Sodium channel diversity in the vestibular ganglion: Na\textsubscript{V}1.5, Na\textsubscript{V}1.8, and tetrodotoxin-sensitive currents

Liu XP, Wooltorton JR, Gaboyard-Niay S, Yang FC, Lysakowski A, Eatock RA. Sodium channel diversity in the vestibular ganglion: Na\textsubscript{V}1.5, Na\textsubscript{V}1.8, and tetrodotoxin-sensitive currents. J Neurophysiol 115: 2536–2555, 2016. First published March 2, 2016; doi:10.1152/jn.00902.2015.—Firing patterns differ between subpopulations of vestibular primary afferent neurons. The role of sodium (Na\textsubscript{V}) channels in this diversity has not been investigated because Na\textsubscript{V} currents in rodent vestibular ganglion neurons (VGNs) were reported to be homogeneous, with the voltage dependence and tetrodotoxin (TTX) sensitivity of most neuronal Na\textsubscript{V} channels. RT-PCR experiments, however, indicated expression of diverse Na\textsubscript{V} channel subunits in the vestibular ganglion, motivating a closer look. Whole cell recordings from acutely dissociated postnatal VGNs confirmed that nearly all neurons expressed Na\textsubscript{V} currents that are TTX-sensitive and have activation midpoints between ~30 and ~40 mV. In addition, however, many VGNs expressed one of two other Na\textsubscript{V} currents. Some VGNs had a current that was sensitive to TTX and high-threshold calcium (Ca\textsubscript{V})-gated K channel blockers (K\textsubscript{Ca} and K\textsubscript{Ca}2), and a relatively depolarized voltage range. In two Na\textsubscript{V}1.8 reporter lines, subsets of VGNs were labeled. VGNs with Na\textsubscript{V}1.8-like TTX-resistance current were identified from other VGNs in the voltage dependence of their TTX-sensitive currents and in the voltage threshold for spiking and action potential shape. Regulated expression of Na\textsubscript{V}1.8 channels in primary afferent neurons is likely to selectively affect firing properties that contribute to the encoding of vestibular stimuli.

vestibular ganglion; vestibular afferent; sodium channel; action potential; Na\textsubscript{V}1.8; Na\textsubscript{V}1.5; tetrodotoxin

THE VESTIBULAR ORGANS OF THE INNER EAR SENSE HEAD ORIENTATION AND MOTION, SENDING SIGNALS TO THE BRAIN THAT DRIVE POWERFUL OCULAR, AUTONOMIC, AND POSTURAL REFLEXES, CONTRIBUTE TO OUR SENSE OF HEADING, AND SUPPORT LEARNING OF COMPLEX TASKS SUCH AS DANCING (REVIEWED IN ANGELAKI AND CULLEN 2008; GOLDBERG ET AL. 2012). NORMALLY, WE ARE UNAWARE OF THESE FUNCTIONS, BUT THEIR SIGNIFICANCE IS REVEALED BY THE VERTIGO, BLURRED VISION, AND DISEQUILIBRIUM OF VESTIBULAR DISORDERS.

Signals from vestibular hair cells are conveyed to the brainstem and cerebellum by the processes of bipolar vestibular ganglion neurons (VGNs). VGNs that contact the distinct central and peripheral zones of the vestibular sensory epithelia differ in such key features as regularity of spiking, response dynamics, expression of calcium-sensing proteins, axonal diameter and conduction velocity, and central projections (reviewed in Goldberg 2000; Lysakowski and Goldberg 2004; Eatock and Songer 2011). Variation in the mechanosensitive hair cells also contributes to afferent diversity: type I and type II vestibular hair cells synapse with, respectively, calyceal and bouton afferent terminals, and vestibular afferents are either calyx-only, bouton-only, or, if they contact both types of hair cell, dimorphic.

Mammalian vestibular afferents are remarkable for their high firing rates and bimodal distribution of spike regularity, either highly regular or highly irregular. Evidence from isolated VGNs suggests that the irregular spike timing of central-zone afferents depends on part on afferent expression of low-voltage-activated K (K\textsubscript{Ca} and K\textsubscript{Ca}2) channels (Iwashita et al. 2008; Kaliluri et al. 2010). Voltage-gated calcium (Ca\textsubscript{V}) channels and Ca\textsubscript{V2}–gated K channels also vary across VGN populations (Limon et al. 2005). Because neuronal firing patterns are affected by differential expression of voltage-gated sodium channel (Na\textsubscript{V}) subunits (Bean 2007), we have examined Na\textsubscript{V} channel expression by VGNs. The pore-forming \alpha subunits of Na\textsubscript{V} channels are categorized according to their sensitivity to block by tetrodotoxin (TTX). Most \alpha subunits are TTX sensitive, with half-blocking doses of ~10 nM, but Na\textsubscript{V}1.5 channels (TTX-insensitive) require over 10-fold larger doses, and Na\textsubscript{V}1.8 and Na\textsubscript{V}1.9 channels (TTX-resistant) are not blocked even by 1 \muM TTX. Previous studies on VGNs have reported only TTX-sensitive Na\textsubscript{V} currents (Chabbert et al. 1997; Risner and Holt 2006). Here we show that the rat vestibular ganglion expresses mRNA for TTX-sensitive Na\textsubscript{V} \alpha subunits, as expected, but also Na\textsubscript{V}1.5, 1.8, and 1.9 subunits. Na\textsubscript{V}1.5 channels are strongly expressed in cardiac muscle (Gellens et al. 1992), where their current drives the upstroke of the cardiac action potential. The Na\textsubscript{V}1.8 current was first described as a slow current specific to small peripheral nociceptive neurons of the dorsal root ganglia (DRG) and trigeminal ganglia (Akopian et al. 1996; Sangameswaran et al. 1996). We show that many...
early postnatal VGNs express Na\textsubscript{v}1.5-like or Na\textsubscript{v}1.8-like current in addition to TTX-sensitive current and that the TTX-sensitive current also differs between VGNs. The expression of different complements of Na\textsubscript{v} channels by VGN subpopulations is likely to contribute to spiking heterogeneity that serves vestibular encoding.

**MATERIALS AND METHODS**

Animals were handled in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all procedures were approved by the animal care committee at Baylor College of Medicine (RT-PCR), the Massachusetts Eye and Ear Infirmary (physiology and fixation of tissue from reporter mice), and the University of Illinois at Chicago (immunocytochemistry). Chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

**RT-PCR**

Long-Evans rats, postnatal day (P) 1 or P21, were anesthetized and decapitated and the temporal bones were placed in our standard dissection and bath solution, “modified L-15” (Table 1): chilled, oxygenated Leibovitz-15 medium supplemented with 10 mM HEPES at pH 7.4, ~325 mol/kg. The superior division of the vestibular ganglion, which innervates the utricle, lateral, and anterior semicircular canals and part of the saccule, was dissected out bilaterally and placed in RNase/DNase-free tubes. Excess liquid was removed and the tissue samples were placed on dry ice. The methods are identical to those described for utricular maculae and cristae in Wooltorton et al. (2007). Briefly, we isolated RNA from one ganglion at a time with the RNaseasy kit (Qiagen, Valencia, CA). The RNA was reverse-transcribed to complementary (c) DNA with the Advantage RT-for-PCR Kit (BD Biosystems, Palo Alto, CA) with Moloney murine leukemia virus (MMLV) reverse transcriptase and random hexamer primers. We controlled for genomic DNA contamination by designing primers to span intron-exon boundaries; homogenizing the tissue to break up genomic DNA; using RNase-cleaning columns, which contain a silica membrane that eliminates most DNA; and treating the column with RNase-free DNase I (Qiagen) for 30 min to remove residual DNA. As the “negative RT” controls, for each primer set and tissue type we substituted water for the reverse transcriptase. No bands were detected on agarose gels with any primer set tested in these control samples (data not shown).

PCR was done on a PTC-100 thermocycler (MJ Research, Reno, NV) with the TAQ enzyme (Applied Biosystems, Foster City, CA), the primer sets (IDT, Coralville, IA), and the PCR protocol described in Wooltorton et al. (2007). PCR products were resolved on 1.2% agarose gels and visualized with ethidium bromide.

The PCR product for each primer set was sequenced at least once; sequenced products for Na\textsubscript{v}1.1–1.7 and β-subunits were from the utricular macula, as reported previously (Wooltorton et al. 2007). Na\textsubscript{v}1.8 and Na\textsubscript{v}1.9 products, which were not present in the macula, were sequenced from vestibular ganglia (SeqWright, Houston, TX). Each primer set was tested on ganglion tissue multiple times. For all but three primer sets (Na\textsubscript{v}1.7, 1.8, and 1.9), we simultaneously tested for expression in standard tissues (brain, heart, or skeletal muscle), as a control for primer quality. Na\textsubscript{v}1.7, 1.8, and 1.9 are weakly expressed if at all in these standard tissues.

**Cell Preparation for Physiology**

VGNs were isolated from Long-Evans rat pups. The superior compartment of the vestibular ganglion was dissected out of the isolated temporal bone and placed in modified L-15 (Table 1). The ganglion tissue was incubated in 0.25% trypsin and 0.05% collagenase for 30 min at 37°C and then dissociated by gentle trituration onto glass-bottomed culture dishes (MatTek, Ashland, MA). The glass was uncoated for acute preparations and coated with poly-D-lysine for overnight cultures.

