh, PA), and cryosectioned at 30 μm in a plane perpendicular to the surface of the retina.

Immunohistochemistry reagents

K74/71, mouse monoclonal anti-Na,1.1 is described in detail in Van Wart et al. (2005). The antibody was generated against a fusion protein GST-Na,1.1 consisting of the C-terminus of rat Na,1.1 (amino acids 1929–2009) and GST carrier (Van Wart et al., 2005). 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, antibody (Van Wart et al., 2005). K74/71 was positive by ELISA against the GST-Na,1 fusion protein, positive by immunohistochemistry in sections of rat brain, and positive by immunofluorescence in COS cells expressing full-length Na,1.2 or Na,1.6 (Van Wart et al., 2005), with expression being confirmed by immunostaining with a pan-specific anti-Na, antibody. In addition, no immunofluorescence with K74/71 was detected in nodes of Ranvier in the adult rat optic nerve (Van Wart et al., 2005), 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 nonspecific blocker, or with K74/71 preincubated with 10× molar excess of the Na,1.1 fusion protein used for its generation (Van Wart et al., 2005). Staining was blocked by preincubation with GST-Na,1.1 fusion protein but not by incubation with GST-Na,1.2 (Van Wart et al., 2005). K74/71 can be obtained from the UC Davis/NINDS/NIMH NeuroMab facility (http://www.neuromab.org).

K58/35, mouse monoclonal pan-specific anti-Na, is described in detail in Rasband et al. (1999). The pan-specific Na, channel mouse IgG1 monoclonal antibody K58/35 (Rasband et al., 1999), referred to here as PAN, was generated against a conserved sequence (TEEQKKY- YNAMKKLGSKK; amino acids 1501–1518 of rat Na,1.1) present in all vertebrate Na,1 isoforms. Immunoreactivity in retina and optic nerve was abolished by preincubation with the immunizing peptide (Boiko et al., 2001).

K11A41, mouse monoclonal anti-neurofascin is described in detail in Schafer et al. (2004). The mouse monoclonal IgG1 antibody was generated against a recombinant GST fusion protein containing amino acids 1066–1174 of rat neurofascin-155 (NF-155), and common to NF-186. The antibody recognizes both major splice variants of neurofascin (NF-155, NF-186) on immunoblots. Preincubation of K11A41 with the immunizing fusion protein abolished all immunoreactivity (Schafer et al., 2004). The staining pattern in brain sections is consistent with that of a combination of NF-155 (Collinson et al., 1998) and NF-186 (Davies et al., 1996). K11A41 can be obtained from the UC Davis/NINDS/NIMH NeuroMab facility (http://www.neuromab.org).

K14/16, mouse monoclonal anti-K,1.2 is described in detail in Bekele-Arcuri et al. (1996). The mouse monoclonal IgG2b antibody was generated against a recombinant GST fusion protein (GST-RACK) containing amino acids 428–499 of rat K,1.2. The binding site was subsequently mapped to within amino acids 463–480. No crossreactivity was observed against other K, family members (K,1.1, K,1.3, K,1.4, K,1.5, and K,1.6) overexpressed in heterologous cells (Bekele-Arcuri et al., 1996). Immunoblots yield a major population of K,1.2 with processed N-oligosaccharides, and a minor population with high mannos oligosaccharides (Bekele-Arcuri et al., 1996). Immunoblot staining was eliminated by preincubation with GST-RACK (Bekele-Arcuri et al., 1996). The staining pattern in brain sections (Bekele-Arcuri et al., 1996; Rhodes et al., 1997; Monaghan et al., 2001) is consistent with published (Tsaur et al., 1992; Veh et al., 1995) and unpublished (K.J. Rhodes) in situ hybridization data, and with published light and electron microscopic level reports of K,1.2 localization (McNamara et al., 1993; Wang et al., 1993, 1994). K14/16 can be obtained from the UC Davis/NINDS/NIMH NeuroMab facility (http://www.neuromab.org).

Mouse monoclonal anti-ankyrin-G antibody described by Jenkins et al. (2001) was purchased (cat. no. 12719; Santa Cruz Biotechnology, Santa Cruz, CA). Rabbit poly-
clonal anti-ankyrin-G described by Johnson et al. (2006) was a gift of Bettina Winckler (University of Virginia Medical School). Immunostaining with both antibodies reproduced the previously published pattern of ankyrin-G concentration at axon initial segments and nodes of Ranvier (Kordeli et al., 1995).

