Voltage-gated sodium channel α-subunits Na$_v$1.1, Na$_v$1.2, and Na$_v$1.6 in the distal mammalian retina

Deb K. Mojumder$^1$, Laura J. Frishman$^1$, Deborah C. Otteson$^1$, David M. Sherry$^{1,2}$

$^1$University of Houston College of Optometry; $^2$University of Houston, College of Pharmacy, Department of Pharmacological and Pharmaceutical Sciences, Houston, TX

Purpose: Recent studies indicate the presence of functional voltage-gated sodium channels (Na$_v$ channels) in the distal retina in several species. This study examined the distribution of Na$_v$ channels in the outer plexiform layer (OPL) of rat, mouse, and rabbit retinas.

Methods: Immunohistochemical and electroretinographic approaches were used.

Results: Antibodies specific for Na$_v$1 α-subunits appropriately labeled retinal ganglion cells, their axons, and amacrine cells that are known to have tetrodotoxin (TTX)-sensitive Na$_v$ channels. Pan-Na$_v$, Na$_v$1.2, and Na$_v$1.6 labeling was found in horizontal cells and processes in all three species. Weaker Na$_v$1.1 labeling was observed in rodent horizontal cells, but some rabbit horizontal cells and processes were prominently labeled. Additional labeling for Na$_v$1.1, Na$_v$1.2, and Na$_v$1.6 that was not attributable to horizontal cells was also present in the OPL. Much of this labeling was diffusely distributed. Some of the additional Na$_v$1.1 labeling was associated with photoreceptor terminals. By exclusion using photoreceptor and horizontal cell markers, some of this labeling could have been associated with bipolar cell dendrites, although colocalization was not directly established due to the diffuse nature of the labeling and limits on anatomical resolution. No Na$_v$1 α-subunit labeling was observed in bipolar cell bodies. Testing for functional Na$_v$ channels was performed by recording full field flash electroretinograms from dark-adapted rats before and after intravitreal injections of TTX, 6-cyano-7-nitroquininaline-2,3-dione (CNQX), or TTX+CNQX. TTX and CNQX+TTX, but not CNQX alone, greatly attenuated the dark-adapted cone-driven b-waves.

Conclusions: Horizontal cells from three different mammalian retinas showed prominent labeling for Na$_v$1 α-subunits. Some additional diffuse Na$_v$1 α-subunit labeling in the OPL was associated with photoreceptor terminals. Na$_v$1 α-subunit labeling also may have been present on bipolar cell dendrites, although it was not possible to establish this localization unequivocally by immunostaining. However, cone-driven b-waves in rats were reduced in maximum amplitude by TTX in the presence of CNQX which blocks synaptic input to horizontal, amacrine, and ganglion cells. This finding is consistent with TTX effects on the b-wave being due to blockade of Na$_v$ channels in cone bipolar cell dendrites in the OPL. The role of Na$_v$ channels in horizontal cells remains to be determined.

Voltage-gated Na$^+$ channels (Na$_v$ channels) are members of a large superfamily of ion channel proteins in membranes of cells that are characteristically involved in electrical signaling [1,2]. The opening of these channels in response to membrane depolarization causes an influx of Na$^+$ ions to the cell that is essential for initiating and propagating sodium-dependent action potentials in neurons and muscle cells [3]. However, the functional role of Na$_v$ channels is not restricted to mediating rapid changes in membrane polarization and action potentials. Recent studies demonstrate that Na$_v$ channels have wider physiological and developmental roles. For example, Na$_v$ channels can load cells with Na$^+$ ions during slow inactivation and thereby modulate intracellular Na$^+$ homeostasis [4]. Na$_v$ channel interactions with tenascin and receptor protein tyrosine phosphatase-suggest these channels also can participate in cell-cell or cell-matrix interactions (for review see [5]). The absence of a specific Na$_v$ α-subunit (Na$_v$1.6) during development leads to deficits in photoreceptor function as evidenced by reduced amplitudes of the electroretinogram (ERG) waves originating from photoreceptors as well as retinal postreceptorial neurons [6].

Na$_v$ channels are composed of a large (about 260 kDa) pore-forming α-subunit (Figure 1A) associated with one or more smaller α-subunits [1,2,7]. The Na$_v$ α-subunit is obligatory for channel function [1,2,5]. There are ten known Na$_v$ α-subunit isoforms (designated Na$_v$1.1 through Na$_v$1.9 and Na$_v$), each encoded by a separate gene [1,8,9]; (gene nomenclature is provided in Table 1). Na$_v$1.1, Na$_v$1.2, Na$_v$1.3, and Na$_v$1.6 are highly expressed in the central nervous system, including the retina [8-15]. These Na$_v$1 α-subunits are all sensitive to tetrodotoxin (TTX) blockade, a property that has been used to demonstrate the presence of functional Na$_v$ channels in various retinal neurons (summarized in Figure 1B). The TTX sensitivity of retinal ganglion cell action potentials is well known.
Several subtypes of amacrine cells, the other major third order retinal neurons, also possess TTX-sensitive Na+ currents (e.g., AII, A17, A18, and starburst amacrine cells); TTX-sensitive Na+ currents also have been reported in other retinal neurons. These currents, however, are not as prominent as those of amacrine and ganglion cells, and their functional significance is not yet fully understood. Recent studies have identified voltage-gated, TTX-sensitive Na+ currents in classes of bipolar cells from a variety of vertebrate retinas: rat [24], goldfish [25], salamander [26], ground squirrel [27], and most recently, human [28,29]. In the goldfish, Na+ channel immunoreactivity was localized to cone bipolar cells of both ON and OFF types [25]. In rat retina, TTX-sensitive voltage-gated Na+ currents were also observed in subsets of ON and OFF cone bipolar cells, but not in rod bipolar cells. TTX-sensitive currents also have been detected in isolated retinal horizontal cells from several species, but have never been identified in intact retinal recordings: catfish [30], white perch [31], cat: A-type horizontal cells [32], rabbit: 81% of the A-type and 90% of the B-type cells [33], and mouse [34]. Voltage-gated Na+ channels also have been found in human rods and cones where their precise role is still unclear [35,36]. Together, these studies suggest a potentially broader role for Na+ channels in the retina than has been appreciated to date.

Detailed knowledge of the distribution of the Na(α)-subunits is important to interpreting the various functional roles of Naα channels and the different Naα-α-subunits in physiology and other processes in the retina. To better understand Naα channels in the mammalian retina, we have examined the distribution of Naα channel α-subunits in the retina using immunohistochemical and ERG approaches, with particular focus on expression of Na1.1, Na1.2 and Na1.6 by second-order neurons. To assess similarities and differences across different mammalian species, we performed experiments that used the retinas of rat, mouse, and, to a lesser extent, rabbit. Each Na1 α-subunit showed a distinctive distribution in the retina. As expected, labeling was present in third-order retinal ganglion cells and amacrine cells. However, labeling for each Na1 α-subunit also was found in horizontal cells and their processes and elsewhere in the outer plexiform layer (OPL). Application of TTX reduced the amplitude of the dark-adapted...
cone-driven b-wave of the ERG, an ERG component known to arise primarily from light-evoked responses of ON cone bipolar cells.

**METHODS**

**Animals for immunohistochemical studies:** Studies were performed using nine adult Brown Norway rats (*Rattus norvegicus*, Charles River Laboratories, Wilmington, MA; six weeks-six months of age), six mice (*Mus musculus*, C57/BL6; Simonsen Laboratories, Gilroy, CA; six weeks-six months of age), and four rabbits (*Oryctolagus cuniculus*, New Zealand White; Myrtles Rabbitry Incorporated, Thompsons Station, TN; six to nine months of age). All animals were maintained on a 12:12 h dark cycle with food and water available ad libitum. All animals were light-adapted at the time of euthanasia. Animals for immunohistochemical studies were anesthetized using an intramuscular injection of ketamine (Vedco Inc., St Joseph, MO; rats, 86 mg/kg; mice, 70 mg/kg; rabbits, 35 mg/kg, obtained from) and xylazine (Vedco Inc.; rats, 6.5 mg/kg; mice, 7 mg/kg; rabbits, 5 mg/kg) and then were euthanized by an intramuscular injection of a lethal dose of pentobarbital (Ft. Dodge Laboratories, Inc., Ft. Dodge, IA; 150-200 mg/kg). All animal procedures and protocols conformed to US Public Health Service and Institute for Laboratory Animal Research guidelines and were approved by the University of Houston Institutional Animal Care and Use Committee.

**Tissue preparation for immunohistochemistry:** To ensure that immunolabeling patterns obtained with each antibody were consistent and accurate we performed immunolabeling in multiple species and in two different tissue preparations. Tissue fixation regimes were optimized accordingly for each preparation and antibody. The effects of fixation and tissue preparation on labeling patterns is discussed further in Results. Following euthanasia, the eyes used for frozen sections were rapidly excised from the head, leaving a portion of the superior rectus muscle to indicate the superior pole of the globe. The corneas were slit with a razor blade, the lens was removed, and eyes were immersed in 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) for 15 min to 2 h at 4 °C. Following fixation, eyes were rinsed in phosphate buffered saline (PBS; pH 7.4) and cryoprotected overnight in 30% sucrose/PBS overnight. The next day, the eyes were embedded in Tissue-Tek OCT Compound (Tissue-Tek, Hatfield, PA) and fast frozen in liquid nitrogen. Vertical cryostat sections of 10-12 μm thickness were taken along the vertical meridian of the eyecup and collected onto Superfrost/Plus microscope slides (Fisherbrand, Fisher Scientific, Pittsburgh, PA). Sections were stored at -20 °C until use.