Usually, VGNs were dissociated from P1–P8 rat pups, allowed to settle at room temperature (20–25°C), and recorded from between 1 and 7 h posttrituration. In a small number of experiments, dissociated VGNs from P1–P11 rats were incubated in culture medium in 5% CO\textsubscript{2}–95% air at 37°C for ~20 h. The culture medium was serum-free minimal essential medium (MEM) with Glutamax (Invitrogen, ThermoFisher, Waltham, MA), supplemented with 10 mM HEPES and 1%

<table>
<thead>
<tr>
<th>Table 1. External and internal solutions</th>
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<tr>
<td><strong>Solution</strong></td>
</tr>
<tr>
<td>External</td>
</tr>
<tr>
<td>Cs\textsuperscript{+} external</td>
</tr>
<tr>
<td>K\textsuperscript{+} external</td>
</tr>
<tr>
<td>NMDG\textsuperscript{+} external</td>
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<tr>
<td>Modified L-15</td>
</tr>
<tr>
<td>Internal</td>
</tr>
<tr>
<td>NaCl</td>
</tr>
<tr>
<td><strong>KCl</strong></td>
</tr>
<tr>
<td><strong>K\textsuperscript{2}SO\textsubscript{4}</strong></td>
</tr>
<tr>
<td><strong>CsCl</strong></td>
</tr>
<tr>
<td><strong>Mg\textsuperscript{2+}</strong></td>
</tr>
<tr>
<td><strong>ATP</strong></td>
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<tr>
<td><strong>LiGTP</strong></td>
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<tr>
<td><strong>Na-cAMP</strong></td>
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<tr>
<td><strong>EGTA</strong></td>
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<tr>
<td><strong>CaCl\textsubscript{2}</strong></td>
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<td><strong>HEPES</strong></td>
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Concentrations in mM. Dissections were done in modified L-15. Most voltage-clamp recordings were done with Cs\textsuperscript{+} internal and Cs\textsuperscript{+} external solutions. Current-clamp recordings of spiking were done with K\textsuperscript{+} internal and modified L-15. Osmolarity was ~325 mol/kgH\textsubscript{2}O for external solutions except for NMDG\textsuperscript{+} external (315 mol/kgH\textsubscript{2}O) and ~315 mol/kgH\textsubscript{2}O for internal solutions except for the perforated patch solution (270 mol/kgH\textsubscript{2}O). pH was adjusted to 7.3–7.4 by adding KOH to K\textsuperscript{+} internal (~17 mM), CsOH to Cs\textsuperscript{+} internal (~17 mM), and NaOH to external solutions (~4 mM), except for external solutions containing 4-AP or NMDG\textsuperscript{+}, which were titrated to pH 7.3–7.4 with HCl. The free internal Ca\textsuperscript{2+} concentration for 800 μM Ca\textsuperscript{2+} and 5 mM EGTA was calculated as 20 mM (MaxChelator WEBMAXC software, http://maxchelator.stanford.edu/). In some early experiments on the voltage dependence and tetrodotoxin (TTX) sensitivity of total Na\textsubscript{v} current, internal CsCl was 130 mM and external NaCl was 65 mM. Inward Na\textsuperscript{+} currents were correspondingly smaller in those cells but no other differences were noted. These data were not included in estimates of reversal potential. *L-15 also contains 1.34 mM Na\textsubscript{2}HPO\textsubscript{4} and 5 mM Na-pyruvate. See http://www.lifetechnologies.com/us/en/home/technical-resources/media-formulation.80.html.
penicillin/streptomycin (Invitrogen). Data from overnight-cultured neurons are excluded from analysis except for the quantitative characterization of their TTX-sensitive Na\textsubscript{v} currents.

**Recording Solutions**

During recording sessions, cells were bathed in preoxygenated L-15 medium. The recorded cell was locally superfused, usually with “Cs\textsuperscript{+} external” solution (Table 1), which minimized K\textsuperscript{+} currents by substituting Cs\textsuperscript{+} for K\textsuperscript{+} and 50–75 mM tetraethylammonium (TEA, a K channel blocker) for equivalent Na\textsuperscript{+}. Replacing external Na\textsuperscript{+} with TEA\textsuperscript{−} also reduced total Na\textsubscript{v} current and series resistance voltage errors. Ca\textsuperscript{2+} currents were minimized by substituting Mg\textsuperscript{2+}, a Ca channel blocker, for Ca\textsuperscript{2+}. Only trace Ca\textsuperscript{2+} was present.

For current-clamp studies, solutions were more physiological. The external solution was modified L-15 and the internal solution was “K internal” (Table 1). After recording in current-clamp mode, we switched to voltage-clamp mode to characterize the Na\textsubscript{v} currents, changing at the same time from modified L-15 to “K internal” solution containing TEA and 4-AP to block K\textsubscript{v} currents. In several recordings, we used the perforated patch method to preserve the intracellular milieu (Horn and Marty 1988). The pore-forming antifungal drug (amphotericin B, 200 µg/ml) was added to an internal solution containing K\textsubscript{2}SO\textsubscript{4} and KCl (Table 1).

**Whole Cell Recordings**

Cells were visualized at ×600 on an inverted microscope equipped with Nomarski optics (IMT-2; Olympus, Lake Success, NY). We chose round cells with diameters >10 µm (range 14–30 µm, most 18–24 µm) and sometimes partly covered by remnants of the enveloping satellite cells. Almost all of these cells had large total Na\textsubscript{v} currents (peak values >2 nA), consistent with their being VGNs. These diameters are within the range reported by Limón et al. (2005) for VGNs from P7–P10 rats, but smaller on average, probably because our recordings were from a younger age range (P1–P8). The mean membrane capacitance was 15.2 ± 0.4 pF (n = 239), similar to the mean values we obtained previously for P1–P7 rat VGNs with either sustained or transient firing patterns (Kalluri et al. 2010). Kalluri et al. (2010) found that in the second postnatal week, VGNs that fired transiently were larger than those that fired tonically. Thus, in our sample, some neurons have not reached their mature sizes.

Data were acquired with a Multiclamp 700B amplifier, Digidata 1440A digitizer, and pClamp 10 software (Axon Instruments, Molecular Devices, Sunnyvale, CA) with low-pass Bessel filtering set at 10 kHz and sampling interval set at 10 µs. Electodes were pulled from borosilicate glass to resistances of 1–4 MΩ in our solutions and coated with a silicone elastomer (Sylgard 184; Dow Corning, Midland, MI) to reduce electrode capacitance. Voltages were corrected for a liquid junction potential between 5 and 6 mV, calculated for each solution with JPCalc software (Barry 1994) in pClamp10.

To document the incidence of each type of Na\textsubscript{v} current, we included all VGNs for which Na\textsubscript{v} currents could be clearly identified based on their kinetics and voltage dependence. For analyses of voltage-dependent properties and current-clamp behavior, cells were accepted if one of the following criteria was met: input resistance ≥0.5 GΩ; a giga-ohm initial seal with a smooth rupture; and a resting potential of at least −58 mV. For the VGNs analyzed for voltage-dependent properties and voltage responses in current clamp, seal resistances exceeded 1 GΩ and series resistance, R\textsubscript{se}, ranged from 4 to 14 MΩ before electronic compensation. For ruptured-patch recordings, R\textsubscript{se} was compensated electronically, usually by 80–90%, and the average residual R\textsubscript{se} was 1.6 ± 0.1 MΩ (n = 198). For analysis of the relatively large total Na\textsubscript{v} current, we reduced residual R\textsubscript{se} to 1 MΩ or less to minimize voltage-clamp errors. The mean clamp rise time, τ\textsubscript{clump} = [R\textsubscript{se} × C\textsubscript{m} × (1-prediction)], where “prediction” is a fraction set with the amplifier, was 54 ± 2 µs (n = 190). Note also that in external solutions for voltage clamp (Table 1), replacement of one-third to one-half of Na\textsuperscript{+} by the K channel blocker TEA\textsuperscript{−} reduced Na\textsubscript{v} current and series resistance voltage errors. The perforated-patch recordings (see above) had, as is typical, higher R\textsubscript{se} values (mean: 29 ± 4.2 MΩ, n = 11, compensated by 50–85% to 5–15 MΩ), and were not used to measure kinetics or activation.

The holding potential (V\textsubscript{H}) in voltage-clamp mode was –70 mV. Most recordings were at room temperature (range 20–25°C, mean 23.9 ± 0.3°C, n = 130), because fast Na\textsubscript{v} currents are difficult to voltage clamp and characterize at body temperature. For some recordings (indicated), we heated the bath to 37°C with a heated platform and temperature controller (TC-344B; Warner Instruments, Hamden, CT). Note that VGNs exhibit similar firing pattern categories (“transient” vs. “sustained”) at room and body temperatures (Kalluri et al. 2010).

**Pharmacology**

Frozen stock solutions of TTX (2 mM or 200 µM in distilled water) were thawed and added to 10 ml of external solution to make the specified concentrations on the day of the experiment. Drugs were applied via controlled local perfusion (Valvelink 8; AutoMate Scientific, Berkeley, CA). Multiple lines were merged at a manifold that flowed into a single tip (~3-mm long, 250-µm diameter). This dead volume provided a temporal separation between any transient mechanical artifacts induced by line switching and the onset of the drug effect. Perfusion of control solution before the test solution provided an additional control for flow effects. To visualize flow, we added 2 µl of latex beads (0.46–µm mean particle size) to 10 ml of the control and test solutions. Cells were confirmed to be in the path of bead movement and the flow was adjusted to a gentle but steady rate. Wash-in and wash-out of the drug were tracked by a protocol that applied a test voltage step at 10-s intervals. Recordings were analyzed only for cells with stable R\textsubscript{se} values in both control and drug solutions. Many cells also provided stable recordings after wash-out of the drug.