Rabbit polyclonal anti-Nav1.1 antibody raised against amino acids 465–481 of rat Nav1.1 was purchased from Chemicon (Temecula, CA; cat. no. AB5204). It was tested in double-label immunofluorescence experiments on rat retina in combination with the monoclonal anti-Nav1.1 antibody, K74/71, which is described above. Although non-specific nuclear staining was present with AB5204 but not K74/71, all other staining with the two antibodies coincided, with no other extraneous labeling above background.

Rabbit polyclonal anti-Nav1.6 is an anti-peptide rabbit polyclonal antibody against Na_v1.6, generated against a synthetic peptide (amino acids 460–477; SEDAIEEEGEDGVGSPRS) corresponding to a unique sequence in the large intracellular domain I-II loop of Na_v1.6 (Caldwell et al., 2000) and was a generous gift of Dr. Rock Levinson. This antibody does not stain nodes of Ranvier in Na_v1.6-deficient medJ mice, and staining in wildtype mice is abolished by prior incubation of the antibody with the antigenic peptide (Caldwell et al., 2000).

Alexa 488-conjugated secondary antibodies (Invitrogen/Molecular Probes, Eugene, OR) were used to detect rabbit polyclonal antibodies, and Cy-3-conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA) were used for visualization of the mouse monoclonal antibodies.

**Immunostaining of cryosections**

Slides with cryosections were thawed at room temperature (RT), washed 3 × 10 minutes in PBS, then incubated for 2 hours at RT with blocking solution consisting of 6% normal goat serum (NGS) in PBST (PBS + 0.3% Triton X-100). Primary antibodies were diluted in blocking solution and applied to sections to be incubated overnight at RT in sealed humidified chambers. Slides were then washed 3 × 10 minutes in PBS. Secondary antibodies were diluted in blocking mix, filtered through 0.22-µm filters, and applied to sections for 45 minutes at RT in the dark. After washing 1 × 10 minutes in PBST and 2 × 10 minutes in PBS, sections were dried for 10 minutes, and

![Fig. 1. Na_v1.6 channel immunofluorescence does not completely colocalize with ankyrin-G or pan-specific Na_v staining at the initial segment. Flat-mount retinas stained for Na_v1.6 (C–F, green) and either ankyrin-G (A, red), or all Na_v isoforms (D, PAN, red). Close examination reveals that Na_v1.6 did not uniformly occupy the entire ankyrin-G-positive IS, with staining tapering off at the proximal end (A–C, arrowhead). Also, PAN staining extended beyond the Na_v1.6-positive subregion of the IS in the proximal direction (D–F, arrowhead). Asterisks mark the cells of origin of the indicated initial segments. Projections span 2 µm (A–C), and 1.5 µm (D–F). Scale bars = 20 µm.](image)
mounted in Vectashield (Vector Laboratories, Burlingame, CA).

**Immunostaining of flat mounts**

Staining was carried out using a protocol similar to that used for the cryosections with the following exceptions. Whole isolated retinas were incubated free-floating in primary-antibody solution for 3–5 days at RT on a nutator in the presence of 3 mM sodium azide, then washed 3 × 10 minutes in PBS. Secondary antibody incubations were done for 1 hour, followed by 1 × 15 minutes PBST and 2 × 15 minutes PBS washes. Several cuts were then made at the edges of the retina toward the optic disc, and retinas were dipped in deionized water to eliminate excess salt. Finally, retinas were spread flat and mounted retinal ganglion cell (RGC) side down onto a coverslip, which was covered with a slide bearing a drop of Vectashield (Vector Laboratories).

**Data acquisition**

Images were acquired using an FV300 laser-scanning confocal microscope (Olympus Optical, Tokyo, Japan), initially processed using Olympus FluoView software, and later exported into Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA) for final processing. No staining above background was detectable in sections incubated with secondary antibody alone or with primary antibody preincubated with blocking peptide. 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, the figures show either planar projections of a series of successive confocal images, or representative, individual confocal sections.

**Image analysis**

For quantification of Na,1.1 and Na,1.6 staining intensity at initial segments, all images were acquired within the linear range of the photomultiplier, and taken from short projections (3–5 optical sections) through the ganglion cell layer of the flat-mount retina. This procedure selected for initial segments that ran tangentially along the ganglion cell layer, thus minimizing variations in background levels. Using NIH ImageJ software (Bethesda, MD), a segmented line was drawn through the length of the IS, as defined by Na, channel staining (levels greater than that of the cell body). Pixel intensity along the line was then measured and exported to Microsoft Excel for further analysis. Background fluorescence was measured by taking the average pixel intensity of each channel within an area of the projection adjacent to the captured IS. These background values were then subtracted from the pixel intensity measurements along the line. The data were then smoothed by calculating a moving average over five consecutive pixels. Counts of Na,1.1-positive initial segments were made using confocal projections taken through the entire ganglion cell and nerve fiber layer of flat-mounted retina. Only initial segments that were entirely located in the field of view were counted. For quantification of ganglion cell number and size, confocal projections were taken through the ganglion cell layer of the central, intermediate, and peripheral one-third of flat-mounted retinas. Background was measured as described previously in each image and background immunofluorescence was subtracted from the images. Positive cells were then counted (104 total), and cell size was measured along the long axis of the cell soma, defined by Na, channel immunostaining.