Retinal wholemounts were prepared by excising the eye and removing the cornea and lens as described in the previous paragraph. The neural retina was rapidly isolated free of the retinal pigmented epithelium in cold Ames’ medium (4 °C, pH 7.4) and then immediately fixed lightly in 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) for 5 min at 4 °C. The vitreous humor was removed, and relaxing cuts were made in the retinal margin to allow the retina to flatten. The retina was rinsed in PBS and immunolabeled free-floating.

**Antibodies and antisera:** The details of all primary antibodies are presented in Table 2. Primary antibodies against NaV 1 α-subunits were mouse monoclonal antibodies raised against relatively short amino acid epitopes based on rat NaV 1 α-subunit sequences and have been used previously for studies of NaV 1 α-subunits in the retina and optic nerve [13,14]. Figure 1A provides a schematic representation of the structure of the NaV 1 α-subunit and shows the locations of the amino acid epitopes used to raise each NaV 1 α-subunit antibody. The specificity of these antisera has been extensively characterized previously using immunoblotting, ELISA and preabsorption assays and immunolabeling of cells and tissues known to contain or lack specific NaV 1 α-subunits [13,14,37] (Dr. James Trimmer, personal communication). A pan-specific antibody that recognizes all NaV 1 α-subunits (Pan-NaV) was raised against a synthetic peptide corresponding to a region of the intracellular III-IV loop conserved in all NaV 1 family α-subunits [37] (Figure 1A for a schematic representation of the structure of the NaV 1 α-subunit). Anti-NaV 1.1 was raised against a fusion protein corresponding to amino acids 1929-2009 of the C-terminal domain of rat NaV 1 [14]. Anti-NaV 1.2 was raised against a fusion protein corresponding to amino acids 1882-2005 of the C-terminal domain of rat NaV 1.2 (Dr. James Trimmer, personal communication). The antibody specific for NaV 1.6 was raised against a synthetic peptide corresponding to amino acids 459-476 of the intracellular interdomain loop I-II of rat NaV 1.6 [13]. Immunoblotting of crude membrane homogenates from mouse retina and brain as described previously [38] confirmed labeling of a single band of appropriate molecular mass by the NaV 1 α-subunit antibodies. A dilution series for each NaV 1 α-subunit antibody was performed to determine the optimal dilution for immunolabeling. The specificity of the NaV 1 α-subunit antibodies and epitopes is assessed further in Results. A panel of

### Table 1. Conservation of peptide immunogens used to generate antibodies against NaV 1.1, NaV 1.2, and NaV 1.6 across rat NaV 1 α-subunit family proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Gene name</th>
<th>Accession number</th>
<th>NaV 1.1</th>
<th>NaV 1.2</th>
<th>NaV 1.6</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaV 1.1</td>
<td>Scn1a</td>
<td>NP_110502</td>
<td>100.0</td>
<td>59.2</td>
<td>38.9</td>
</tr>
<tr>
<td>NaV 1.2</td>
<td>Scn2a</td>
<td>NP_036779</td>
<td>50.6</td>
<td>100.0</td>
<td>50.0</td>
</tr>
<tr>
<td>NaV 1.3</td>
<td>Scn3a</td>
<td>NP_037251</td>
<td>48.1</td>
<td>73.4</td>
<td>33.3</td>
</tr>
<tr>
<td>NaV 1.4</td>
<td>Scn4a</td>
<td>NP_037310</td>
<td>21.0</td>
<td>40.0</td>
<td>22.2</td>
</tr>
<tr>
<td>NaV 1.5</td>
<td>Scn5a</td>
<td>NP_037257</td>
<td>25.3</td>
<td>42.0</td>
<td>33.3</td>
</tr>
<tr>
<td>NaV 1.6</td>
<td>Scn8a</td>
<td>NP_062139</td>
<td>18.5</td>
<td>44.4</td>
<td>100.0</td>
</tr>
<tr>
<td>NaV 1.7</td>
<td>Scn9a</td>
<td>NP_579823</td>
<td>38.3</td>
<td>68.5</td>
<td>27.8</td>
</tr>
<tr>
<td>NaV 1.8</td>
<td>Scn10a</td>
<td>NP_058943</td>
<td>16.5</td>
<td>32.3</td>
<td>27.8</td>
</tr>
</tbody>
</table>

Table showing the protein names, gene names and accession numbers for members of the rat NaV 1 (α-subunit) family of sodium channels. To determine the percent amino acid identity, the amino acid sequences of the three peptide immunogens used to generate the antibodies against NaV 1.1, NaV 1.2, and NaV 1.6 were aligned pairwise with the full length sequence of each of the NaV 1 proteins using AligX.
additional primary antibodies and antisera directed against a number of well-characterized cell- and synapse-specific markers also were used (see Table 2).

Immunoblotting of crude membrane homogenates from mouse retina and brain was used to confirm labeling of a single band of appropriate mass by the Na\(\alpha_1\)-subunit antibodies. For immunoblotting, neural retinas were isolated, or brain removed, and crude membrane homogenates were prepared by sonication and centrifugation [38]. Proteins were loaded onto 10% polyacrylamide gels and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred to polyvinylidene difluoride (PVDF) membranes for visualization. PVDF membranes were blocked using 2% normal goat serum in 0.1 M tris-buffered saline +0.05% tween-20 (TBS-Tween, pH 7.2), and then incubated overnight in primary antibody against Na\(\alpha_1.1\), Na\(\alpha_1.2\), or Na\(\alpha_1.6\) at 4 °C. Blots were rinsed in TBS-Tween followed by TBS alone and incubated for 2 h at room temperature in goat anti-mouse-horseradish peroxidase (1:2000). Blots were rinsed in TBS, and labeled protein bands were visualized using an enhanced chemiluminescence kit according to the manufacturer’s instructions (Santa Cruz Biotechnology, Santa Cruz, CA). All antibodies were diluted in 2% normal goat serum in TBS-Tween.

Binding of primary antibodies was detected using fluorescent secondary antisera. Secondary antisera were raised in goat and were specific for mouse, rabbit or sheep, immunoglobulins and were conjugated to Cy3 or Cy5 (diluted 1:200; Jackson ImmunoResearch Laboratories, West Grove, PA) or conjugated to AlexaFluor488 or AlexaFluor 546 (diluted 1:200; Molecular Probes, Eugene, OR). Double labeling was performed with an appropriate combination of secondary antibodies conjugated to different fluorescent tags.

**Immunolabeling:** In most experiments, immunolabeling of frozen sections employed immunofluorescent methods [38-40]. Briefly, frozen sections were thawed, immersed in 4% formaldehyde for 10-15 min at room temperature to improve adherence, and rinsed in deionized water. They were then treated with 1-2% NaBH\(_4\) for 1-2 min at room temperature to reduce non-specific autofluorescence, rinsed in deionized water, and exchanged to PBS. Non-specific labeling was blocked with 10% normal goat serum + 5% bovine serum albumin + 0.5-1% fish gelatin + 0.1-0.5% Triton X-100 in PBS (blocker). Excess blocker was removed, and primary antibody was applied for 2 days at 4 °C. For double labeling experiments, a combination of primary antibodies was applied simultaneously. Sections were rinsed, blocked for 30 min at room temperature, and secondary antibody was applied for 1 h at room temperature. For double-labeling experiments, an appropriate combination of secondary antisera was applied simultaneously. Sections were rinsed and coverslipped in a fade-retardant mounting medium (either Vectashield, Vector Labs, Burlingame, CA, or Prolong Gold, Molecular Probes, Eugene, OR) and examined in the microscope. Sections processed substituting normal rabbit serum for rabbit polyclonal primary antisera, non-specific mouse IgGs, or in the absence of primary antibodies showed no labeling, as appropriate. All primary and secondary antibodies were diluted in blocker.

To obtain consistent immunolabeling in frozen sections using the Pan-Na\(\alpha_1\) antibody, an antigen retrieval step was required [41]. For immunolabeling of frozen sections using the Pan-Na\(\alpha_1\) antibody, thawed sections were heated to 90 °C in PBS at pH 7.4-8.3. They were allowed to cool to room temperature and then were processed as described in the previous paragraph for frozen sections, without any further exposure of the sections to fixatives (See Results for further discussion).

Rat retinal wholemounts were immunolabeled free-floating. Wholemounts were treated with 1-2% NaBH\(_4\) for 1-2 min, rinsed in deionized water followed by PBS and incubated in blocker solution for 1 h at room temperature to block non-specific labeling. Retinas were incubated in primary antibody for 5 days at 4 °C. They were then rinsed in PBS for 2 h at room temperature and incubated free-floating in secondary antibody at room temperature for 1 h. Retinas were rinsed in PBS for 2 h at room temperature, flattened onto microscope slides with the ganglion cell side up, coverslipped with Prolong Gold and examined in the confocal microscope.

**Imaging:** Images were acquired using a Leica TCS SP2 confocal microscope and LCS software (Leica Microsystems, Exton, PA). Images were captured using 20x (NA, 0.7), 63x oil (NA, 1.32), or 63x water immersion (NA, 1.2) objective lenses.