**Analysis**

Analysis was performed in Matlab (The MathWorks, Natick, MA) and Clampfit (Axon Instruments, Molecular Devices). Figures were prepared with Origin software (versions 8–15; OriginLab, Northampton, MA). Means ± SE are presented. The voltage protocol comprised, in sequence, a 25- to 80-ms hyperpolarizing prepulse (from V\textsubscript{H} to −120, −125, or −130 mV) to relieve channel inactivation; a test step of iterated voltage (duration determined by the time course of the current under study, voltage iterated from −125 mV to +30 mV), and a brief tail step at a constant voltage (stepping from the test step to −15, −20, or −30 mV). The voltage dependence of activation and inactivation was analyzed only when R\textsubscript{se} voltage error was <5 mV at peak current and τ\textsubscript{clamp} was <100 µs. For the comparatively small TTX-insensitive and TTX-resistant currents, R\textsubscript{se} voltage errors were typically <3 mV and were not corrected post hoc.

Activation curves of conductance, g, vs. voltage, V, were generated by plotting the peak current (I) against step voltage, fitting the linear upper region of the V- or U-shaped I-V curve (typically between 0 and +30 mV) to find the reversal potential (E\textsubscript{rev}) and then dividing the peak current by the driving force (V-E\textsubscript{rev}) to obtain g. The activation curves were fit by a Boltzmann function:

\[
g(V) = \frac{g_{\text{max}}}{1 + e^{(V-V_{1/2})/s}}
\]

where g\textsubscript{max} is maximum conductance, V\textsubscript{1/2} is voltage of half-maximal activation, and s is the slope factor. Inactivation curves were generated by plotting the peak current produced by a test pulse (to −30, −20, or −10 mV) against the iterated prepulse voltage and fitting with a Boltzmann function:
with $V_{\text{inact}}$ the voltage of half-maximal inactivation.

We fit drug dose-response curves with the Hill equation with Hill coefficient of 1:

$$y = \frac{[\text{drug}]}{K_{d1} + [\text{drug}]}$$

where $y$ is the fraction of channels blocked by drug. This equation assumes simple single-site block with an excess of drug relative to sodium channels and no conductance in the blocked state. It is successful at approximating TTX dose-response relations for NaV channels (e.g., Ritchie 1979; Du et al. 2009). Rearranging the Hill equation allows us to calculate the dissociation constant ($K_d$) from the block at a single drug dose:

$$K_d = [\text{drug}] \times \left( \frac{1 - y}{y} \right)$$

For TTX block of total current in cells with both TTX-sensitive and TTX-insensitive currents, we fit the dose-response relation with:

$$g(V, [\text{drug}]) = \left(1 - \frac{[\text{drug}]}{K_{d1} + [\text{drug}]^2}\right) \left( \frac{A[\text{drug}]}{1 + e^{(V-V_{\text{max}})/s_1}} \right) + \left(1 - \frac{[\text{drug}]}{K_{d2} + [\text{drug}]^2}\right) \left( \frac{1 - A[\text{drug}]}{1 + e^{(V-V_{\text{max}})/s_2}} \right)$$

$V_{\text{inact}}$ and $s$ values for each current were taken from their average values in Table 2. $A$ and the $K_d$ values were estimated by fitting dose-response data from VGNs (see RESULTS).

**NaV1.8 Reporter Lines**

We looked for evidence of NaV1.8 expression in two reporter mice: 1) an NaV1.8 reporter mouse generated by crossing the BAC transgenic “SNS-Cre” line (Agarwal et al. 2004) with a reporter line Rosa26SL-tdTomato (Jackson Laboratory) in which a loxP-flanked STOP cassette prevented transcription of tdTomato, a variant of red fluorescent protein, under the CAG promoter (Madisen et al. 2009); and 2) the NaV1.8-Cre line, in which targeted knockin of Cre replaces the NaV1.8 sequence at its endogenous locus (Stirling et al. 2005). The mice used were hemizygous for SNS-Cre or heterozygous for NaV1.8-Cre and heterozygous for tdTomato. As a positive control for NaV1.8 expression and confirmation of genotyping, we dissected the trigeminal ganglia and DRG from the same animals; NaV1.8 is well documented in somatosensory neurons, including trigeminal ganglion cell bodies (Thun et al. 2009).

Reporter mouse tissues were also labeled with antibodies against calretinin, neurofilament 200 (NF200), and β-III tubulin, as described next.

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**Table 2. Electrophysiological properties of NaV currents in vestibular ganglion neurons.**

<table>
<thead>
<tr>
<th>Current/VGN Subpopulation</th>
<th>$V_{0.5}$, mV</th>
<th>$G_{max}$, nS/pF</th>
<th>$t_{\text{inact}}$, 0 mV, ms</th>
<th>$t_{\text{ramp}}$ – 15 mV, ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaV (total)</td>
<td>-76.7 ± 0.15 (13)</td>
<td>-9.3 ± 0.4 (13)</td>
<td>2.3 ± 0.3 (13)</td>
<td>100 ± 6.2 (13)</td>
</tr>
<tr>
<td>NaV with NaS</td>
<td>-76.4 ± 0.27 (12)</td>
<td>-9.3 ± 0.10 (12)</td>
<td>2.6 ± 0.12 (12)</td>
<td>102 ± 6.1 (12)</td>
</tr>
<tr>
<td>NaV with INaR</td>
<td>-76.3 ± 0.17 (13)</td>
<td>-9.3 ± 0.5 (13)</td>
<td>2.2 ± 0.7 (13)</td>
<td>101 ± 6.1 (13)</td>
</tr>
<tr>
<td>NaV with NaI</td>
<td>-76.5 ± 0.20 (13)</td>
<td>-9.3 ± 0.3 (13)</td>
<td>2.5 ± 0.2 (13)</td>
<td>101 ± 6.1 (13)</td>
</tr>
<tr>
<td>NaV with NaI + NaS</td>
<td>-76.8 ± 0.20 (13)</td>
<td>-9.3 ± 0.3 (13)</td>
<td>2.5 ± 0.2 (13)</td>
<td>101 ± 6.1 (13)</td>
</tr>
<tr>
<td>NaV with NaI + INaR</td>
<td>-76.8 ± 0.20 (13)</td>
<td>-9.3 ± 0.3 (13)</td>
<td>2.5 ± 0.2 (13)</td>
<td>101 ± 6.1 (13)</td>
</tr>
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Values are means ± SE (n). Sample sizes were smaller for activation parameters because higher recording quality is required. Room temperature, Cs internal, and Cs external solutions (Table 1), VGN.

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**Immunohistochemistry**

Long-Evans rats (P3–P21) were deeply anesthetized with gaseous isoflurane or Nembutal (sodium pentobarbital, 80 mg/kg ip) and then either decapitated (younger animals, ages ≤ P6) or transcardially perfused (older animals, ages > P6) with 2 ml/g body wt of an aldehyde fixative [4% paraformaldehyde, 1% acrolein, 1% picric acid, and 5% sucrose in 0.1 M phosphate buffer (PB), pH 7.4]. For young animals, the vestibular ganglia were quickly exposed and then tissue was transferred to 4% paraformaldehyde fixative for 20 min followed by rinses in phosphate-buffered saline (PBS, 0.01 M).

For SNS-Cre reporter mice, vestibular ganglia were dissected in L-15 and then fixed in 4% paraformaldehyde for 2–3 h. For NaV1.8-Cre reporter mice, similar results were obtained whether the tissue was first dissected in L-15 and transferred to fix or fixed by transcardial perfusion of aldehyde fixative. Background fluorescence was reduced by incubating the tissue in a 1% aqueous solution of sodium borohydride for 10 min.

For NaV1.5 immunoreactivity and tissue from SNS-Cre mice, frozen sections (35 μm) were cut with a sliding microtome. NaV1.8-Cre ganglia were studied as whole mounts. Antibodies were from Chemicon (Temecula, CA) unless otherwise specified. Immunocytochemistry was performed on free-floating sections, permeabilized with Triton X-100 in a blocking solution of 0.5% fish gelatin and 1% BSA in PBS. Samples of vestibular tissues were incubated with Triton X-100 at concentrations that depended on age: P1: 0.3% overnight at 4°C; P3: 0.5% for 3 h at room temperature; P8: 2% for 1 h, room temperature; and P21 and adult: 4% for 1 h, room temperature. Samples were then incubated with a cocktail of two primary antibodies: goat anti-calretinin and rabbit anti-NaV1.5 (1:200 in the blocking solution) for 2 days at 4°C with 0.1, 0.3, and 0.5% Triton X-100 for P1, P3, and P8-adult sections, respectively. Specific labeling was revealed with a cocktail of two secondary antibodies: fluorescein-conjugated donkey anti-goat and rhodamine-conjugated donkey anti-rabbit or Alexa Fluor 594-conjugated donkey anti-mouse (from Molecular Probes, Eugene, OR), all at 1:200 in the blocking solution. Sections were rinsed with PBS between and after incubations and mounted on slides in Mowiol (Calbiochem, Darmstadt, Germany). Final image processing was done with Adobe Photoshop CS4 software (San Jose, CA).

For NaV1.5 immunostaining, two kinds of control reactions were done: 1) no primary antibody; and 2) primary antibody preincubated with the antigenic peptide (10 μg/ml, per kg) for 1 h at room temperature. Images comparing labeling in control and test conditions were acquired and digitally processed identically.

In addition, Western blots, performed as described in Wooltorton et al. (2007), yielded a band at the appropriate size (227 kDa) for inner ear and control tissue (heart) prepared from adult rats.