**RESULTS**

**Multiple Na,1 channel isoforms are present within the IS**

Retinal ganglion cells express mRNAs for four different Na, channel α subunit isoforms: Na,1.1, Na,1.2, Na,1.3,
and Nav1.6 (Fjell et al., 1997). We showed previously that in adult rat GCs a particular isoform, Nav1.6, is present at greater than 95% of axon initial segments, defined by ankyrin-G staining, while Nav1.2 is expressed uniformly throughout the axon (Boiko et al., 2003). Ankyrin-G, a cytoskeletal linker protein found at high density in the IS and at nodes of Ranvier, is believed to anchor the high density of Nav1.6 channels at these sites. However, we noticed that while ankyrin-G consistently labeled all the Nav1.6-positive IS in the ganglion cell layer, there was a short region at the proximal end of the IS with strong ankyrin-G staining, but where Nav1.6 staining was either reduced or absent. This can be seen in flat-mount retinas double-labeled for Nav1.6 and ankyrin-G (Fig. 1A–C). To verify that this observation was not an artifact of immunostaining, retinas were also stained with anti-Na,1.1 antibodies and a PAN-specific Na, channel antibody that recognizes all Na,1 isoforms (Dugandzija-Novakovic et al., 1995) (Fig. 1D–F). As with the ankyrin-G immunofluorescence, PAN staining also extended beyond the region of bright Na,1.6 staining, revealing a gap at the proximal IS where a high density of Na, channels is present, but Na,1.6 is not the dominant isoform. As described previously, Na,1.2 channels are expressed uniformly along the mature GC axon and IS (Boiko et al., 2003), and since Na,1.3 channels are expressed primarily during prenatal and early postnatal development (Beckh et al., 1989), we hypothesized that Na,1.1 channels may account for the additional PAN staining that does not coincide with Na,1.6 immunoreactivity.

**Nav1.1 expression in retinal ganglion cells**

Throughout the nervous system, Na,1.1 channels are mainly localized to neuronal somata and dendrites, where they are believed to be involved in signal integration (Westenbroek et al., 1989, Gong et al., 1999, Whitaker et al., 2001). Consistent with this pattern, many ganglion cells throughout the retina exhibited detectable Na,1.1 immunostaining in their cell bodies and dendrites (Figs. 2, 3). However, fascicles of unmyelinated ganglion cell axons passing across the surface of the retina also contained moderate levels of Na,1.1 staining, but the brightest

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**Fig. 3.** Na,1.1 immunofluorescence is also present in GC somata and axon fascicles, as well as the proximal dendrites of a subset of large GCs. A–C: Adult flat-mount retinas were stained for Na,1.1, and confocal projections through the GC layer were taken from central (A), intermediate (B), and peripheral retina (C), with respect to the optic nerve head. D: Summary of soma diameters of Na,1.1-positive ganglion cells, measured along the long axis. E: The density of Na,1.1-positive ganglion cells was measured in central, intermediate, and peripheral retina. Scale bars = 40 μm in A–C.
Nav1.1 immunoreactivity was observed in short regions (5–15 μm) of the proximal IS of the axon (Fig. 2B). This staining was noticeably more intense than that of the passing GC nerve fibers and was comparable to that of discrete sites of Nav1.1 clustering in the inner plexiform layer (IPL) (Van Wart et al., 2005). In larger axons, like that shown in Figure 2B, the Nav1.1 staining appeared to be concentrated along the membrane of the putative initial segment. Nav1.1 immunostaining in the IS was found consistently throughout the GC layer and was not restricted to cells of a particular size or location, although the brightest somatodendritic staining was detected in large GCs (soma diameter ≥20 μm) and their proximal dendritic membranes (Figs. 2, 3). These large cells made up ~18% of the total Nav1.1-positive GC population. In the periphery, where the density of cells is lower, we could usually distinguish one to four primary dendrites that visibly stained for Nav1.1, and then bifurcated as the staining tapered away. Thus, Nav1.1 channels are actually found throughout the GC and its processes, but appear to be enriched at the proximal initial segment of the axon.