### Table 2. Primary Antibodies and Antisera

<table>
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<tr>
<th>Antigen and accession number</th>
<th>Host</th>
<th>Dilution</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na(\alpha_1)-subunit, pan-specific (Pan-Na(\alpha_1))</td>
<td>Mouse</td>
<td>1:500-1:1000</td>
<td>Sigma-Aldrich, St. Louis, MO (Catalog number S8609; Clone 68/35)</td>
<td>Rasband et al., 1999 [37]</td>
</tr>
<tr>
<td>Na(\alpha_1.1)-subunit (NP_110502)</td>
<td>Mouse</td>
<td>1:500-1:1000</td>
<td>G. Matthews (SUNY-Stony Brook), J. Trimmer (University of California Davis); Antibodies, Inc./NeuroMab, Davis, California (Catalog number 75-023; Clone 87A/31)</td>
<td>Van Wart et al., 2003 [14]</td>
</tr>
<tr>
<td>Na(\alpha_1.2)-subunit (NP_056779)</td>
<td>Mouse</td>
<td>1:500-1:1000</td>
<td>J. Trimmer (University of California Davis); Antibodies, Inc./NeuroMab, Davis, California (Catalog number 75-024; Clone K69/3)</td>
<td>Van Wart et al., 2003 [14]</td>
</tr>
<tr>
<td>Na(\alpha_1.6)-subunit (NP_062339)</td>
<td>Mouse</td>
<td>1:500-1:1000</td>
<td>J. Trimmer (University of California Davis); Antibodies, Inc./NeuroMab, Davis, CA (Catalog number 75-026; Clone K87A/15)</td>
<td>Boiko et al., 2003 [13]</td>
</tr>
<tr>
<td>Calbindin-28 KDa</td>
<td>Rabbit</td>
<td>1:100-1:5000</td>
<td>SWANT, Bellinzona, Switzerland (Catalog number CB30)</td>
<td>-</td>
</tr>
<tr>
<td>αGLuR6</td>
<td>Rabbit</td>
<td>1:500</td>
<td>Antibodies Inc., Davis, California (Catalog number 61-181)</td>
<td>-</td>
</tr>
<tr>
<td>Ch e-10</td>
<td>Sheep</td>
<td>1:50</td>
<td>Exelixis Biologics, Inc., Watertown, Massachusetts (Catalog number XI180F)</td>
<td>-</td>
</tr>
<tr>
<td>Protein Kinase C, α-subunit (PKC)</td>
<td>Rabbit</td>
<td>1:100</td>
<td>Calbiochem, EMD Biosciences, Inc., San Diego, California (Catalog number 539601)</td>
<td>-</td>
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<tr>
<td>Vesicular glutamate transporter 1 (VGluT1)</td>
<td>Rabbit</td>
<td>1:500-1:1000</td>
<td>Synaptic Systems, Göttingen, Germany (Catalog number 135-302)</td>
<td>Sherry et al., 2003 [39]</td>
</tr>
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</table>

Specificity, host, dilution, and source information for the primary antibodies and antisera used in these studies.
lenses. Stacks of serial optical sections ranging from 1.5-6 µm thickness in the Z-plane were collected using step sizes of 0.15-0.6 µm thickness. Assessment of labeling in single optical planes was performed using 63x objectives to achieve a maximal Z-plane resolution of approximately 0.3 µm. Images in each fluorescent channel were collected sequentially with laser power and detector sensitivity adjusted to prevent bleedthrough of signals between fluorescence channels. The absence of bleedthrough between channels was confirmed in sections treated with a single primary antibody and a combination of secondary antibodies to verify that only the channel corresponding to the primary antibody showed labeling.

Figures were prepared using Adobe Photoshop 6.0 (Adobe Systems, Inc., Mountain View, CA). Image scale was calibrated and image brightness and contrast were adjusted to highlight specific labeling, if needed.

**Bioinformatics and sequence analysis:** Sequences of orthologous Na\(_{\alpha}\) x subunit proteins from *Rattus norvegicus* (rat), *Mus musculus* (mouse), *Canis familiaris* (dog), *Bos taurus* (cow), and *Homo sapiens* (human) were obtained from the NCBI protein database. Amino acid sequences for rat-specific peptides used as immunogens to generate the monoclonal antibodies against Na\(_{\alpha}\)1.1, Na\(_{\alpha}\)1.2 and Na\(_{\alpha}\)1.6 were obtained from the NeuroMab (Davis, CA). Protein sequences for *Oryctolagus cuniculus* (rabbit) were obtained by employing full length rat Na\(_{\alpha}\)x cDNA or protein sequences as queries for tBlastn or tBlastx searches of the translations of the predicted transcripts (Ab-initio cDNAs/Genscan/SNAP) in the Ensembl rabbit Genome Database (v42.1-Dec2006). Amino acid sequences were aligned using AlignX software (Vector NTI Advance 10.1; Invitrogen Corporation, Carlsbad, CA), using the rat peptides as the consensus sequence. For proteins within a species that were represented by more than one sequence in the database, multiple protein sequences were aligned to determine the consensus sequence. Rat peptide sequences were also used as a query for Basic Local Alignment Search Tool for Proteins (BlastP) and tBlastn searches against the protein, non-redundant and expressed sequence tag (EST) databases (NCBI), limiting search to mammalian sequences [42].

**Electroretinograms:** Rats were dark-adapted overnight and prepared for recording under red illumination (LED, >620 nm). The individual drug experiments were performed on 11 rats of similar ages. They were initially anesthetized with an intramuscular injection of 86 mg/kg ketamine and 6.5 mg/kg xylazine into the hamstring muscles. For recording, animals were placed on a thermostatically controlled electrical heating blanket (CWE, Inc., Ardmore, PA) and anesthesia was maintained at 36-37 °C. Animals generally recovered after the sessions; however, after they were given intravitreal injections in both eyes, they were killed using 100 mg/kg, intraperitoneal injection of sodium pentobarbital. Pupils were dilated to 5 mm in diameter with topical 0.5% atropine and 2.5% phenylephrine. A drop of proparacaine hydrochloride (0.5%) was used for corneal anesthesia. The animal’s head was held steady, in order to reduce noise originating from respiratory and other movements, using an aluminum head holder with a hole for the upper incisors. This head holder also served as the ground.

Recording sessions lasted 4 to 8 h. ERGs were recorded differentially between Dawson, Trick, and Litzkow (DTL) fiber electrodes [43], moistened with saline and placed on both eyes. The stimulated eye was covered with a clear contact lens heat monitored from ACLAR (Ted Pella, Inc., Redding, CA), and the control eye was covered with an opaque plastic contact lens. Both lenses were placed over a cover of 1% methylcellulose sodium (Celluvisc, Allergan Inc., Irvine, CA). Signals were amplified (DC to 300 Hz), digitized at 1 KHz with a resolution of 2 µV, and sent to the computer for averaging, display, storage, and subsequent analysis.

ERGs were recorded using brief full field flashes (λ\(_{\text{max}}\): 462 nm; -5.8 to 1.9 log sc td s) under fully dark-adapted conditions, in most cases, following a conditioning flash, to suppress rod-driven activity as described in Results. Intervals between flashes were adjusted so that the response returned to baseline before another stimulus was presented. A digital 60 Hz notch filter was applied offline.

Stimulus calibration was performed by measuring scotopic luminance with a scotopically corrected (for humans) photometer (International Light, model IL1700, Newburyport, MA) [44]. The LEDs in the stimulator were activated with a 1 kHz train of current pulses each having a duration of 100 µs and the luminance was measured. The luminance-time product for each of the single flash stimuli was expressed in sc cd s m\(^{-2}\) and converted to sc td s by multiplying the product by the pupil area.

**Intravitreal injections:** Intravitreal injections were performed under a binocular operating microscope (magnification: 10x), under dim red illumination (>620 nm). A small hole (about 0.5 to 1 mm) was punctured behind the limbus using a 27 gauge needle to allow penetration at 45 °C of the underlying sclera, choroid, and retina by a fine glass micropipette (tip about 20 µm; diameter: 1 mm) fixed on a 10 µl Hamilton microsyringe (Hamilton Company, Reno, NV) for injecting pharmacological agents. A single about 2 µl injection was given slowly over 1 min in the vitreous. The intravitreal drug concentrations used were about 3 µM TTX and 200 µM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), based on an estimated vitreous chamber volume of 40 µl in the rat [45,46]. After injection, the ERG was monitored until no further change in response was seen, generally about one hour, before taking data.

**Electroretinogram analysis:** ERG amplitudes before and after drug administration were measured at a fixed time of 50 ms after the stimulus flash, corresponding to the time of peak of the cone-driven b-wave and plotted against the flash energy to generate stimulus response curves.

**RESULTS**

**Na\(_{\alpha}\) x-subunit epitope sequence analysis and specificity:** The anti-Na\(_{\alpha}\)1 x-subunit-specific monoclonal antibodies used in this study were generated using rat-specific peptides...
located within C-terminus (Na\textsubscript{v} 1.1 and Na\textsubscript{v} 1.2) or a non-conserved region near the sixth transmembrane domain (Na\textsubscript{v} 1.6; Figure 1A). Because we were also using a more distantly related species (rabbit) that was not used in previous studies, we wanted to predict whether these monoclonal antibodies would recognize orthologous Na\textsubscript{v} 1 \(\alpha\)-subunit proteins. To this end, we used a bioinformatics approach to examine sequence conservation of these peptide immunogens in Na\textsubscript{v} 1.1, Na\textsubscript{v} 1.2 and Na\textsubscript{v} 1.6 across mammalian species (Table 3). To identify the most highly conserved sequences, we used AlignX software to do pair-wise alignments of each of the peptides with the orthologous Na\textsubscript{v} 1 \(\alpha\)-subunit proteins from each species and calculated the percent amino acid identity. We found that the Na\textsubscript{v} 1.1, Na\textsubscript{v} 1.2, and Na\textsubscript{v} 1.6 peptide immunogens were each highly conserved among rat, mouse, rabbit, dog, bovine, and human proteins (Figure 2). Na\textsubscript{v} 1.1 and Na\textsubscript{v} 1.2 peptides were 94–100% identical with their respective orthologs across all species and the shorter Na\textsubscript{v} 1.6 peptide was 100% identical between rat and mouse and 88–94% between rat and non-redundant Na\textsubscript{v} 1.6 sequences. Based on this high level of sequence identity, each of the monoclonal antibodies was predicted to recognize their orthologous proteins in mammals.