**RESULTS**

**Expression of NaV Channel Subunits in the Vestibular Ganglion**

RT-PCR revealed robust expression of NaV pore-forming (α) subunits and accessory (β) subunits in the superior compartment of the vestibular ganglion, which contains neuronal cell bodies and their satellite cells (Fig. 1). Data were obtained from the ganglion at P1 and at P21. At P1, all known NaV α-subunits were detected; at P21 all were detected except NaV1.4, the skeletal muscle subunit. Most α-subunits (NaV1.1–1.4, 1.6, and 1.7) carry TTX-sensitive currents. NaV1.5 carries TTX-insensitive current and is prevalent in cardiac muscle but also reported in neurons, including somatosensory ganglion neurons (Renganathan et al. 2002), and in hair cells of the rat utricular macula (Wooltorton et al. 2007). NaV1.8 and 1.9 carry TTX-resistant current in somatosensory ganglion neurons. All four β-subunits were expressed.

The positive results for NaV1.5, NaV1.8, and NaV1.9 primers were remarkable given previous reports that all NaV current in VGNs was TTX sensitive. These RT-PCR results on vestibular ganglia were obtained in parallel with RT-PCR results for vestibular (utricular and semicircular canal) epithelia and controls that are reported in Wooltorton et al. (2007). Water (no template) controls and − RT (no reverse transcriptase) controls were negative. In vestibular epithelia, all β-subunits and most α-subunits were detected at P1 and/or P21, but NaV1.8 and NaV1.9 subunits were never detected. Correspondingly, no TTX-resistant currents were recorded in utricular hair cells. These clear negative results serve as an additional control for the strong NaV1.8 and NaV1.9 expression of the vestibular ganglia (Fig. 1B). Positive support for the NaV1.5 result in vestibular ganglia is provided by strong NaV1.5-like immunoreactivity in the distal calyceal terminals (Wooltorton et al. 2007; Lysakowski et al. 2011) of VGNs. In this report, we provide further support for expression of NaV1.5 and NaV1.8 subunits in vestibular ganglion cell bodies in the form of whole cell currents, immunostaining of the cell bodies (NaV1.5), and results with reporter mice (NaV1.8).

To look for functional expression of NaV channels in vestibular afferents, we dissociated VGNs from P1–P8 rats and recorded whole cell currents (n = 239 VGNs) during 7 h of dissociation. Dissociated neuronal somata are more accessible than neuronal terminals in situ and allow better space clamp. Recordings were restricted to the early postnatal period because increasing myelination of vestibular ganglion cell bodies (Dechesne et al. 1987) interferes with patching the neuronal membrane at later ages. In some previous reports, VGNs have been studied after overnight culturing, which reduces myelin and satellite cell coverage of the cell bodies. As described later, we found that culturing overnight may affect expression of NaV channels and have excluded data from overnight-cultured neurons except where indicated.

In all VGNs, fast inactivating inward current was evoked by depolarizing voltage steps following a hyperpolarizing prepulse (Fig. 2). Most recordings were made in solutions designed to minimize K+ and Ca2+ currents (Cs+ internal solution and Cs+ external solution with TEA and only trace Ca2+; Table 1). Experiments described later confirm that these are Na+ currents. We refer to the total voltage-dependent Na+ current as $I_{Na,V}$. Application of the classic NaV channel blocker TTX revealed that $I_{Na,V}$ comprised currents with three different TTX sensitivities in different combinations. TTX-sensitive current ($I_{Na,T}$) was present in all VGNs. In 32 of 109 (29%) VGNs, the total inactivating current was fully blocked by 300 nM TTX (Fig. 2A). Currents that were not TTX sensitive fell into two distinct categories. In many VGNs (69/111, 62%), a fast inward TTX-insensitive current ($I_{Na,I}$) remained in 300 nM TTX (Fig. 2B). $I_{Na,I}$ was a small proportion of total $I_{Na,V}$ at ~20 mV (Fig. 2B, left) but a much larger proportion at ~58 mV (Fig. 2B, right), indicating that $I_{Na,I}$ had a negatively shifted activation range compared with $I_{Na,T}$. Other VGNs (47/178, 26%) had $I_{Na,R}$, a TTX-resistant inward current that was not blocked by micromolar levels of TTX and had slower kinetics.
and a less negative voltage range than either \( I_{\text{NaS}} \) or \( I_{\text{NaI}} \) (Fig. 2C). (Sample sizes differ because different conditions were used to detect different \( \alpha \text{NaV} \) components.) In no case did we detect both \( I_{\text{NaI}} \) and \( I_{\text{NaR}} \) in a single cell, although small levels of expression cannot be ruled out. Evidence on the identities of the channels carrying \( I_{\text{NaI}} \) and \( I_{\text{NaS}} \) was provided in sequence in the following sections. In addition, we show that the properties of \( I_{\text{NaS}} \) differed depending on whether \( I_{\text{NaR}} \) was also present.

**\( I_{\text{NaI}} \) Is Carried by \( \alpha \text{NaV,1.5} \) Channels**

\( I_{\text{NaI}} \) resembled current carried by \( \alpha \text{NaV,1.5} \) channels in its TTX sensitivity, voltage dependence and kinetics, and sensitivity to block by divalent cations.

Reported \( K_{d} \) values for TTX block are 1–10 nM for TTX-sensitive currents and ~100-fold higher for \( \alpha \text{NaV,1.5} \) current (Cribbs et al. 1990). To look for \( \alpha \text{NaV,1.5} \) current, we applied 300 nM TTX, which would block most TTX-sensitive current while significantly sparing any \( \alpha \text{NaV,1.5} \) current. The small current remaining in 300 nM TTX (\( I_{\text{NaI}} \)) activated and inactivated at substantially more negative voltages than total \( I_{\text{NaN}} \) in 0 TTX did (Fig. 2B; Fig. 3A and B), indicating that \( I_{\text{NaI}} \) was a distinct current component. \( I_{\text{NaI}} \) was blocked by a higher concentration of TTX (5 \( \mu \)M) and eliminated by replacing \( \text{Na}^{+} \) with the impermeant ion NMDG\(^{+} \) (Fig. 3C), confirming that it was a \( \text{Na}^{+} \) current. Even in VGNs with \( I_{\text{NaI}} \), 300 nM TTX
block total $I_{NaV}$ by $\sim 90\%$ (at $-25$ to $-35$ mV, Fig. 3A3), indicating that most of the $NaV$ current was TTX sensitive. In control conditions, therefore, $I_{NaV}$ was masked by the much larger $I_{NaS}$, such that the voltage dependence of total $I_{NaV}$ was similar to that of TTX-sensitive channels (Fig. 3, B1 and B2, and Table 2). The masking of $I_{NaV}$ by $I_{NaS}$ in inactivation curves in 0 TTX is explored in a later section.

The activation and inactivation midpoints of $I_{NaV}$ were, respectively, $-10$ and $-25$ mV negative to the midpoints for the total current (Table 2; $P = 0.009$ relative to $I_{NaS}$ in cells without TTX). Reported $V_{inact}$ values for $NaV$ (Fig. 2) range from $-106$ to $-82$ mV (DiFrancesco et al. 1985; Frelin et al. 1986; Sheets and Hanck 1992, 1999; Yamamoto et al. 1993; Wang et al. 1996; Cummins et al. 1998; O’Leary 1998;
Zhang et al. 1999; Kuo et al. 2002; Wooltorton et al. 2007), compared with −35 to −78 mV for TTX-sensitive channels (Noda et al. 1986; Sangameswaran et al. 1997; Dietrich et al. 1998; Smith and Goldberg 1998; Chen et al. 2000; Moran et al. 2003) and approximately −30 mV for NaV1.8 channels (discussed later). Thus the \( V_{\text{inact}} \) value of \( I_{\text{Na}} \) is consistent with NaV1.5 and no other NaV α-subunits.

Since \( I_{\text{Na}} \) activated at significantly more negative voltages than other NaV currents, it contributed significantly to Na+ current evoked by steps from hyperpolarized voltages to near resting potential (Fig. 2B, right). The averaged inactivation and activation curves cross over at −71.5 mV for \( I_{\text{Na}} \) and −56.0 mV for \( I_{\text{NaV}} \), with crossover conductance levels at 6% of peak and correspondingly small window currents (Fig. 3B). Resting membrane potentials range from −75 to −45 mV in isolated VGNs of the first postnatal week (present study and Kalluri et al. 2010) and could be less negative in vivo under the influence of excitatory synaptic input from hair cells. For resting potentials positive to −70 mV, our results indicate that \( I_{\text{Na}} \) channels would be mostly inactivated. We investigated two factors that might negatively shift the voltage range of NaV channels in our experiments: temperature and second messenger availability. Increasing temperature from room to 37°C can shift the inactivation range of NaV channels positively (Ruppersberg et al. 1987; Oliver et al. 1997). For \( I_{\text{Na}} \) in our cells, however, \( V_{\text{inact}} \) was similar at 37°C (−103.3 ± 1.5 mV, \( n = 6 \)) and room temperature (−100.6 ± 1.6 mV, \( n = 15 \); \( P = 0.34 \); Fig. 3B).