To verify that the sites of Nav1.1 clustering were within the IS itself, as opposed to the axon hillock or a dendrite from which the IS may emerge, retinas were double-labeled for Nav1.1 and the IS marker, ankyrin-G (Fig. 4). We found that ankyrin-G staining indeed coincided with the region of bright Nav1.1 staining in the axon. However, Nav1.1 was only concentrated within approximately one-third of the ankyrin-G-defined IS, in the zone proximal to the cell body. Thus, Nav1.1 channels, in addition to Nav1.6 channels, accumulate at the ganglion cell IS, but the cluster of Nav1.1 channels is restricted to the proximal IS, as defined by ankyrin-G staining. Consistent with these results, Nav1.6 immunofluorescence was either reduced (in large diameter axons) or absent (small diameter axons) in the region of bright Nav1.1 staining in the proximal IS, while Nav1.1 channels were undetectable along the distal...
Fig. 6. Pan-Na$_v$ immunofluorescence is approximately constant in the Na$_v$1.1 and Na$_v$1.6 domains within GC initial segments. A: Na$_v$1.1 immunofluorescence increased as Na$_v$1.6 levels declined at the proximal IS. Pixel intensities of Na$_v$1.1 and Na$_v$1.6 staining were measured along the GC IS shown in Figure 5. Na$_v$1.1 staining hovered around background levels at the distal IS, where Na$_v$1.6 immunofluorescence was high. As Na$_v$1.1 staining increased toward the cell body, Na$_v$1.6 immunofluorescence fell to background levels. B: In a GC IS double-labeled with pan-specific anti-Na$_v$ (PAN) and anti-Na$_v$1.6 antibodies, pixel intensity of PAN staining was approximately constant within the proximal IS, where Na$_v$1.6 staining declined to background level. C: Pixel intensity of pan-specific and Na$_v$1.1 immunofluorescence along a double-labeled GC IS.

Fig. 5. Na$_v$1.1 and Na$_v$1.6 immunofluorescence occupy mutually exclusive domains within GC initial segments. Flat-mount retinas stained for Na$_v$1.1 (A,B, red) and Na$_v$1.6 (B,C, green). Na$_v$1.1 staining (arrowhead) was detected adjacent to Na$_v$1.6 staining at the majority of IS (219 of 259), and Na$_v$1.6 was either absent or reduced in the Na$_v$1.1-positive subregion. Projections span 3 μm. Scale bar = 20 μm.
two-thirds of the IS, where bright Na\textsubscript{v}1.6 staining was observed (Figs. 5, 6A). Na\textsubscript{v}1.1 immunostaining was detected at 85% (219 of 259) of Na\textsubscript{v}1.6-labeled GC initial segments. Although Na\textsubscript{v}1.6 immunostaining declined in the proximal one-third of the IS, the intensity of pan-specific Na\textsubscript{v} staining in this subregion remained at approximately the same level as in the distal IS, as illustrated in Figure 6B. Similarly, the decline in Na\textsubscript{v}1.1 staining in the distal IS was not accompanied by a change in pan-specific Na\textsubscript{v} immunofluorescence (Fig. 6C). The fact that the intensity of pan-Na\textsubscript{v} staining was approximately constant throughout the initial segment, while Na\textsubscript{v}1.1 and Na\textsubscript{v}1.6 intensities were complementary (Fig. 6A), suggests that Na\textsubscript{v}1.1 and Na\textsubscript{v}1.6 channels largely account for the Na\textsubscript{v} staining in the proximal and distal subregions of the IS, respectively. Thus, the subregions of Na\textsubscript{v}1.6 and Na\textsubscript{v}1.1 accumulation were spatially segregated in the IS, revealing an unexpected molecular substructure within the high-density clusters of ankyrin-G and the total Na\textsubscript{v} channel pool as revealed by staining with pan-Na\textsubscript{v} antibodies.