The first 36 amino acids of the Na\textsubscript{v} 1.1 and Na\textsubscript{v} 1.2 peptides differed at only three residues (Figure 2B); therefore, we did pair-wise alignments of each peptide immunogen with all members of the Na\textsubscript{v} 1 family in rat to determine the extent of this sequence similarity (Table 1). These analyses revealed that over its entire length, the Na\textsubscript{v} 1.2 peptide immunogen, respectively shared 64%, 73%, and 71% amino acid identity with rat Na\textsubscript{v} 1.1, Na\textsubscript{v} 1.3, and Na\textsubscript{v} 1.7. Although this level of sequence conservation raises the possibility that the anti-Na\textsubscript{v} 1.2 monoclonal antibody could potentially crossreact with these other Na\textsubscript{v} 1 family proteins, this is unlikely. If the anti-Na\textsubscript{v} 1.2 antibody recognizes a portion of the epitope that is highly conserved across these Na\textsubscript{v} 1 family members, then the Na\textsubscript{v} 1.2 antibody would label all structures or cells that express Na\textsubscript{v} 1.1, Na\textsubscript{v} 1.3, or Na\textsubscript{v} 1.7. However, this is not the case, as the Na\textsubscript{v} 1.2 antibody produces immunolabeling patterns distinct from other Na\textsubscript{v} 1 family members. The Na\textsubscript{v} 1.2 immunolabeling pattern is distinct from the Na\textsubscript{v} 1.1 pattern in rat cerebellar granule cells [47] and axon initial segments of Purkinje neurons in Na\textsubscript{v} 1.6 null mice [15] and differential labeling by anti-Na\textsubscript{v} 1.2 and Na\textsubscript{v} 1.3 antibodies has been reported in the myenteric nervous system [48]. Therefore, despite the modest sequence conservation of the amino acid sequence in the peptide used to raise the Na\textsubscript{v} 1.2 antibody, the antibody is unlikely to cross react with other Na\textsubscript{v} 1 \(\alpha\)-subunit family members. Direct testing of the Na\textsubscript{v} 1.2 antibody shows no crossreactivity with Na\textsubscript{v} 1.1, Na\textsubscript{v} 1.3, or Na\textsubscript{v} 1.6 (Dr. James Trimmer, personal communication).

As an additional evaluation of the specificity of the anti-Na\textsubscript{v} 1 \(\alpha\)-subunit antibodies, we used peptide immunogen sequences for BlastP and tBlastn searches of the SwissProt, non-redundant and EST databases (NCBI) to determine if they shared sequence identity with other known or predicted mammalian proteins. In Blast searches, each match is scored by an E-value that indicates the number of matches predicted to occur by chance alone, therefore, we examined all matches with an E value of less than 1 [42]. For each of the searches, all matches identified were for Na\textsubscript{v} 1 family proteins. Therefore, no other mammalian proteins were predicted to contain the peptide epitopes used to generate these monoclonal antibodies, consistent with our conclusions that they were specific for the different Na\textsubscript{v} 1 \(\alpha\)-subunit proteins.

**Localization of Na\textsubscript{v} 1 \(\alpha\)-subunit:** We first assessed the overall distribution of Na\textsubscript{v} 1 \(\alpha\)-subunits in the retina in comparison to the distribution of Na\textsubscript{v} channels reported in previous physiological and anatomical studies. These studies have clearly established the presence of Na\textsubscript{v} 1 \(\alpha\)-subunits in third-order amacrine and ganglion cells, as discussed in the Introduction. Each Na\textsubscript{v} 1 \(\alpha\)-subunit antibody used in these studies produced the expected labeling of amacrine and ganglion cells. In addition, each Na\textsubscript{v} 1 \(\alpha\)-subunit antibody produced a characteristic pattern of labeling that included cells and processes in the distal retina, extending the findings of the previous studies.

**Pan-Na\textsubscript{v} labeling:** Labeling using the Pan-Na\textsubscript{v} antibody revealed a characteristic pattern of Na\textsubscript{v} 1 \(\alpha\)-subunit distribution that was similar in rat and mouse retina (Figure 3, rat retina shown). In vertical cryosections of retina, Pan-Na\textsubscript{v} labeling was present in ganglion cell bodies and axons and in processes in the inner plexiform layer (IPL), consistent with previous studies (Figure 3A,B). Two distinct sets of cell bod-

### Table 3. Na\textsubscript{v} 1 amino acid sequences used for sequence analysis

<table>
<thead>
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<th>Protein gene</th>
<th>Na\textsubscript{v} 1.1 Scn1a</th>
<th>Na\textsubscript{v} 1.2 Scn2a</th>
<th>Na\textsubscript{v} 1.6 Scn8a</th>
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<td>1639</td>
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<td>XP_535941</td>
<td>2009</td>
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<td>Bos taurus</td>
<td>XP_879829</td>
<td>2009</td>
<td>XP_879182</td>
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<tr>
<td>Homo sapiens</td>
<td>NP_008851</td>
<td>1998</td>
<td>NP_001035232</td>
</tr>
</tbody>
</table>

RefSeq Protein sequences were obtained from NCBI protein database, publicly available at NCBI. Predicted transcripts of *Oryctolagus cuniculus* (rabbit) were obtained using BlastP search of rat and bovine sequences against the low coverage rabbit genome database; Dec2006, publicly available at Ensembl. In this table aa means amino acids.
ies in the inner nuclear layer (INL) were also labeled. One set of cells corresponded to amacrine cells located near the border of the IPL and INL. The second set consisted of large flat cells at the distal edge of the INL that corresponded to horizontal cells (see next section). Little Pan-Na<sub>a</sub> immunoreactivity was observed in the mid-INL, where the cell bodies of bipolar and Müller glial cells reside, or in the outer nuclear layer (ONL), where the photoreceptors reside. Relatively little Pan-Na<sub>a</sub> labeling was present in the IPL or outer plexiform layer (OPL) in frozen sections. However, discretely labeled processes similar to the “morphologically distinct processes” described previously [14] were observed.

Labeling using the Pan-Na<sub>a</sub> antibody was highly sensitive to fixation and processing of the tissue and this was particularly labile in the plexiform layers. For frozen sections of retina that were fixed for more than 15 min total, antigen retrieval procedures were required to obtain Pan-Na<sub>a</sub> labeling. In contrast, wholemounted retinas that were only lightly fixed (5 min) and never subjected to freezing did not require antigen retrieval for immunostaining with the Pan-Na<sub>a</sub> antibody (Figure 3C-H, single optical planes focused at different retinal levels shown). Immunolabeling of lightly fixed rat retinal wholemounts confirmed the pattern of Na<sub>a</sub>,<sub>1</sub> α-subunit distribution observed in frozen sections and revealed additional,
fixation-sensitive immunoreactivity that was not detected in frozen sections. Pan-Na\textsubscript{v} labeling was found in the cell bodies of ganglion, amacrine, and horizontal cells, similar to labeling seen in vertical frozen sections. The identity of these cell types was confirmed by double labeling for Pan-Na\textsubscript{v} and the markers calbindin and calretinin which label ganglion, amacrine and horizontal cells (data not shown). Ganglion cell axons and axon initial segments showed strong Pan-Na\textsubscript{v} labeling, as expected. Much more pronounced Pan-Na\textsubscript{v} labeling was present in the plexiform layers in wholemounts than in frozen sections. In the OPL of retinal wholemounts, small puncta of Pan-Na\textsubscript{v} labeling were embedded on a background of more diffuse labeling. The IPL of retinal wholemounts showed abundant diffuse Pan-Na\textsubscript{v} labeling at all levels in addition to intensely labeled varicose processes similar to the morphologically distinct processes described previously. Photoreceptor

Figure 3. Pan-Na\textsubscript{v} labeling in the rat retina. A-B: Pan-Na\textsubscript{v} immunolabeling in a shown in a projection of confocal images through a vertical cryosection (projection of 44 optical planes; 6.27 \( \mu \)m total thickness). A: Differential interference contrast (DIC) image showing retinal layers. B: Pan-Na\textsubscript{v} immunolabeling was present in the cell bodies of amacrine cells (A) in the proximal inner nuclear layer (INL) and putative horizontal cells (H) at border of the INL and the outer plexiform layer (OPL). Labeling was also present in large, morphologically distinct processes (arrows) in the inner plexiform layer (IPL). Cell bodies of ganglion cells (G) and axons (arrowheads) also showed labeling. Photoreceptor cell bodies in the outer nuclear layer (ONL) did not show labeling. C-H: Pan-Na\textsubscript{v} immunolabeling at different levels in retinal wholemount is shown (in single optical sections). C: At the level of the ONL/OPL border only diffuse labeling was present. D: At the OPL/INL border, cell bodies and processes of putative horizontal cells (H) and processes showed Pan-Na\textsubscript{v} labeling. Additional diffuse Pan-Na\textsubscript{v} labeling also was present. E: The cell bodies of bipolar and Müller cells in the mid-INL did not show Pan-Na\textsubscript{v} labeling. F: Many amacrine cells (A) at the INL/IPL border show Pan-Na\textsubscript{v} labeling. Strong Pan-Na\textsubscript{v} labeling was present in morphologically distinct processes coursing in the distal IPL (arrows). Substantial diffuse Pan-Na\textsubscript{v} labeling was associated with other processes in the distal IPL. G: Deeper in the IPL, a similar distribution of strongly labeled processes (arrows) and diffuse labeling was evident. H: Pan-Na\textsubscript{v} labeling at the border of the ganglion cell layer (GCL) and nerve fiber layer (NFL) is shown. Many cells in the GCL show Pan-Na\textsubscript{v} labeling, including a prominently labeled set of large ganglion cells (G). Ganglion cell axon bundles (NF) in the nerve fiber layer (NFL) also showed strong Pan-Na\textsubscript{v} labeling. Structures similar in appearance to axon initial segments also showed strong labeling (arrows). Scale bars equal 20 \( \mu \)m for all panels.
cell bodies in the ONL and bipolar and Müller cell bodies in the mid-INL showed scant Pan-Na\(_{\alpha}\) labeling, similar to results seen in cryosections.