Alternatively, loss of intracellular signaling molecules during ruptured-patch recording might shift \( V_{\text{inact}} \) negatively (e.g., Penniman et al. 2010). To test for such washout effects, we recorded from three VGNs with the perforated-patch method. The inactivation range of \( I_{\text{Na}} \) was still very negative (\( V_{\text{inact}} = −101.8 ± 4.1 \) mV; no significant difference from ruptured-patch recordings, \( P = 0.78 \); Fig. 3B). Ruptured-patch data in Fig. 3D further indicate that the negative inactivation range was not a washout or rundown artifact: 1) the negative inactivation range could be seen within seconds of patch rupture (red inverted triangles); 2) in 0 TTX, the inactivation range of total current, \( I_{\text{NaV}} \), did not shift negatively in ∼10 min following patch rupture (purple, cyan, and green curves show data from 3 cells); 3) removing TTX from the external solution moved the inactivation range of total NaV current positively with time (compare black squares, taken early in TTX, and gray circles, taken later from the same VGN after removing TTX).

Another property of NaV1.5 currents that distinguishes them from TTX-sensitive currents is a relatively high sensitivity to block by Cd2+ and Zn2+ (Backx et al. 1992; Chen et al. 1992; Heinemann et al. 1992; Satin et al. 1992). Reported half-blocking concentrations (IC50) of Cd2+ for NaV1.5 current are 50–250 μM (DiFrancesco et al. 1985; Scornik et al. 2006; Wooltorton et al. 2008), in contrast to 5 mM for TTX-sensitive currents (Frelin et al. 1986; Roy and Narahashi 1992). Like NaV1.5 current, \( I_{\text{Na}} \) was ∼half-blocked by 200 μM Cd2+ (Fig. 3E), a dose that barely affects TTX-sensitive current. We first isolated \( I_{\text{Na}} \) by recording in Cs+–based external solution with 300 mM TTX and then added 200 μM CdCl2 to the external solution (Fig. 3E). In 13 cells, 200 μM Cd2+ reversibly blocked \( I_{\text{Na}} \) at −20 mV by 53 ± 4%. Assuming that the block is of a single current, the measured block yields a \( K_d \) of 179 μM (Eq. 4), consistent with the reported range for NaV1.5 channels. (Note that the \( K_d \) for Cd2+ may be lower in 0 TTX because Cd2+ competes with TTX for a common binding site; Doyle et al. 1993; Renganathan et al. 2002.) \( I_{\text{Na}} \) at −20 mV was negligibly blocked by 200 μM Cd2+ in three of three cells (block of 0.6 ± 4.7%). The current blocked by 200 μM Cd2+ resembled the total current in 300 nM TTX (\( I_{\text{Na}} \)) in its kinetics (Fig. 3E, normalized currents) and voltage dependence of inactivation (Fig. 3E), supporting the interpretation that \( I_{\text{Na}} \) is dominated by a Cd2+-sensitive inward current with fast kinetics and relatively negative voltage range.

Given that \( I_{\text{Na}} \) was partly blocked by 200 μM Cd2+, a known blocker of voltage-gated Ca2+ channels, how can we be certain that it was not carried by CaV channels? This conclusion is based on multiple arguments: 1) \( I_{\text{Na}} \) was almost entirely blocked at 5–10 μM TTX (\( n = 7 \); Fig. 3C). In two cells, we were able to test for reversibility and in both cases the additional block by 5–10 μM TTX could be reversed and reapplied multiple times. The difference current obtained by subtracting current in 5 or 10 μM TTX from current in 300 nM TTX had similar biophysical properties to \( I_{\text{Na}} \), consistent with \( I_{\text{Na}} \) being primarily a single TTX-blockable current. 2) \( I_{\text{Na}} \) was eliminated by replacing external Na+ with the impermeant ion NMDG+ (Fig. 3C). This effect was almost fully reversible (>95%, 3 VGNs). 3) The biophysical properties of \( I_{\text{Na}} \) were not consistent with those of inactivating T-type Ca2+ currents. \( I_{\text{Na}} \) inactivated with a time constant of ∼300 μs at 0 mV and was half-inactivated at approximately −100 mV (Table 2), in contrast to T-type Ca2+ currents, which have inactivation time constants ranging from ten to hundreds of milliseconds (depending on voltage and subunit) and \( V_{\text{inact}} \) values from −47 to −86 mV (Coulter et al. 1989; Monteil et al. 2000; Chemin et al. 2001; Komora et al. 2002; Diaz et al. 2005; Nelson et al. 2005; Vitko et al. 2005; Emeric et al. 2006; Zhong et al. 2006). The fast inactivation of L-type Ca2+ current is relatively slow, with time constants of ∼15 ms or more (Beuckelmann et al. 1991; Mewes and Ravens 1994; Magyar et al. 2000) and is Ca2+ dependent, with reduced extent and speed of inactivation when Ba2+ or Na+ (in μM Ca2+) is the charge carrier (Breym and Eckert 1978; Lee et al. 1985; Ferreira et al. 2003; Brunet et al. 2009). In contrast, we observed rapid and near complete inactivation of \( I_{\text{Na}} \) when Na+ was the charge carrier and current was eliminated by Na+ replacement (Fig. 3C). 4) \( E_{\text{rev}} \) for \( I_{\text{Na}} \) was consistent with a Na+ current. For our usual recording solutions (Cs+ internal and Cs+ external, Table 1), we estimated \( E_{\text{rev}} \) from \( I-V \) curves (see MATERIALS AND METHODS) as +49 ± 2 mV (\( n = 10 \)) for total NaV current and +50 ± 5 mV (\( n = 6 \)) for \( I_{\text{Na}} \). \( E_{\text{Na}} \) calculated from the nominal Na+ concentrations (Table 2) equaled +62 mV but \( E_{\text{rev}} \) is expected to be lower because of nonnegligible Cs+ permeability, \( P_{\text{Cs}} \). Ratios of \( P_{\text{Cs}} \) to \( P_{\text{Na}} \) for mammalian cardiac NaV currents, which \( I_{\text{Na}} \) closely resembles, range from 0.007 to 0.06, with a mean across studies of 0.02 (reviewed in Kurata et al. 1999). In our solutions, the predicted \( E_{\text{rev}} \) for a permeability ratio of 0.02 is +53 mV, close to the value (+50 mV) extrapolated from the \( I_{\text{Na}} \) \( I-V \) relations. 5) Our external solutions for recording NaV currents contained only trace (no added) Ca2+. CaV channels do become permeable to Na+ at concentrations of Ca2+ <10 μM (Almers and McCleskey 1984; Hess and Tsien 1984), but to achieve such low concentrations usually requires chelators, which we did not include. Moreover, the voltage dependence of CaV channels is similar.
whether the current is carried by Na⁺ or Ca²⁺ (Fukushima and Hagiwara 1985), and, as already noted, the voltage dependence of I₅₀ is very different from that of T-type channels. Our external solution included 3.5 mM Mg²⁺ to help counter the effects of low Ca²⁺ on apparent voltage dependence (Frankenhaeuser and Hodgkin 1957) and to block CaV channels (Fukushima and Hagiwara 1985; Kurejová et al. 2007). It is improbable that I₅₀, which is as large as 2 nA (below), was carried by trace Ca²⁺ or by Na⁺ through partly blocked CaV channels.

**Sizes and Proportions of I₅₀, I₅0 and Iₐν Current**

Total peak current (I₅₀) for cells without I₅₀ was −13.1 ± 1.8 nA (n = 6; range −5.7 to −17.0 nA) in K⁺ internal solution and K⁺ external solution (100 mM Na⁺) and −11.7 ± 0.9 nA (n = 21) in Cs⁺ internal solution and Cs⁺ external solution (80 mM Na⁺). [Note that external Na⁺ was reduced from physiological levels (−145 mM) to reduce current size and associated voltage-clamp errors.] The mean peak current in 300 nM TTX (I₅₀) was −1.2 ± 0.2 nA (n = 6) (range −6.0 to −2.2 nA) for K⁺ internal and K⁺ external solutions and −834 ± 115 pA (n = 24) for Cs⁺ internal and Cs⁺ external solutions. Peak I₅₀, estimated by subtracting the current in 300 nM TTX from the control condition, was −12.3 ± 1.6 nA (n = 14; all solutions). Because I₅₀ was partly blocked in 300 nM TTX, these estimates underestimate I₅₀ and overestimate I₅₀.

We estimated proportions of I₅₀, I₅₀ and I₅₀ in VGNs with both currents from the dose-response curve for the total Na⁺ current at −15 mV. A dose-response curve of 11 normalized data points pooled from five cells (Fig. 4A) is better fit by a two-component model (Eq. 5) than a one-component model (Eq. 3); the deviation of the data from a single-component fit exceeds the variability between the data points. According to the two-component fit, I₅₀ was 8.4% of the total current and had an IC₅₀ of 774 nM; I₅₀ constituted the remainder with an IC₅₀ of 8 nM. These values are consistent with the range reported for Na₅₀ currents (150 nM to 2 µM; reviewed in Woolferton et al. 2008) and the range of 1–10 nM typical of TTX-sensitive currents.

If I₅₀ is present within the total Na⁺ current, why is it not visible as a separate component in the inactivation curves? To explore this question, we used Eq. 6 to simulate, for various TTX concentrations, the inactivation curve for total Na⁺ current in a cell with 10% I₅₀ plus 90% I₅₀ and K₆ of 800 and 5 nM, respectively, similar to the IC₅₀ of the two-component fit in Fig. 4A. The inactivation midpoints and slopes of the two inactivation components in Eq. 6 were set to the average values for I₅₀ and for I₅₀ in VGNs with I₅₀ (Table 2). The effect of I₅₀ on the shape of the simulated inactivation curve at different TTX doses (Fig. 4B1) reproduces data (Fig. 4, B2 and B3) well and shows that in such a mixture, I₅₀ cannot be distinguished as a separate component, especially at 0 TTX where I₅₀ dominates. At moderate TTX doses (100–300 nM), V₅₀ shifted negatively and the slope decreased, consistent with a more balanced contribution of two currents. The mean s value for the inactivation curve was 7.4 ± 0.2 mV (n = 27) at 0 TTX; in contrast, s values were between 12.4 and 13.4 in four of five cells in 100 nM TTX. At high TTX doses (1 µM), I₅₀ dominated.