Na\textsubscript{v}1.1 also clusters in initial segments of a subset of hippocampal CA3 neurons

Previous studies have shown that Na\textsubscript{v}1.6 is the major Na\textsubscript{v} channel of nodes of Ranvier and IS in the brain (Caldwell et al., 2000; Jenkins and Bennett, 2001), while Na\textsubscript{v}1.1 channels are present in the somata and/or dendrites of many CNS neurons, including cortical pyramidal neurons, cerebellar Purkinje cells, and pyramidal and granule neurons of the hippocampus (Westenbroek et al., 1989; Gong et al., 1999; Schaller and Caldwell, 2000; Whitaker et al., 2001). Because our finding in initial segments of GCs is the first reported instance of an accumulation of Na\textsubscript{v}1.1 sodium channels within axons, we asked whether this arrangement is specific to GCs or a common characteristic of axon initial segments in the CNS. In sections of rat brain double-stained for ankyrin-G and Na\textsubscript{v}1.1, we observed an Na\textsubscript{v}1.1-dense subregion of the IS only in a subset of initial segments (13 of 317; 4.1%) adjacent to or within the pyramidal cell layer of hippocampal area CA3 (Fig. 7A, arrow). As with GCs, the clusters of Na\textsubscript{v}1.1 were located at the proximal end of the IS nearest the soma in this subset of CA3 neurons. We did not observe Na\textsubscript{v}1.1 staining coinciding with ankyrin-G at initial segments of cerebellar Purkinje cells (Fig. 7B, arrowheads), cells of the granule cell layer of the cerebellum (Fig. 7B), granule cells of the dentate gyrus (Fig. 7C), CA1 hippocampal neurons (Fig. 7D), or cortical pyramidal cells (Fig. 7F). Initial segments of most pyramidal neurons in hippocampal CA2/3 also did not contain Na\textsubscript{v}1.1 (Fig. 7E).

**Fig. 7.** Na\textsubscript{v}1.1-enriched subdomain is present at initial segments of a subset of hippocampal neurons, but not in cerebellar or cortical neurons. Rat brain sections stained for ankyrin-G (red) and Na\textsubscript{v}1.1 (green). Na\textsubscript{v}1.1 immunostaining was detected at the proximal IS of a subset of neurons in CA3 of the hippocampus (A, arrow). However, Na\textsubscript{v}1.1 was not detectable at initial segments of cerebellar Purkinje neurons (B, arrowheads), neurons of the granule layer of the cerebellum (B), hippocampal dentate granule cells (C), hippocampal CA1 pyramidal neurons (D), most hippocampal CA3 pyramidal neurons (E), and cortical pyramidal cells (F). Scale bars = 10 μm.
Thus, although an IS subregion enriched in Nav1.1 is rare among brain axons, this substructure is not completely unique to retinal GCs.

**Subdomain is established independent of Nav1.1 expression**

We showed previously that by the time of eye opening in rats (at P14), Na1.6 is already detectable at 80% of initial segments and >90% of nodes of Ranvier (Boiko et al., 2001, 2003). However, when we examined Na1.1 immunostaining at this age (Fig. 8A) and earlier (not shown), we found only very low levels of Na1.1 immunoreactivity had appeared in GC somata, and staining could not be detected in GC dendrites, initial segments, or axon fiber bundles (Fig. 8A, "f"). This is consistent with previous reports that during retinal development Na1.1 protein levels are low at birth, and do not rise until the third postnatal week (Wollner et al., 1988). By contrast, as

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Fig. 8. Ankyrin-G defines the site of subsequent Na1.1 channel clustering in the developing retina. A: In a section of P14 retina stained for Na1.1, staining was prominent in processes of the IPL, but was absent from the nerve fiber layer (f). B,C: In the flat-mount P14 retina stained for ankyrin-G (red) and Na1.6 (green), Na1.6 was located nonuniformly along the ankyrin-G-positive segment. Arrowheads indicate region of low Na1.6 immunofluorescence in the proximal IS. D: In sections from retinas at age P19, Na1.1 immunostaining was present in the IPL, as well as ganglion cell bodies and their axons (f). E,F: In flat-mount P20 retinas stained for Na1.1 (red) and Na1.6 (green), Na1.1 immunostaining in the proximal IS (arrowhead, E) was adjacent to the bright Na1.6 staining in the more distal IS. Na1.6 staining was reduced in the proximal IS (arrowhead, F) compared to the distal IS. G-I: Flat-mount retina at P14 stained with pan-specific anti-Na1.6 (PAN; red) and anti-Na1.6 (green). Arrowheads indicate the proximal initial segment, where the intensity of Na1.6 immunostaining was reduced (panel I). Arrows indicate the distal initial segment, where Na1.6 immunostaining was brighter (panel I). Pan-specific Na1.1 immunostaining was present in both proximal and distal portions of the initial segment. Tick marks indicate IPL borders in A and D. Images span 2 μm (A,D), 3 μm (B,C,G–I), 1 μm (E,F). Scale bars = 20 μm.
Figure 9
reported previously (Van Wart et al., 2005), Na\(_{\text{v}}\)1.1 channel clusters can already be detected at P14 in morphologically distinct IPL processes (Fig. 8A, arrowhead), which serve as an internal control for the effectiveness of immunostaining.