**Labeling with isoform-specific Na\(_{\alpha}\) \(\alpha\)-subunit antibodies:** Each isoform-specific Na\(_{\alpha}\) \(\alpha\)-subunit antibody produced a characteristic labeling pattern consistent with the overall distribution of Na\(_{\alpha}\) \(\alpha\)-subunit shown by the Pan-Na\(_{\alpha}\) antibody (Figure 4; mouse retina illustrated). In contrast to Pan-Na\(_{\alpha}\) antibody, labeling using the isoform-specific Na\(_{\alpha}\) \(\alpha\)-subunit antibodies was not fixation-sensitive and did not require antigen retrieval.

**Na\(_{\alpha}\) 1.1:** As expected, labeling for Na\(_{\alpha}\) 1.1 was found in cell bodies in the GCL, ganglion cell axons, a subpopulation of amacrine cells along the INL/IPL border and strongly labeled processes in the IPL (Figure 4A). In addition, Na\(_{\alpha}\) 1.1 immunoreactivity was found in the OPL, although little labeling was present in bipolar or Müller cell bodies in the mid-INL or photoreceptor cell bodies in the ONL. A similar distribution of Na\(_{\alpha}\) 1.1 labeling was seen in wholemounts, which also revealed strong labeling of structures similar in appearance to ganglion cell axon initial segments (Figure 4D).

**Na\(_{\alpha}\) 1.2:** Labeling for Na\(_{\alpha}\) 1.2 also was distributed as expected in the inner retina (Figure 4B). Cell bodies in the GCL and along the INL/IPL border showed labeling. Although some strongly labeled processes were observed in the IPL, labeling tended to be more diffuse, and strongly labeled processes were seen less often than with Na\(_{\alpha}\) 1.1 labeling. In addition to labeling in the inner portion of the retina, strong Na\(_{\alpha}\) 1.2 labeling was found in a set of cell bodies located along the OPL/INL border and diffuse labeling was present in the OPL. Little Na\(_{\alpha}\) 1.2 labeling was found in the mid-INL or ONL. Labeling for Na\(_{\alpha}\) 1.2 in wholemounts was similar, although labeling of ganglion cell axons and of structures with the appearance of ganglion cell axon initial segments was more obvious than in vertical sections (Figure 4E).

**Na\(_{\alpha}\) 1.6:** The pattern of Na\(_{\alpha}\) 1.6 labeling also was consistent with the labeling pattern seen with the Pan-Na\(_{\alpha}\) antibody (Figure 4C). As expected, Na\(_{\alpha}\) 1.6 immunolabeling was found in cell bodies in the GCL. Na\(_{\alpha}\) 1.6 labeling was also seen in a subset of amacrine cells at the INL/IPL border and diffusely distributed in the IPL. Ganglion cell axons also showed Na\(_{\alpha}\) 1.6 labeling as appropriate, particularly structures with the appearance of ganglion cell axon initial segments (Figure 4F). Labeling for Na\(_{\alpha}\) 1.6 also was present in the OPL and a population of horizontal cells along the distal border of the INL. There was little Na\(_{\alpha}\) 1.6 labeling in the mid-INL or ONL, similar to the Pan-Na\(_{\alpha}\), Na\(_{\alpha}\) 1.1, and Na\(_{\alpha}\) 1.2 labeling patterns.

Together, these results confirm that amacrine and ganglion cells, their processes in the IPL and ganglion cell axons, express Na\(_{\alpha}\) 1.1, Na\(_{\alpha}\) 1.2 and Na\(_{\alpha}\) 1.6 \(\alpha\)-subunit, consistent with Pan-Na\(_{\alpha}\) labeling and previous reports [13-15]. These results also indicated that there was little expression of Na\(_{\alpha}\) 1 \(\alpha\)-subunits in the cell bodies of photoreceptors in the ONL or bipolar and Müller cells in the mid-INL, although labeling was present in the OPL and a population of cells along the OPL/INL border.

**Na\(_{\alpha}\) 1 \(\alpha\)-subunit immunolabeling was not detected in bipolar cell bodies:** Recent patch-clamp studies have indicated the presence of functional Na\(_{\alpha}\) channels in isolated rat bipolar cells [24,49], although we observed little labeling for any Na\(_{\alpha}\) 1 \(\alpha\)-subunit in the mid-INL where bipolar cell bodies reside. To better understand the distribution of Na\(_{\alpha}\) 1 \(\alpha\)-subunits and their potential functional roles in bipolar cells, we performed triple labeling in the rat retina using the Pan-Na\(_{\alpha}\) antibody in conjunction with antibodies against protein kinase C and Chx-10 that label rod bipolar cells or all types of bipolar cells, respectively [50,51]. These studies definitively showed that the cell bodies along the distal border of the INL showing Na\(_{\alpha}\) 1 \(\alpha\)-subunit labeling were not bipolar cells and that the Na\(_{\alpha}\) 1 \(\alpha\)-subunit-positive cells in the innermost portion of the INL represented amacrine cell populations. No colocalization of Pan-Na\(_{\alpha}\) labeling with either protein kinase C or Chx10 labeling was present, indicating that Na\(_{\alpha}\) 1 \(\alpha\)-subunits were either absent from bipolar cell bodies or expressed at a level too low to detect (Figure 5).

**Horizontal cells and other cell types ramifying in the outer plexiform layer express Na\(_{\alpha}\) 1 \(\alpha\)-subunits:** The finding that the cells in the distal INL showing Na\(_{\alpha}\) 1 \(\alpha\)-subunit labeling did not express bipolar cell markers suggested that these cells might be horizontal cells. To test this, we performed double labeling for Pan-Na\(_{\alpha}\) and calbindin, a marker for horizontal cells in rat, mouse and rabbit retina [52-54]. Horizontal cell bodies and processes showed Pan-Na\(_{\alpha}\) labeling in rat, mouse, and rabbit retina (Figure 6). However, additional diffuse Pan-Na\(_{\alpha}\) labeling also was present throughout the OPL that did not correspond to horizontal cell processes, indicating that Na\(_{\alpha}\) 1 \(\alpha\)-subunit expression in the OPL was not limited strictly to horizontal cells. In the rabbit OPL, specifically, discrete annular clusters of Pan-Na\(_{\alpha}\) labeling were present in addition to diffuse labeling. Together, these results indicate that Na\(_{\alpha}\) 1 \(\alpha\)-subunits were present on horizontal cells in addition to the processes of other cell types that ramify in the OPL.

**Na\(_{\alpha}\) 1 \(\alpha\)-subunit isoforms are differentially distributed within horizontal cells and the outer plexiform layer:** To better understand the distribution of Na\(_{\alpha}\) 1 \(\alpha\)-subunits in the outer retina, we performed a series of double labeling experiments to characterize further the distribution of Na\(_{\alpha}\) 1.1, Na\(_{\alpha}\) 1.2, and Na\(_{\alpha}\) 1.6 subunits within horizontal cells and the OPL.

In both rat and mouse retinas, horizontal cell bodies and processes showed scant Na\(_{\alpha}\) 1.1 labeling, while appreciable Na\(_{\alpha}\) 1.1 labeling was present in the OPL distal to the horizontal cell processes (Figure 7A-C; rat shown). Rabbit horizontal cell bodies and processes, however, showed distinct labeling for Na\(_{\alpha}\) 1.1 (Figure 7D-F). Labeling for Na\(_{\alpha}\) 1.1 in the rabbit OPL also showed discrete annular clusters similar to those observed in the rabbit OPL using the Pan-Na\(_{\alpha}\) antibody. The Na\(_{\alpha}\) 1.1 clusters in the rabbit OPL did not colocalize with calbindin, suggesting that they arose from cells other than horizontal cells, consistent with the pattern seen using the Pan-Na\(_{\alpha}\) antibody.

Na\(_{\alpha}\) 1.2 and Na\(_{\alpha}\) 1.6 labeling was closely associated with horizontal cells in all three species, colocalizing extensively
with calbindin labeling and with comparatively little additional labeling for Na\textsubscript{1.2} or Na\textsubscript{1.6} detected elsewhere in the OPL. Horizontal cell bodies and processes, including the dendritic tips, showed prominent Na\textsubscript{1.2} labeling (Figure 7G-I; rat shown). In contrast, labeling for Na\textsubscript{1.6} in horizontal cells was appreciably stronger in the cell body and proximal processes than in the dendritic tips (Figure 7J-L; rat shown). Na\textsubscript{1.2} and Na\textsubscript{1.6} labeling patterns in the horizontal cells and OPL were similar in the retinas of rat, mouse, and rabbit.