Although I₅₀ was just 10% of total Na⁺ current at −15 mV, its maximum size of ~200 to ~2,000 pA in 100 mM external Na⁺ (and larger in physiological Na⁺ concentrations) is well above current thresholds for spiking, particularly in VGNs of the first postnatal week (10–100 pA, Kalluri et al. 2010). The mean I₅₀ conducance density was 1.0 ± 0.2 nS/P (n = 9), 8% of the mean total NaV conducance density per cell (13.0 ± 2.6 nS/P, n = 9).

Our results suggest several reasons that Na₅₀ currents has not been reported previously in VGNs. First, even in cells with I₅₀, the total current had similar biophysical properties to I₅₀ as predicted for cells with a ~10% contribution of I₅₀ (Fig. 4B). Second, the very negative inactivation range of I₅₀ required a deeply hyperpolarized prepulse (to −130 mV in Fig. 3A) to relieve inactivation and fully reveal the full conducance. Using less negative prepulses (Chabbert et al. 1997; Risner and Holt 2006) would reduce the pool of Na₅₀ channels available for activation. Third, the practice of culturing ganglion neurons overnight to reduce satellite cell coverage may suppress I₅₀ expression either by the conditions or the passage of time ex vivo: in 14 VGNs that we recorded from after overnight culture, I₅₀ was small or undetectable. We also did not observe I₅₀ in cultured cells but did not specifically...
test for it. Fourth, it is not uncommon in NaV channel studies to add Cd²⁺ to block CaV channels, and this would block NaV1.5 channels.

Finally, VGN maturation may play a factor in whether INaR is detected in VGNs. In our sample, the incidence of INaR in VGN cell bodies decreased toward the end of the first postnatal week (Fig. 5, dark bars). To estimate the incidence of INaR, we counted only cells that had total peak INaV larger than 4 nA in 0 TTX; for smaller INaV, the voltage dependence of the much smaller TTX-insensitive component was difficult to analyze. INaI was identified by its TTX insensitivity or Cd²⁺ sensitivity and its kinetics and voltage range. For VGNs dissociated on P1–P5, 60–85% of the recorded VGNs had detectable INaI. By P6–P8, the incidence dropped to 30–40%.

NaV1.5-Like Immunoreactivity in VGN Somata

The calyceal terminals of VGNs on type I hair cells in vestibular epithelia are strongly stained by NaV1.5 antibody during development (Wooltorton et al. 2007) and in adult (Lysakowski et al. 2011). With the same antibody, we also observed immunoreactivity in cell bodies within the ganglion, examined from ages P3–P21 (Fig. 6). Use of calretinin antibody at P8 and P21 showed that some NaV1.5-immunoreactive VGNs were also immunoreactive for calretinin, a marker for the large, irregularly-firing “calyx-only” afferents that form calyceal terminals on type I hair cells in the central zones of vestibular epithelia (Desmadryl and Dechesne 1992; Kevetter and Leonard 2002; Leonard and Kevetter 2002; Desai et al. 2005a,b).

INaR Is Carried by NaV,1.8 Channels

In approximately one-quarter (47/178) of VGNs, depolarizing voltage steps in 300 nM TTX evoked a relatively slow inactivating inward current, INaR, which resembled currents carried by NaV,1.8 (SNS, PN3) channels, first identified in DRG (Akopian et al. 1996; Sangameswaran et al. 1996). Distinctive properties of NaV1.8 current include TTX resistance, relatively positive voltage dependence, slow time course, and rapid recovery from inactivation. INaR was readily detected in the presence of other NaV currents by its distinctive voltage range and time course (Figs. 2 and 7, A–C). In all cases of INaR recorded in 0 TTX (n = 20), INaS was also present. In all cases of INaR recorded in 300 nM TTX to block most INaS, either no fast component (9 cells) or only a very small fast component (12 cells) could be discerned. Where it was possible to analyze that fast component (5 cells), it resembled INaS rather than INaR. Thus we recorded no examples of INaR and INaI in the same cell.

For analyses of biophysical properties, we used a subset of 23 VGNs that satisfied two criteria: almost all INaS was blocked, and unblocked outward current that overlapped in time and voltage dependence with INaR was at least 15-fold smaller than the peak inward INaR. The voltage dependence (Fig. 7B) and time course (Fig. 7, A and C) of INaR (Table 2) were similar to reported values for NaV1.8 current in somatosensory afferent neurons.

Inactivation and activation curves of INaR were well fit by single Boltzmann functions (Fig. 7B, bottom left), with midpoints of −31 and −16 mV (Table 2), comparable to values between −25 to −35 mV and −15 to −20 mV, respectively, for NaV1.8 currents in DRG neurons (Akopian et al. 1996; Cummins and Waxman 1997; Sangameswaran et al. 1997; Leffler et al. 2005). NaV1.9 channels (reviewed in Dib-Hajj et al. 2015) have substantially more negative midpoints of activation and inactivation (−50 to −60 mV) and a larger window current. Relative to INaI and INaS, the time to peak, Ipeak, for INaR was three- to fourfold slower at 0 mV and eightfold slower at −15 mV (Table 2) and consistent with Ipeak values for NaV1.8 channels of ~2 ms at ~0 mV (Elliott and Elliott 1993; Scholz et al. 1998). Inactivation of INaR was well fit by a single
exponential with a time constant of $\sim 2.5$ ms at 0 mV and $\sim 8$ ms at $-15$ mV, slower than $I_{NaS}$ and $I_{NaI}$ (Table 2 and Fig. 7C) and close to inactivation time constants of $NaV_{1.8}$ currents (3.5 ms at 0 mV, 9 ms at $-15$ mV; Renganathan et al. 2002).

$I_{NaR}$ was highly resistant to TTX. In Fig. 7D, increasing the TTX concentration from 300 nM to 5 $\mu$M, a dose that blocked most of $I_{NaI}$, produced only 7% additional block of $I_{NaR}$. Solving Eq. 4 for the effect yields a $K_d$ of 61 $\mu$M, within the expected range for either $NaV_{1.8}$ channels or $NaV_{1.9}$ channels ($IC_{50}$ values 40 to >100 $\mu$M; Akopian et al. 1996; Sangameswaran et al. 1996; Rabert et al. 1998; Cummins et al. 1999).

$I_{NaR}$ was carried by Na$^+$. Substitution of external Na$^+$ with NMDG$^+$ reversibly eliminated the current (Fig. 7E; eliminated in 13/13 cells; reversed in 7 of 7 cells that were tested for reversibility). $E_{rev}$ for $I_{NaR}$ in Cs$^+$ external solution was $+50 \pm $
2 mV (n = 9), similar to $E_{\text{rev}}$, estimates for total $I_{\text{NaV}}$ and $I_{\text{NaI}}$ (49–50 mV, above).

$Na_{1.8}$ current recovers from fast inactivation an order of magnitude faster than TTX-sensitive current recovers at the same voltage (Elliott and Elliott 1993), consistent with the general observation that time constants for macroscopic transitions of channel states become faster away from the midpoint of the inactivation range. The fast recovery time constant of $I_{\text{NaR}}$ from inactivation at −90 mV (1.0 ± 0.2 ms, n = 3, Fig. 7F, left) is consistent with recovery time constants for $Na_{1.8}$ currents in small DRG neurons (∼1 ms at −100 mV; Cummins and Waxman 1997) and 4 ms at −67 mV (Elliott and Elliott 1993) and one-quarter the recovery time constants for TTX-sensitive current in small DRG neurons (Everill et al. 2001).

In addition to the inactivation described above, $Na_v$ channels can enter into a distinct slow inactivated state, lasting seconds, which probably involves a different channel conformation (reviewed in Ulbricht 2005). $Na_{1.8}$ is unusual among $Na_v$ subunits for its rapid entry into slow inactivation from which it recovers slowly, even for relatively short depolarizations, contributing to adaptation of responses to physiological stimuli (Blair and Bean 2003). In our protocols probing recovery from inactivation (Fig. 7F, left), only ~75% of $I_{\text{NaR}}$ recovered with the fast time constant. The remainder of the recovery occurred with a slow time constant (1,060 ± 196 ms, n = 4), even for brief inactivating steps (Fig. 7F, right).

In summary, the properties of $I_{\text{NaR}}$ are consistent with previous reports of current through $Na_{1.8}$ channels. As noted for $I_{\text{NaI}}$, it is very unlikely that $Ca_v$ channels were involved, for similar reasons: $I_{\text{NaR}}$ is carried by $Na^+$, there was only trace $Ca^{2+}$ in the external medium, and the voltage dependence and time course differed substantially from reported values for T-type ($Ca_{v3.1}$) $Ca^{2+}$ currents, including T-type $Ca^{2+}$ currents in immature mouse vestibular ganglion cells (Desmadyrl et al. 1997; Chambard et al. 1999; Autret et al. 2005; Limón et al. 2005). Furthermore, the fast time constant for recovery from inactivation was much faster for $I_{\text{NaR}}$ than for T-type current (1 ms at −90 mV, Fig. 7F, vs. 25–50 ms at −100 mV; Hering et al. 2004).