Interestingly, in flat-mount retinas from animals age P14, Na\(_{\text{v}}\)1.6 channels are not distributed uniformly throughout the IS (Fig. 8B,C), despite the absence of Na\(_{\text{v}}\)1.1 in the proximal portion of the IS. Arrowheads indicate the proximal region of the IS, defined by ankyrin-G staining, where Na\(_{\text{v}}\)1.6 immunostaining is dimmer than in the distal IS. Na\(_{\text{v}}\)1.1 immunofluorescence rises in the ganglion cell and nerve fiber layers during the third postnatal week, and by P19 this staining is evident in GC somata and their axon fibers (Fig. 8D, “f”). At this time we can also detect Na\(_{\text{v}}\)1.1 immunofluorescence adjacent to Na\(_{\text{v}}\)1.6 channels within the proximal IS (Fig. 8E,F), resembling the adult pattern of immunostaining. These data suggest that a subregion exists within the proximal IS before the presence of Na\(_{\text{v}}\)1.1 channels at this site, and that as Na\(_{\text{v}}\)1.1 protein levels increase in the GCs, Na\(_{\text{v}}\)1.1 channels preferentially occupy this proximal zone of the IS. Although it is possible that a small number of Na\(_{\text{v}}\)1.1 channels are present at P14 but undetectable with our antibodies, it is clear that Na\(_{\text{v}}\)1.6 channels are preferentially enriched at the distal region of the IS, even at a time when Na\(_{\text{v}}\)1.1 levels are low. Therefore, it is unlikely that newly expressed Na\(_{\text{v}}\)1.1 channels are outcompeting Na\(_{\text{v}}\)1.6 channels for binding partners in the proximal IS during week 3, and more likely that most Na\(_{\text{v}}\)1.6 channels are instead actively excluded from this region, even prior to detectable Na\(_{\text{v}}\)1.1 expression.

Although the density of Na\(_{\text{v}}\)1.6 channels was lower in the proximal than in the distal IS at P14, despite the absence of Na\(_{\text{v}}\)1.1, pan-specific Na\(_{\text{v}}\) immunostaining was present throughout the IS at this age (Fig. 8G–I), indicating that another Na\(_{\text{v}}\) isoform occupies the proximal IS. Na\(_{\text{v}}\)1.2 channels are present in GC initial segments both early in development and in adulthood (Boiko et al., 2003), and at P14 Na\(_{\text{v}}\)1.2 immunostaining was evenly distributed along the IS, which was marked by ankyrin-G staining (data not shown). Therefore, Na\(_{\text{v}}\)1.2 channels likely account for the pan-specific Na\(_{\text{v}}\) staining observed in the proximal IS at P14, prior to the appearance of Na\(_{\text{v}}\)1.1 at that site.