Figure 4. Labeling for Na\textsubscript{1.1}, Na\textsubscript{1.2} and Na\textsubscript{1.6} is isoform-specific and consistent with the distribution of Pan-Na\textsubscript{v} labeling. A-C: Shown are labeling for Na\textsubscript{1.1}, Na\textsubscript{1.2}, and Na\textsubscript{1.6} in vertical retinal sections. Rat and mouse retina showed similar labeling patterns (mouse retina shown). Labeling of blood vessels (bv) was non-specific. A: Na\textsubscript{1.1} labeling in the outer plexiform layer (OPL) was characterized by discrete puncta surrounded by more diffuse labeling. A subset of putative amacrine cells (A) in the proximal inner nuclear layer (INL) also showed labeling. Large, intensely labeled processes (arrows) and diffuse labeling were present in the inner plexiform layer (IPL). Cells in the ganglion cell layer (GCL) were lightly labeled (*). Ganglion cell axons in the nerve fiber layer (NFL) also showed labeling. B: Na\textsubscript{1.2} labeling in the OPL was diffuse, but a few presumptive horizontal cell bodies (H) in the distal INL showed strong labeling. Some presumptive amacrine cells (A) in the proximal INL also showed Na\textsubscript{1.2} labeling. The IPL showed diffuse labeling. Cells in the GCL (*) also showed labeling. C: Na\textsubscript{1.6} labeling in the OPL was diffuse. Presumptive horizontal cell bodies (H) in the distal INL showed prominent labeling. A few presumptive amacrine cells (A) in the proximal INL showed labeling for Na\textsubscript{1.6}. As expected, cells in the GCL (*) also were labeled. D-F: Confocal images of labeling for Na\textsubscript{1.1}, Na\textsubscript{1.2}, and Na\textsubscript{1.6} in wholemounted rat retina at the level of the GCL and NFL are illustrated. D: Labeling for Na\textsubscript{1.1} was present in ganglion cell axons (arrowhead) and putative initial segments (arrows). Weakly labeled cell bodies in the GCL also were visible. E: Labeling for Na\textsubscript{1.2} at this level was most prominent in ganglion cell axons (arrowheads) and structures with the appearance of initial segments (arrows). F: Structures corresponding to the putative initial segments of ganglion cell axons showed strong labeling for Na\textsubscript{1.6} (arrows), but relatively little labeling was present elsewhere in the axons or cell bodies of the cells in the GCL. Scale bars equal 20 µm for all panels.
These results indicate that Na\textsubscript{v} 1.1, Na\textsubscript{v} 1.2, and Na\textsubscript{v} 1.6 \(\alpha\)-subunits are all expressed by mammalian horizontal cells. Although horizontal cells expressed Na\textsubscript{v} 1.1, much of the Na\textsubscript{v} 1.1 labeling in the OPL did not colocalize with calbindin, suggesting that Na\textsubscript{v} 1.1 also was present on processes from other cell types. In contrast, Na\textsubscript{v} 1.2 and Na\textsubscript{v} 1.6 in the distal retina were closely associated with horizontal cells and their processes, indicating that other cell types ramifying in the OPL expressed little Na\textsubscript{v} 1.2 and Na\textsubscript{v} 1.6.

Na\textsubscript{v} 1.1 is diffusely expressed by multiple cell types in the outer plexiform layer: Imaging of rat retinal wholemounts double-labeled for Na\textsubscript{v} 1.1 and calbindin confirmed that much of the diffuse Na\textsubscript{v} 1.1 labeling in the OPL was not localized to horizontal cell processes themselves (Figure 8A-C; single optical section shown). The presence of additional Na\textsubscript{v} 1.1 labeling in the OPL that did not correspond to horizontal cell processes indicated that Na\textsubscript{v} 1.1 was expressed by other cell types ramifying in the OPL, such as bipolar cells or photoreceptors. To better understand the distribution of Na\textsubscript{v} 1.1 in the OPL, we performed double labeling for Na\textsubscript{v} 1.1 and markers for photoreceptor terminals and the tips of ON bipolar cell dendrites and examined them by confocal microscopy in single optical sections.

To determine whether Na\textsubscript{v} 1.1 labeling might be associated with photoreceptor terminals, we double labeled sections for Na\textsubscript{v} 1.1 and vesicular glutamate transporter 1 (VGluT1), which is expressed in both rod and cone terminals [39,55]. Examination of single optical sections in the OPL showed appreciable overlap of Na\textsubscript{v} 1.1 and VGluT1 labeling, suggesting that some Na\textsubscript{v} 1.1 labeling in the OPL was associated with photoreceptor terminals (Figure 8D-F). However, much of the Na\textsubscript{v} 1.1 labeling in the OPL did not colocalize with photoreceptor terminals.

The presence of Na\textsubscript{v} 1.1 labeling that did not colocalize with either horizontal cell or photoreceptor terminals suggested that Na\textsubscript{v} 1.1 also might be expressed on the processes of other cells ramifying in the OPL. To test whether Na\textsubscript{v} 1.1 was associated specifically with ON-cone bipolar and rod bipolar cell

![Image of retinal wholemounts](https://www.molvis.org/molvis/v13/a247/)

**Figure 5. Pan-Na\textsubscript{v} labeling was absent from bipolar cell bodies.** A-D: Triple labeling for Pan-Na\textsubscript{v} (green), the rod bipolar cell marker protein kinase C (PKC, blue) and the pan-bipolar cell marker Chx-10 (red), showed no colocalization of Pan-Na\textsubscript{v} labeling with bipolar cell markers. A: Pan-Na\textsubscript{v} labeling was present in many amacrine cell bodies (A) in the proximal inner nuclear layer (INL) and a few cell bodies in the distal INL (arrows). B: Rod bipolar cells showed labeling for PKC throughout the cell. C: Labeling for Chx-10 is present in the nuclei of all bipolar cells. Overlay of panels A-C showing that Pan-Na\textsubscript{v} labeling did not colocalize with labeling for either PKC or Chx-10. Images are a projection of 53 optical planes; 7.2 \(\mu\text{m}\) total thickness. Rat retina is shown. The following abbreviations were used: outer nuclear layer (ONL); inner plexiform layer (IPL). Scale bar equal 20 \(\mu\text{m}\).
dendrites at the point of contact with the synaptic ribbons of cone and rod terminals, we double labeled sections for \( \text{Na} \, \alpha \)-subunits in horizontal cells and rod bipolar cell dendrites at ribbon synapses [56], and examined them by confocal microscopy. Examination of single optical planes (approximately 0.3 \( \mu \text{m} \) thickness) revealed that \( \text{Na} \, \alpha \)-subunits and mGluR6 labeling showed little colocalization, although they were often found in close proximity (Figure 8G-I). Thus, \( \text{Na} \, \alpha \)-subunits did not specifically colocalize with mGluR6 on bipolar cell dendrites within the ribbon synapse complexes.

**Effects of tetrodotoxin on the rat electroretinogram:** Given the extensive labeling for \( \text{Na} \, \alpha \)-subunits in horizontal cells and the OPL, we were interested in looking more closely at the effects on outer retinal function of blocking retinal \( \text{Na} \) channels. Similar to past studies in rats [57], intravitreal injections of TTX to block \( \text{Na} \) channels severely attenuated the potentials in the dark-adapted ERG known to arise from amacrine cells and retinal ganglion cells (p-STR and n-STR; Figure 9B,). Past studies indicate that TTX does not alter the amplitude of waves of the dark-adapted ERG originating more distally, the a-wave from rods and the b-wave from rod bipolar cells. However, under photopic conditions, TTX reduces the amplitude of the cone-driven b-wave, a signal generated primarily by cone bipolar cells [57]. In the present study we recorded ERGs from rats under dark-adapted conditions, but with rod signals active or suppressed to study more closely...

![Figure 6](http://www.molvis.org/molvis/v13/a247/)

**Figure 6.** Horizontal cells showed Pan-Na\(_\alpha\) labeling. Double labeling for Pan-Na\(_\alpha\) (green) and the horizontal cell marker calbindin (red) confirmed that horizontal cells (H) and their processes (arrows) expressed Na\(_\alpha\)-subunits in rat retina (A-C: vertical section; D-F: wholemount). Mouse retina (G-I), and rabbit retina (J-L). Additional Pan-Na\(_\alpha\) labeling that is not associated with calbindin-positive horizontal cell processes also is present in the outer plexiform layer (OPL) and was particularly visible in whole mounted retina (arrowheads in D-F). Distinctive annuli of Pan-Na\(_\alpha\) labeling (arrowheads in J-L) were noted in the rabbit OPL. Labeling of blood vessels (bv) in mouse and rat retina was non-specific. In the figure inner nuclear layer is abbreviated INL. Scale bars equal 10 \( \mu \text{m} \) for A-F; 20 \( \mu \text{m} \) for G-L.
the effects of TTX on the rod vs. cone circuits under similar conditions of light-adaptation (Figure 9).

ERG responses to low stimulus energies up to those that saturated the dark-adapted b-wave (e.g., response to -1.3 sc td s in Figure 9A), are believed to originate from the sensitive rod to rod bipolar cell circuit (e.g., [58-60]). Following TTX blockade, we observed, that the b-wave was unaffected over the range of these stimulus intensities, as illustrated here for a representative result (unpublished observations). This finding indicates that cells of origin of the b-wave in the rod to rod bipolar cell circuit were hardly affected by TTX. B-waves in response to higher stimulus energies in the dark-adapted ERG (1.3 and 1.6 log sc td; Figure 9A) were reduced by TTX. Dark-adapted ERG responses to strong stimuli were mixed rod-cone responses (e.g., [58,61]), meaning cone circuits were active.