**Size, Proportion, and Incidence of $I_{\text{NaR}}$**

The average peak $I_{\text{NaR}}$ was −2,962 ± 642 pA (n = 4; range −1,620 to −4,470 pA) or −109 ± 16.6 pA/pF in K$^+$ internal and K$^+$ external solutions and $−1,978 ± 231$ pA or $−78 ± 9.4$ pA/pF (n = 7) in Cs$^+$ internal and Cs$^+$ external solutions. Peak $I_{\text{NaR}}$ conductance was $32.9 ± 4.0$ nS and peak conductance density was $1.47 ± 0.25$ nS/pF (n = 7; Cs$^+$ internal and Cs$^+$ external solutions). Since we excluded cells for which peak $I_{\text{NaR}}$ was <15 times the residual (unblocked) outward current, these values may be larger than the true mean.

In five cells, we could segregate $I_{\text{NaR}}$ and $I_{\text{NaS}}$ pharmacologically by applying high doses of TTX to reveal $I_{\text{NaR}}$ and subtracting to obtain $I_{\text{NaS}}$. In five other cells, we estimated the ratio of peak $I_{\text{NaR}}$ to peak $I_{\text{NaS}}$ by comparing peak $I_{\text{NaR}}$ at −15 mV after a prepulse to −120 mV, which inactivated neither current, and after a prepulse to −40 mV, which inactivated $I_{\text{NaS}}$ fully but $I_{\text{NaR}}$ only by 15% (method illustrated in Fig. 7A1). To calculate proportions of each current, we corrected for the differences in percent maximal activation at the test voltage of −15 mV (100% for $I_{\text{NaS}}$, vs. 76 ± 4%, n = 13, for $I_{\text{NaR}}$, Fig. 7B) and for the 15% inactivation of $I_{\text{NaS}}$ at −40 mV. The method was validated by applying it to VGNs for which we also had pharmacological isolation of the currents, which showed that similar isolation was obtained (compare the red curves in Fig. 7, A2 and A3). The ratios of peak $I_{\text{NaR}}$ to peak $I_{\text{NaS}}$ did not differ significantly between data collected with and without TTX and were pooled, yielding a mean value of 3.8 ± 0.6 (range 1.8 to 7.5). Thus peak $I_{\text{NaR}}$ was at least twice as large as $I_{\text{NaS}}$ in a given cell. Peak current ratios may underestimate the relative influence of the TTX-resistant current, which, being slow, prolongs depolarization and its influence over other voltage-gated channels and calcium influx.

The incidence of $I_{\text{NaR}}$ in our sample as a function of postnatal age increased rapidly from zero at P1 to ~30% by P3–P4 (Fig. 5, hatched bars), in contrast to the decreasing incidence of $I_{\text{NaI}}$. Thus, by the latter part of the first postnatal week, approximately one-third of cells expressed $I_{\text{NaR}}$ and $I_{\text{NaS}}$, one-third expressed $I_{\text{NaS}}$ and $I_{\text{NaR}}$, and one-third expressed only $I_{\text{NaS}}$.

**TTX-Sensitive Currents Differed Between VGNs With and Without TTX-Resistant Current**

$I_{\text{NaS}}$ was isolated by subtraction of records in 100–300 nM TTX from control records (0 TTX). $I_{\text{NaS}}$ in VGNs with $I_{\text{NaR}}$ differed from $I_{\text{NaS}}$ in VGNs with $I_{\text{NaI}}$ (Fig. 8 and Table 2) in

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**Fig. 7.** Some VGNs expressed a TTX-resistant $Na_v$ current ($I_{\text{NaR}}$) with properties of $Na_{1.8}$ channels. A: in responses to an inactivation protocol with $V_{\text{holding}} = −70$ mV, 80-ms prepulse, and test step to −15 mV, $I_{\text{NaR}}$ was distinguished from $I_{\text{NaS}}$ by its less negative voltage dependence and slower time course for P6 VGN. Vertical scale bar applies to A–F2; A1 has a shorter time scale than A2 and A3. A1 and A2: control data (K$^+$ internal, K$^+$ external). A3’s expanded time scale shows separation of $I_{\text{NaS}}$ (blue dashed line) and $I_{\text{NaR}}$ (red dashed line) with voltage and time, allowing estimation of their amplitudes in 0 TTX (see RESULTS). After a prepulse to −40 mV (red traces), $I_{\text{NaS}}$ was almost completely inactivated but $I_{\text{NaR}}$ was minimally inactivated; thus peak current after a −40-mV prepulse provided an estimate of $I_{\text{NaS}}$. Conversely, the difference current (blue dashed line) between −120- and −40-mV prepulses was almost entirely $I_{\text{NaR}}$. This method was validated by blocking $I_{\text{NaS}}$ with 300 nM TTX (A3), which did not affect current after the −40-mV prepulse (red trace, compare to control), confirming that it was $I_{\text{NaS}}$. B: voltage ranges of activation and inactivation. Solutions: Cs$^+$ internal, Cs$^+$ external. Top: peak V-I-relations in control and 300 nM TTX, for the cell in A. Bottom left: activation data (circles) and inactivation data (triangles) for $I_{\text{NaR}}$ obtained by subtracting traces in 300 nM TTX from control traces (black) and for $I_{\text{NaS}}$ obtained in 300 nM TTX (red), for the cell in A. Data were fitted with Eqs. 1 and 2. $I_{\text{NaR}}$ inactivation curve: $V_{\text{m,act}} = −32.1$ mV, $s = 5.4$ mV, activation curve: $V_{\text{m,act}} = −14.8$ mV, $s = 5.4$ mV, $g_{\text{m,act}} = 51.1$ nS. $I_{\text{NaS}}$ inactivation curve: $V_{\text{m,act}} = −63.6$ mV, $s = 11.9$ mV; activation curve: $V_{\text{m,act}} = 33.4$ mV, $s = 4.3$ mV, $g_{\text{m,act}} = 106.0$ nS. For B, g was normalized to $g_{\text{m,act}}$. Bottom right: superimposed activation and inactivation curves from individual fits (thin gray lines, room temperature) and average fits (thick black lines are shown). Average parameter values are in Table 2. Thin red lines, data at 37°C, not in average. C: time constants of fast inactivation of $I_{\text{NaS}}$ decreased 10-fold to −2 ms as voltage was made less negative from the range of approximate $V_{\text{m,act}}$ to +15 mV; prepulse was −125 mV, 50 ms. Numbers of values averaged (one per VGN) are indicated above each data point. Inset: most smaller averaged values for $I_{\text{NaR}}$ and $I_{\text{NaS}}$ on an expanded ordinate. D: $I_{\text{NaR}}$ was blocked only slightly more (7%) by 5 μM TTX than by 300 nM TTX. Data are from P7 VGN. E: $I_{\text{NaR}}$ was completely and reversibly eliminated by replacing external Na$^+$ with NMDG$^+$. Data are from P5 VGN. F: $I_{\text{NaR}}$ recovery at −90 mV from inactivation during 25-ms steps at 0 mV, in 4 VGNs. $I_{\text{NaR}}$ recovered −75% within ms (left) but took seconds to recover fully (right). Solutions were Cs$^+$ internal and Cs$^+$ external except for blue triangles: K$^+$ internal, K$^+$ external (P7). Open circles, P7 (same VGN at left and right). Red inverted triangles, P2. Black triangles, P5.
voltage dependence and fast recovery from inactivation of 

After recording firing patterns, we switched to voltage-clamp 
effects of washout of endogenous molecules on firing patterns. Table 1) and no ion channel blockers. Current-clamp record-

Boltzmann parameters, numbers of cells in each group, and means 

size (about half) and voltage dependence: less negative ($P = 0.005$ for $V_{\text{inact}}$; $P = 0.004$ for $V_{\text{act}}$) and broader inactivation range ($s_{\text{inact}}, P = 0.0007$) and was similarly distinct from $I_{\text{NaS}}$ in cells with neither $I_{\text{NaR}}$ nor $I_{\text{NaR}}$ (Table 2). These differences might arise from the expression of different combinations of 

Firing Properties of Cells with $I_{\text{NaR}}$

Eighty VGNs were studied in current-clamp mode with more physiological solutions (K$^+$ internal and modified L-15, Table 1) and no ion channel blockers. Current-clamp record-
ings were made immediately upon rupture to reduce possible 
effects of washout of endogenous molecules on firing patterns. After recording firing patterns, we switched to voltage-clamp mode and K$^+$ external solution with K channel blockers and no added Ca$^{2+}$ (Table 1) to determine whether $I_{\text{NaR}}$ was present. $I_{\text{NaR}}$ was recognized by its distinctive voltage and time depend-
ence.

The most obvious differences in firing were between VGNs that expressed $I_{\text{NaR}}$ ($n = 11$ VGNs) and VGNs that lacked $I_{\text{NaR}}$ ($n = 29$ VGNs). Differences in other currents, including the differences in $I_{\text{NaS}}$ described above, may contribute. Nevertheless, certain firing properties of VGNs with $I_{\text{NaR}}$ resemble those in NaV1.8-expressing DRG neurons. The depolarized voltage dependence and fast recovery from inactivation of NaV1.8 channels are thought to contribute to tonic firing during sustained depolarizations. The slower time course produces broad spikes with an infection or hump during the repolariza-
tion phase (reviewed in Rush et al. 2007). These features were observed in spikes from VGNs with $I_{\text{NaR}}$, as shown in Fig. 9.