**Na\(_{\text{v}}\)1.1 also occupies a separate microdomain from K\(_{\text{v}}\)1.2 potassium channels**

The finding that Na\(_{\text{v}}\)1.6 is restricted to the distal IS before the expression of Na\(_{\text{v}}\)1.1 in the proximal IS suggests that there is a complex substructure to the IS, which may exist independent of Na\(_{\text{v}}\) channel isoform expression. Since high levels of K\(_{\text{v}}\)1.1 delayed rectifier potassium channels are also found diffusely along unmyelinated axons within the retina (Boiko et al., 2001) and at juxtaparanodes of optic nerve nodes of Ranvier (Chiu and Ritchie, 1980; Wang et al., 1993), we examined the distribution of K\(_{\text{v}}\)1 channels in relation to the newly uncovered substructure within the initial region of the axon. In the myelinated portion of GC axons, Na\(_{\text{v}}\)1.6 channels at nodes of Ranvier are separated from a high density of juxtaparanodal K\(_{\text{v}}\)1 channels by paranodal axoglial junctions. In contrast, we found that the high levels of K\(_{\text{v}}\)1.2 staining found throughout the unmyelinated intraretinal axons also penetrated into the IS, as defined by both ankyrin-G and neurofascin labeling (Fig. 9A–F). This staining is consistent with reports of K\(_{\text{v}}\)1.1 and K\(_{\text{v}}\)1.2 channels being present within the first 20 µm of axons in the medial nucleus of the trapezoid body (Dodson et al., 2002), as well as reports of K\(_{\text{v}}\)1.1 channels at the IS of avian vestibular axons (Popratiloff et al., 2003). Although anti-K\(_{\text{v}}\)1.2 antibodies also labeled the GC bodies and occasionally their dendrites, only trace amounts of K\(_{\text{v}}\)1.2 staining were detected between the cell body and the cluster of Na\(_{\text{v}}\)1.1 staining in the IS (Fig. 9G–M). Na\(_{\text{v}}\)1.1 membrane staining was seen in the proximal axon, whereas lighter K\(_{\text{v}}\)1.2 puncta were also detected. At the distal termination of the bright Na\(_{\text{v}}\)1.1 immunofluorescence, where Na\(_{\text{v}}\)1.6 channels cluster, K\(_{\text{v}}\)1.2 channels also began to accumulate and appeared to be associated with the membrane. Thus, like Na\(_{\text{v}}\)1.6, K\(_{\text{v}}\)1.2 channels were largely excluded from the proximal IS subregion, where Na\(_{\text{v}}\)1.1 accumulates, and increased in density in the distal IS (Fig. 10). Distal localization of K\(_{\text{v}}\)1.2 channels like that shown in Figures 9 and 10 has also been reported in the axon initial segment of cortical pyramidal neurons (Inda et al., 2006).
Interestingly, the axon diameter tapers along the Na$_v$ channel-enriched IS, and in thicker axons, such as that depicted in Figure 9 M, the Na$_v$1.1-enriched portion can be nearly double the diameter of the Na$_v$1.6/K$_v$1.2-labeled segment. Because anti-Na$_v$1.1 antibodies also diffusely label the cell body and hillock, it is apparent that the Na$_v$1.1-dense region of the initial segment is located some distance from the cell body (>20 μm) and axon hillock (Fig. 9J–L), and that this distance is highly variable from cell to cell. With this in mind, caution should be taken in future studies in defining the initial segment, at least in GCs, strictly by distance from the cell body.

In summary, while the initial segment is generally defined by a dense complex of interacting proteins such as Na$_v$ channels, ankyrin-G, and neurofascin, we have found that a complex substructure exists within this domain where specific membrane proteins are selectively assigned. The precise juxtaposition of Na$_v$1.1 relative to Na$_v$1.6 and K$_v$1.2 channels within the IS may be an important determinant of the integrative capacity and firing characteristics of GCs and other brain neurons.

**DISCUSSION**

Na$_v$1.1 targeting and microdomain formation

The cytoskeletal linker protein ankyrin-G is a particularly important component of the IS, required for clustering of NrCAM, neurofascin-186, and Na$_v$ channels (Zhou et al., 1998), as well as KCNQ channels (Pan et al., 2006). Since ankyrin-G binds a motif conserved in all Na$_v$ channel isoforms (Lemailliet et al., 2003), it can likely anchor all three adult CNS channel subtypes (Na$_v$1.1, Na$_v$1.2, Na$_v$1.6) at sites of high-density channel clustering. In agreement with this expectation, we have seen here and in previous studies that ankyrin-G does in fact colocalize with each of the three channels within the GC axon at different times during development (Boiko et al., 2003), or at other sites in the retina (Van Wart et al., 2005). However, we have also found that within the stretch of ankyrin-G and Na$_v$ channel accumulation in the IS, two Na$_v$ channel isoforms coexist side-by-side yet spatially segregated, revealing previously unsuspected subcompartments within the IS.

If ankyrin-G does play a role in determining the localization of Na$_v$ channels, how are Na$_v$1.1 and Na$_v$1.6 channels prevented from binding to ankyrin-G in the distal and proximal portion of the IS, respectively? It is unlikely that protein levels / mass action alone can account for the differential clustering of Na$_v$ channel isoforms with ankyrin-G, because Na$_v$1.1 and Na$_v$1.6 are simultaneously localized at high density adjacent to one another in the adult IS. Also, Na$_v$1.6 channels are restricted largely to the distal region of ankyrin-G staining even before Na$_v$1.1 levels rise during development (see Fig. 8). Simultaneous colocalization of different Na$_v$ isofoms with ankyrin-G in neighboring subregions of the mature IS suggests either selective insertion/retrieval of particular channel types in the different membrane zones, or segregation of another binding partner within the IS. With respect to the former, a possible endocytic domain has been found within the Na$_v$ channel C-terminus, which was shown to modulate the surface expression of chimeras containing the Na$_v$1.2 C-terminus (Garrido et al., 2001). However, this domain influenced retrieval only from the somatodendritic region, and only for the Na$_v$1.2 isofrom. Overall, although ankyrin-G serves multiple purposes at the IS, acting as a diffusion barrier for maintaining neuronal polarity (Winckler et al., 1999; Nakada et al., 2003) and an anchor for maintaining Na$_v$ channels, the simplest explanation is that ankyrin-G is not the primary determinant for the differential distribution of Na$_v$ isofoms within the IS.