We investigated the hypothesis that the TTX-mediated attenuation of the b-wave was occurring in cone circuits in the mixed rod-cone ERG by isolating dark-adapted cone-driven responses using a cone isolation protocol. Briefly, as described in studies by other investigators [62,63], an initial conditioning flash (1 s duration) was used to suppress the rod photocurrent and then, at an appropriate time interval for recovery of cone but not rod responses, a test flash was presented [61,62,64,65]. In rodents, an 800 ms interval between conditioning and test flashes allows full recovery of cone signals while rod signals are still suppressed [62,66]. Using an interstimulus interval of 800 ms, we were able to isolate and study dark-adapted cone-driven responses over a range of stimulus energies. As shown in Figure 9C, the isolated cone-driven b-wave was reduced by TTX for all stimulus energies that elicited a response (more fully documented in unpublished observations). The effect on the cone-driven response was more than sufficient to account for the reduction in the dark-adapted mixed rod-cone ERG.

In the next phase of the experiment, the dark-adapted cone-isolation protocol was repeated following blockade with CNQX of α-amino-5-hydroxy-3-methyl-4-isoxazole propionic acid (AMPA) and kainate (KA) receptors [67]. In the retina, these receptors are located on the dendrites of horizontal, OFF bipolar, amacrine, and ganglion cells. Finally, the experiment was repeated again after intravitreal injection of TTX in the eyes already injected with CNQX (Figure 10). Representative

Figure 7. Distribution of Na\textsubscript{v} 1.1, Na\textsubscript{v} 1.2, and Na\textsubscript{v} 1.6 in horizontal cells and the outer plexiform layer. Double labeling using isoform-specific antibodies directed against Na\textsubscript{v} 1.1, Na\textsubscript{v} 1.2, or Na\textsubscript{v} 1.6 (green) and calbindin (red) was used to assess Na\textsubscript{v} a-subunit localization in horizontal cells. A-C: Labeling for Na\textsubscript{v} 1.1 in the rat and mouse retina was most prominent in the distal outer plexiform layer (OPL; at retina shown). Rat and mouse horizontal cells and their processes showed only weak labeling for Na\textsubscript{v} 1.1. D-F: Rabbit horizontal cell bodies (H) and processes showed distinct Na\textsubscript{v} 1.1 labeling, in contrast to rat and mouse horizontal cells. Distinctive annuli of Na\textsubscript{v} 1.1 labeling were present in the rabbit OPL (arrowheads). G-I: Labeling for Na\textsubscript{v} 1.2 was present in horizontal cell bodies (H) and processes (arrows). Rat, mouse, and rabbit horizontal cells all showed similar labeling for Na\textsubscript{v} 1.2 (rat retina shown). J-L: Much of the Na\textsubscript{v} 1.6 labeling in the outer retina corresponded closely to horizontal cell bodies (H) and processes (arrows), although some additional diffuse labeling in the OPL was also present. Rat, mouse and rabbit horizontal cells all showed similar labeling for Na\textsubscript{v} 1.6 (rat retina shown). Labeling of blood vessels (bv) is non-specific. The following abbreviations were used: outer nuclear layer (ONL); an inner nuclear layer (INL). Scale bars equal 10 μm for all panels.
ERG traces from a single animal (Figure 10A) show that CNQX did not affect the cone-driven ERG. In contrast, CNQX+TTX produced a large reduction of b-wave amplitude. B-wave amplitudes were measured at a fixed time, (50 ms; the cone-driven b-wave time to peak), and plotted as a function of flash energy (Figure 10B). The plot confirms that blockade of AMPA/KA receptors with CNQX left the average b-wave amplitude unchanged, whereas CNQX+TTX produced a large attenuation of the b-wave (n= 6 eyes from 4 rats). The average percent reduction of the maximum b-wave amplitude after CNQX+TTX was very similar to that produced by TTX alone in other eyes (CNQX+TTX: 59%±14.3%, n=6, this study; TTX: 67.6%±17.3%, n=7 eyes in 7 rats, unpublished observations). These results indicate that the blockade of AMPA/KA receptors on neurons in cone circuits located proximal to photoreceptors did not alter the TTX-mediated attenuation of the cone-driven b-wave and suggest that the effect of the TTX was directly on Nav  channels in cone bipolar cells. The nearly parallel downward shift of the b-wave energy-amplitude function in Figure 10B after TTX was injected indicates an effect on response amplitude, more than on sensitivity. This also is consistent with an effect on Na_\text{v}  channels in cone bipolar cells, rather than the cone photoreceptors that provide input to the bipolar cells. Some of these results have been published previously in abstract form [66].

**DISCUSSION**

Previous studies in the rat retina have shown specific distributions of Na_\text{v} 1.1, Na_\text{v} 1.2 and Na_\text{v} 1.6 in the retinal ganglion cells and their axons [12,13,15] and in the IPL [14]. Our studies confirm these findings in the inner retina and demonstrate that each of these Na_\text{v} 1 \alpha-subunits also are present more distally in the retina, particularly in the OPL and horizontal cells. In addition, while Na_\text{v} 1.1, Na_\text{v} 1.2 and Na_\text{v} 1.6 show substantial overlap in our study, the distribution of each subunit in the retina is unique.

A striking finding in our studies is the localization of Na_\text{v} 1 \alpha-subunits to the OPL and horizontal cells and their processes. The localization of Pan-Na_\text{v} labeling and labeling for Na_\text{v} 1.1, Na_\text{v} 1.2 and Na_\text{v} 1.6 \alpha-subunits to rat, mouse, and rabbit horizontal cells is consistent with a previous report of Na_\text{v} 1 \alpha-subunit immunolabeling in horizontal cells in the cat and monkey retina [68]. Furthermore, the current findings are consistent with previous functional studies demonstrating TTX-sen-
sitive Na$^+$ currents in isolated horizontal cells in several species: catfish: [30]; white perch: [31], cat: [32], rabbit: [33].

Na$\text{v}_1$ $\alpha$-subunit labeling of horizontal cells was most prominent in the cell body, but was also present in the processes. Our studies using antibodies directed against specific Na$\text{v}_1$ $\alpha$-subunits indicate that rat, mouse, and rabbit horizontal cells express at least two types of Na$\text{v}_1$ $\alpha$-subunits: Na$\text{v}_1.2$ and Na$\text{v}_1.6$. Additionally, rabbit horizontal cells show substantial labeling for Na$\text{v}_1.1$. Co-expression of multiple Na$\text{v}_1$ $\alpha$-subunit within individual neurons has been reported previously in retinal ganglion cells [13,15] and neurons in the adult brain [69-71]. All horizontal cells in the rat and mouse retina showed similar expression of Na$\text{v}_1.2$ and 1.6, consistent with the presence of only a single type of axon-bearing horizontal cell in the rodent retina [54]. In contrast, rabbit horizontal cells did not show uniform labeling for Na$\text{v}_1$ $\alpha$-subunits, suggesting heterogeneous Na$\text{v}_1$ $\alpha$-subunit expression among rabbit horizontal cells (Figure 6J-L and Figure 7D-F). The large size of the Na$\text{v}_1$ a immuno-positive horizontal cells in rabbit suggests that at least the axonless A-type horizontal cells that exclusively contact cones in the rabbit retina express Na$\text{v}_1$ $\alpha$-subunits [72,73].

The strong labeling of the processes and cell bodies of horizontal and amacrine cells is consistent with somatic labeling of neurons in the adult mammalian brain reported previously [74,75]. Voltage-gated Na$^+$ channels containing Na$\text{v}_1.1$ in the somato-dendritic compartment of neurons in rat brain have been proposed to play a key role dendritic excitability [75]. Somato-dendritic labeling also may represent cytoplasmic Na$\text{v}_1$ $\alpha$-subunit intermediates in the biosynthetic pathway [76] or storage pools. The intensity of Pan-Na$\text{v}$ labeling seen in horizontal and amacrine cell bodies also may reflect the expression of more than one Na$\text{v}_1$ $\alpha$-subunit in these cells. In addition, the cells may possess other Na$\text{v}_1$ $\alpha$-subunits that we did not test.

The results of our single and double immunolabeling analyses suggest that Na$\text{v}_1$ $\alpha$-subunits in the OPL are not restricted only to horizontal cells. Pan-Na$\text{v}$ labeling was diffusely

Figure 9. Tetrodotoxin blockade of Na$^+$ channels in the retina attenuated the cone bipolar cell driven b-wave but did not affect the amplitude of the rod bipolar cell driven b-wave. The response before tetrodotoxin (TTX) is indicated by the black traces and after TTX by the grey traces. A: TTX did not alter the b-wave amplitude for low flash energies that elicit rod-driven b-waves (-1.9 and -1.3 log sc td s). However, TTX decreased b-wave amplitude for higher flash energies (1.3 and 1.6 log sc td s) that produced mixed rod+cone-driven electoretinograms (ERGs). B: TTX blockade of Na$^+$ channels attenuated the negative and positive scotopic threshold responses (STR), potentials of inner retinal origin in the dark-adapted ERG that occured in response to low flash energies (-4.0 log sc td s in the figure). C: For the same animal, TTX produced a severe attenuation of the dark-adapted cone-isolated b-wave (grey traces) compared to the cone-isolated b-wave before TTX administration (black traces). Note that discernable cone-driven b-waves were produced only for flash energies higher than those that saturated the amplitude of the dark-adapted ERG shown in the left panel (-1.3 log sc td).
distributed throughout the OPL, consistent with the band of Na\textsubscript{v} 1.1 labeling found mainly distal to the large processes of the horizontal cells and the weaker, diffuse labeling for Na\textsubscript{v} 1.2 and Na\textsubscript{v} 1.6 present in the OPL. This diffuse Na\textsubscript{v} α-subunit labeling could not be accounted for by the processes of any single cell type ramifying in the OPL, suggesting that Na\textsubscript{v} α-subunits may be present on the processes of multiple cell types that ramify in the OPL.

Our data suggest that some of the additional Na\textsubscript{v} α-subunit labeling in the OPL is associated with photoreceptor terminals. Double labeling for Na\textsubscript{v} 1.1 and VGluT1, a photoreceptor terminal marker [39,55], indicates that at least Na\textsubscript{v} 1.1 is present at the level of the photoreceptor terminals. The patchy distribution of Na\textsubscript{v} 1.1 labeling in the OPL of flatmounted retinas would be consistent with the spatial arrangement of cone terminals (see Figure 8A-C). However, the limits of optical resolution in our confocal images (approximately 0.3 µm), the anatomical complexity of the processes in and around the photoreceptor terminals, and the diffuse nature of the labeling preclude unequivocal assignment of Na\textsubscript{v} 1.1 labeling at the level of the photoreceptor terminals to specific cell types. Other studies also support expression of functional voltage-gated Na\textsuperscript{+} channels by photoreceptors. Patch clamp recordings of human photoreceptors in isolation or in retinal slices have identified TTX-sensitive Na\textsuperscript{+} currents [35,36] and knockout of the gene for Na\textsubscript{v} 1.6 in mouse has been shown to impede the physiological maturation of photoreceptors [6]. Interestingly, in human photoreceptors, single-cell PCR showed Na\textsubscript{v} 1.2-rather than Na\textsubscript{v} 1.1-transcripts [28,36].

Although Na\textsubscript{v} α-subunits were clearly present in horizontal cells and at the level of photoreceptor terminals, these structures could not account for all Na\textsubscript{v} α-subunit labeling in the OPL. One distinct possibility is that some of the diffuse Na\textsubscript{v} α-subunit labeling was associated with bipolar cell dendrites.

Figure 10. Effect of tetrodotoxin on the cone-driven electroretinogram arising from the cone to ON cone bipolar cell circuit. A: Dark-adapted isolated cone-driven responses are shown before intravitreal drug injections (black trace), after 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; dashed trace) and after tetrodotoxin (TTX) added following CNQX (CNQX+TTX; grey trace). B: Average electroretinogram (ERG) amplitudes are shown for all eyes for which the control condition, as well as both drug conditions were available (six eyes from four subjects). Amplitudes of ERGs were measured at 50 ms after the flash, the time to peak of the cone-isolated b-wave and plotted as a function of flash energy for the control dark-adapted cone-isolated b-wave (black circle), after CNQX (grey circle) and after CNQX+TTX (circle) plotted on a log-log scale.
Several pieces of evidence support this possibility: depression of the cone-driven b-wave of the ERG, which arises primarily from bipolar cells, by TTX blockade of Na_\text{v} 1.2 channels (this study); patch-clamp recordings of voltage-gated sodium currents in individual bipolar cells [24,25,28,49], and previous reports of Na_\text{v} 1.2 channel gene or protein expression by bipolar cells [25,28,68]. The diffuse nature of the labeling and the limits of detection and anatomical resolution associated with the immunolabeling techniques in the current study do not permit the direct assignment of Na_\text{v} 1.2-subunits to bipolar cell dendrites. However, the current studies do suggest that Na_\text{v} 1.2-subunits are not concentrated at the tips of bipolar cell dendrites contacting photoreceptor ribbons as there was little colocalization of Na_\text{v} 1.2-subunit and mGlur6 labeling. The current studies also indicate that Na_\text{v} 1.2-subunits are not present in large quantities in the cell bodies of bipolar cells.

An interesting finding of our study was that the extent of labeling by the Pan-Na_\text{v} antibody was highly sensitive to fixation time and processing of the tissue, especially in the plexiform layers. This indicates that increased fixation and processing interferes with immunodetection of Na_\text{v} 1.2-subunits, when the Pan-Na_\text{v} antibody is used. The Na_\text{v} 1.2-subunit-specific antibodies did not show the same sensitivity to fixation, although labeling in the lightly fixed wholemounts was stronger than in frozen sections. Antigen retrieval procedures were required to obtain Pan-Na_\text{v} labeling in frozen sections that had been exposed to fixative for as short as 30 min. Even with antigen retrieval procedures, Pan-Na_\text{v} labeling in the plexiform layers in frozen sections remained limited. In contrast, in wholemounts that were lightly fixed for only five minutes and were never subjected to freezing, Pan-Na_\text{v} labeling was intense and readily detectible in the plexiform layers and neuronal cell bodies without antigen retrieval. The \(\alpha\)-subunit-specific antibodies also produced high levels of labeling in the plexiform layers, consistent with the high levels of Pan-Na_\text{v} labeling seen in the lightly fixed wholemounts. Together, these results suggest that the distribution of Na_\text{v} 1.2-subunits in the plexiform layers is much wider than has been appreciated previously and that use of light fixation enhances immunohistochemical detection of Na_\text{v} 1.2-subunits.

It is unlikely that labeling for Na_\text{v} 1.2-subunits in the outer retina, neuronal cell bodies and the plexiform layers is due to non-specific labeling. The specificity of the antibodies used in the current study has been extensively characterized using immunoblotting, ELISA and preadsorption assays, and immunolabeling of cells and tissues known to either contain or lack specific Na_\text{v} 1.2-subunits [13,14,37]; this report. Our further analysis of epitope sequence homology across Na_\text{v} 1.2-subunits and other mammalian protein sequences showed that these epitopes are unique to Na_\text{v} 1.2-subunits and, in the case of the \(\alpha\)-subunit specific antibodies, are isoform specific as well. The Pan-Na_\text{v}, Na_\text{v} 1.1, Na_\text{v} 1.2, and Na_\text{v} 1.6 labeling patterns observed in our studies precisely corroborate previous reports localizing these subunits in the inner retina [12-15]. Finally, Na_\text{v} 1.2-subunit labeling patterns in the outer retina are consistent with emerging physiological and molecular data indicating that Na_\text{v} 1.2-subunits and functional Na_\text{v} 1.2 channels are present in outer retina [24-26,28,35,36,49].

The dark-adapted rod-driven a-waves were unaffected by TTX, implying that rod photocurrents were unaffected (Figure 9 and unpublished observations). Cone photoreceptor contributions to a-waves were negligible in our experiments, making it difficult to evaluate effects of TTX on cone photocurrents, but it is unlikely that the photocurrents were affected. Labeling for Na_\text{v} 1.1 \(\alpha\)-subunit has not been observed in photoreceptor outer segments where photoreceptor a-waves are generated. However, the dark-adapted cone-driven b-waves were reduced in amplitude by TTX, raising the possibility that there were effects on cone terminals, cone bipolar cells, or other components of the cone circuits. In addition to the ON cone bipolar cells, the cone-driven b-wave also can be shaped by the activity of OFF cone bipolar cells, horizontal cells, as well as more proximal retinal neurons and Müller cells [77-79]. Blockade of inputs to these additional neuronal contributors to the b-wave by CNQX did not alter the cone-isolated ERGs. This reduces the likelihood that TTX effects were mediated by horizontal cells or any of the other neurons in the cone circuit with synaptic input via AMPA/Kainate receptors. However, the b-wave amplitude after CNQX+TTX was reduced and the amount of attenuation was similar to that caused by TTX alone. From these data, we infer that the ON cone bipolar cells themselves are the most likely locus of the TTX effects. Cone inner segments or terminals are also a logical possibility. TTX-sensitive currents have been observed in isolated human photoreceptors, but predominantly in rods (68% of those studied) rather than cones (19%) [35,36]. However, the physiological role of these currents in light-evoked signaling is unclear and in response to flashed stimuli, photoreceptor membranes would be too hyperpolarized to allow Na_\text{v} 1.2 channels to open [80], making it unlikely that cones contribute to the effects of TTX reported here. The ERG results would be consistent with the presence of Na_\text{v} 1.2 channels in the dendritic compartment of ON cone bipolar cells. Past studies also indicate that TTX-sensitive Na_\text{v} 1.2 currents are present in the somato-dendritic compartment of the rat cone bipolar cells [24].

The role of Na_\text{v} 1.2 channels in horizontal cells is puzzling. Under dark-adapted conditions horizontal cells undergo tonic depolarization by glutamate release from the photoreceptors that keep the resting membrane potential around -35 mV (for review see [81]), thereby causing voltage-dependent inactivation of any Na_\text{v} 1.2 channels. A large hyperpolarization of the horizontal cells due to a high energy flash might potentially relieve this inactivation. Voltage-gated Na_\text{v} 1.2 channels could allow inward Na_\text{v} 1.2 currents in horizontal cells after offset of a strong stimulus when the membrane repolarizes [82,83]. The impact of this in retinal processing would need further study.

The role of Na_\text{v} 1.2 channels in mammalian ON-cone bipolar cells also needs elucidation. It has been suggested that Na_\text{v} 1.2 channels in bipolar cells may augment subthreshold depolarizing potentials in the dendrites and cell body of bipolar cells [25] in a manner similar to those seen in CNS neurons [84]. The Na_\text{v} 1.2 channels may play a special role in circuits carrying...
transient signals through the retina [26,27]. Our results in other studies suggest that the TTX-sensitive augmentation of the cone-driven b-wave is maximal under the dark-adapted conditions used in the present study and decreases with increasing background illumination (unpublished observations). This result is similar to the finding in salamander retina, where TTX-sensitive Na+ currents in transient ON bipolar cells were maximal under the dark-adapted condition and decreased with light adaptation [85]. Future studies will provide more insight about the diverse roles that Na+ channels play in retinal processing.

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