Other possible mediators of differential Na$_v$ channel targeting include the four Na$_v$ auxiliary β subunits, which modulate the cell-surface expression of Na$_v$ α subunits and can interact with proteins found at the IS, including ankyrin-G (Malhotra et al., 2002), neurofascin (Ratcliffe et al., 2001), and tenasin C and R (Srinivasan et al., 1998). However, no preferential association between specific α and β subunit isoforms has been described, nor are the various β subunits known to cluster differentially at distinct membrane domains. This raises the possibility that Na$_v$ α subunits possess additional intrinsic targeting signals, beyond those previously described, that underlie their restricted localization within the IS. It is also possible that IS microdomains are formed by a second diffusion barrier or macromolecular sieve, which could also explain our finding that K$_v$ channels, like Na$_v$1.6 channels, are present in the distal zone of the IS but kept apart from Na$_v$1.1 channels in the proximal zone. A similar selective localization of K$_v$1.2 channels to the distal but not the proximal initial segment has been reported in cortical pyramidal cells (Inda et al., 2006). This pattern is reminiscent of the arrangement of K$_v$ channels adjacent to Na$_v$ channels at developing nodes of Ranvier, before K$_v$ channels are sequestered to the juxtaparanodes by the formation of mature paranodal septate junctions (Vabnick et al., 1999). Disruption of the septate junctions allows K$_v$ channels to invade the paranodes, where they remain segregated from the nodal Na$_v$ channels, suggesting a passive barrier at this site (Dupree et al., 1999).

Paranodal axoglial interactions are critical for the transition to Na$_v$1.6 expression at nodes of Ranvier (Boiko et al., 2001; Rashband et al., 2003; Rios et al., 2003), highlighting a possible role for glial signaling in the clustering of different Na$_v$ isoforms at nodes. However, normal myelination is not required for the developmental switch from Na$_v$1.2 to Na$_v$1.6 at the IS (Boiko et al., 2003), nor is any glial contact necessary for clustering of Na$_v$ channels at the IS of neurons in vitro (Alessandri-Haber et al., 1999, 2002). Although myelinating oligodendrocytes do not enter the retina, each GC soma and proximal axon is ensheathed by Müller glial cell endfeet, and the IS beyond the endfeet receives extensive astrocytic contacts (Stone et al., 1995). These interactions with nonmyelinating glia could play a role in establishing or maintaining microdomains of ion channels within the IS.

**Functional significance of microdomains at the IS**

Because the axon initial segment is important in spike generation and propagation, the differential distribution of Na$_v$ and K$_v$ channels within the IS likely influences local excitability, either within the IS itself or in the soma. It has been reported that the initial segments of GC axons receive GABAergic contacts from amacrine cells displaced in the GC layer (Koontz, 1993). The relative location of Na$_v$ and K$_v$ channels within the IS may alter the effectiveness of such inhibitory inputs in modulating action
potential initiation, as has been suggested for the GABAergic inputs from chandelier cells onto the distal IS of cortical cells (Inda et al., 2006). In addition, heterogeneous channel distributions, combined with the morphology of the axon itself, would likely be important for back propagation of action potentials into the soma and dendrites (Fohlmeister and Miller, 1997; Sheasby and Fohlmeister, 1999). Modeling and electrophysiological studies suggest that action potentials in GCs likely arise at a “thin segment,” where the axon narrows substantially before expanding again into the axon proper (Carras et al., 1992; Fohlmeister and Miller, 1997; Sheasby and Fohlmeister, 1999). This thin segment may correspond to the region of high Na\textsubscript{1.6} density we report here in the distal IS. We suggest that the high density of Na\textsubscript{1.1} channels we have described, positioned where the axon increases in diameter from the distal thin segment to the proximal IS and axon hillock, could help to overcome an impedance mismatch for action potentials propagating back from the axon thin segment into the soma/dendrites. Thus, the accumulation of Na\textsubscript{1.1} channels in the proximal microdomain during postnatal week 3 may contribute to a developmental increase in the efficiency of action potential reproduction in the soma. It is not yet clear why Na\textsubscript{1.1} channels, and not some other isoform, are selectively expressed in the proximal IS in GCs. However, given the many potential roles of channel microdomains in the IS, these novel domains of specialized Na\textsubscript{a} and K\textsubscript{o} channel localization are likely to be important determinants of the integrative capacity and intrinsic firing capabilities of mammalian neurons.

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LITERATURE CITED


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AXON INITIAL SEGMENT ION CHANNEL SEGREGATION