The current pulses we used to evoke action potentials were brief (500 µs) to minimize the influence of injected current on the action potential (Fig. 9A); large currents were required to achieve threshold with such brief pulses. The current amplitude was increased in 100-pA increments until a spike was evoked. The properties of this spike were compared for five VGNs with $I_{\text{NaR}}$ and six VGNs without $I_{\text{NaR}}$ at 23–25°C. The voltage threshold of the action potential (voltage at which $dV/dt$ reached 4 mV/ms, chosen to be clearly above noise) was 10 mV less negative in cells with $I_{\text{NaR}}$ ($-44.2 \pm 2.5$ mV) than in cells without $I_{\text{NaR}}$ ($-53.8 \pm 1.1$ mV, $P = 0.0003$). The peak $dV/dt$ on the upstroke of the action potential was twice as fast for cells without $I_{\text{NaR}}$ (207 ± 24 mV/ms) than for cells with $I_{\text{NaR}}$ (99 ± 11 mV/ms; $P = 0.004$). These differences may reflect the smaller size and less negative activation range of $I_{\text{NaS}}$ in cells with $I_{\text{NaR}}$, combined with a strong contribution from $I_{\text{NaR}}$ once voltage exceeds spike threshold. Spikes in VGNs with $I_{\text{NaR}}$ were of similar height to, but broader than, spikes in VGNs without $I_{\text{NaR}}$ (Fig. 9A). Spike height, measured from the spike peak to the afterhyperpolarization trough, was 105.5 ± 2.1 mV for cells with $I_{\text{NaR}}$ and 102.9 ± 3.1 mV for cells without $I_{\text{NaR}}$. Spike width at half-height was ~2–4 ms (2.64 ± 0.31 ms) for cells with $I_{\text{NaR}}$ and ~1 ms (1.07 ± 0.08 ms, $P = 0.006$) for cells without $I_{\text{NaR}}$. The slower kinetics and more depolarized voltage dependence of inactivation of $I_{\text{NaR}}$ may broaden spikes, as reported in DRG and nodose ganglion neurons that have NaV1.8 current (Ritter and Mendell 1992; Djouhri et al. 2003). In addition to being broader, spikes in every VGN with $I_{\text{NaR}}$ had a shoulder on the repolarization

![Fig. 8](http://jn.physiology.org/doi/10.1152/jn.00902.2015/fig8) - Fig. 8. The TTX-sensitive current differed between cells with TTX-resistant 
current and cells without TTX-resistant current. $I_{\text{NaS}}$ in VGNs with $I_{\text{NaR}}$ (red) had a less negative voltage dependence and broader inactivation range than $I_{\text{NaS}}$ in VGNs with only $I_{\text{NaS}}$ (black), and in VGNs with $I_{\text{NaR}}$ (blue). Thin lines, Boltzmann fits for individual cells; thick lines, mean curves. See Table 2 for 

![Fig. 9](http://jn.physiology.org/doi/10.1152/jn.00902.2015/fig9) - Fig. 9. Spike shape and firing pattern differed between VGNs with $I_{\text{NaR}}$ and 
VGNs without $I_{\text{NaR}}$. K$^+$ internal, modified L-15 external. A: representative examples of voltage responses to 500-µs current steps are shown at firing 
threshold: 1.150 pA for a VGN with $I_{\text{NaR}}$ and 400 pA for a VGN without $I_{\text{NaR}}$. VGNs with $I_{\text{NaR}}$ had broader action potentials with less negative voltage 
thresholds and a shoulder during repolarization (arrow). Both cells: P7. Presence of $I_{\text{NaR}}$ for each VGN was determined in voltage clamp after 
changing to K$^+$ external with K channel blockers. B: firing patterns evoked by depolarizing current steps, 200 ms ($B1$) or 400 ms ($B2, B3$) long. VGNs with $I_{\text{NaR}}$ fired either 1–2 spikes ($B1$) or multiple spikes ($B2, B3$). For multispiking 
VGNs, the presence of $I_{\text{NaR}}$ was correlated with constant spike height throughout 
the train. Current steps of 160 pA ($B1$), 240 pA ($B2$), and 130 pA ($B3$) were selected to yield similar steady-state voltage. Insets: individual first spikes 

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negative activation range, TTX-sensitive currents may dominate the initial phase of the spike around threshold, but TTX-resistant currents should contribute strongly later in the spike (Blair and Bean 2002). In this way, inactivation of TTX-sensitive currents may increase spike threshold until spiking stops without much affecting spike height.

Despite a significant overlap of activation and inactivation ranges (a “window current”), the activation range of $I_{NaR}$ was positive relative to most resting potentials. Mean resting potentials were $-68.2 \pm 2.1$ mV ($n = 6$) for cells with $I_{NaR}$ and $-71.6 \pm 0.7$ mV ($n = 26$) for cells without $I_{NaR}$ ($P = 0.19$). Thus the transient component of the current played little role in resting potential in these isolated neurons, although persistent current might (Han et al. 2015). We observed only two spontaneously firing VGNs in this study, neither of which had $I_{NaR}$.

**Na$_{v}$1.8 Expression in Reporter Mice**

Our electrophysiological data suggest that TTX-resistant currents carried by Na$_{v}$1.8 channels are expressed by a VGN subpopulation that also differs from other neurons in the properties of TTX-sensitive current and spikes. To visualize this subpopulation, we examined the vestibular ganglia of two mouse lines in which cells that expressed Na$_{v}$1.8 (SNS) also expressed the red fluorescent protein tdTomato (see MATERIALS AND METHODS). We refer to the BAC transgenic line as SNS-Cre and the targeted knockin as Na$_{v}$1.8-Cre (Stirling et al. 2005).

Confocal images in Fig. 10 illustrate the tdTomato signal in three vestibular ganglia and one DRG. Figure 10A shows an SNS-Cre/Rosa26LslTdTdTmto reporter mouse line in which the targeted knockin and the red fluorescent protein tdTomato are coexpressed. A confocal image shows the tdTomato signal in the DRG (Fig. 10B). In 2 Na$_{v}$1.8 reporter mouse lines, different percentages of vestibular ganglion neurons are labeled. A and B: 35-μm sections from the superior vestibular ganglia (VG) of 4- to 6-wk SNS-Cre reporter mice, showing tdTomato (red) in many VGNs. Scale bars (50 μm) in A4 and B2 apply to A1-A4 and B1-B2. A1: SNS-Cre VG colabeled with antibodies to calretinin (blue) and β-III tubulin (green). A2-A4: color channels of A1 shown separately. Calretinin-positive cells (A3) are relatively large and not tdTomato-positive (e.g., arrow). B1: SNS-Cre section from a different mouse, labeled with NF200 antibody (green). Intensely NF200-positive cells (e.g., arrow) are often large and not tdTomato-positive. B2: same section showing tdTomato alone. C: tdTomato expression in VG (C1) and DRG (C2) of a 5- to 6-wk Na$_{v}$1.8-Cre reporter mouse. Scale bars = 50 μm. C1: maximum intensity projection image of the VG. C2: whole mount DRG from the same animal. Despite strong tdTomato label in the DRG, fewer VGNs are positive in this reporter line than in the SNS-Cre mouse.
only afferents, and β-III tubulin, which labels most VGNs (Perry et al. 2003). The staining patterns for tdTomato, calretinin, and β-III tubulin for this section are shown individually in Fig. 10, A2–A4. Figure 10B1 shows a second SNS-Cre vestibular ganglion with tdTomato and immunostaining for the heavy neurofilament NF200; Fig. 10B2 is the same section showing tdTomato alone. NF200-like immunoreactivity is strongest in large VGN somata (Ylikoski et al. 1990; Usami et al. 1993a,b), similar to the pattern in the DRG (Lawson and Waddell 1991). tdTomato expression was largely nonoverlapping with intense immunostaining in large VGNs for calretinin and for NF-200; arrows point to an exemplar large VGN that is positive for calretinin (tissue in Fig. 10A) or NF200 (tissue in Fig. 10B) but not tdTomato.

In vestibular ganglia of the NaV1.8-Cre mouse line, far fewer neurons were tdTomato-positive (Fig. 10C1), although DRGs from the same animals (Fig. 10C2) had many tdTomato-positive neurons, as did DRGs in the SNS-Cre line (not shown). Differences between transgenic and knockin reporter lines are not unexpected; which is more representative of NaV1.8 expression requires more study.

**DISCUSSION**

**NaV Channel Diversity in the Vestibular Ganglion**

As reported previously, rodent vestibular ganglion cell bodies have large TTX-sensitive currents (Chabbert et al. 1997; Risner and Holt 2006). A closer look has uncovered heterogeneity. RT-PCR of whole vestibular ganglia revealed mRNA expression of all known NaV channel subunits, including TTX-insensitive and TTX-resistant α-subunits. Whole cell recordings then provided evidence for functional expression of multiple NaV currents.

In acutely dissociated VGNs between P3 and P8, before maturation of the inner ear, we isolated TTX-insensitive and TTX-resistant currents that resembled currents carried by NaV1.5 and NaV1.8 α-subunits, respectively, in their kinetics and pharmacology. The expression of these subunits was further supported by NaV1.5-like immunoreactivity and by labeling in two kinds of Nav1.8 reporter mice. Whole cell recordings suggest that NaV1.5 current is widely expressed in neonatal afferent cell bodies but declines over the first postnatal week, while NaV1.8 is upregulated in some VGNs over the same period. We further found that the TTX-sensitive Na+ currents are heterogeneous, having smaller amplitude, less negative voltage dependence, and less steep voltage dependence of inactivation in VGNs with TTX-resistant current than in other VGNs. The RT-PCR results suggest multiple candidates for the pore-forming α-subunits that carry TTX-sensitive currents, and it is possible that VGNs with TTX-resistant current express a different complement of TTX-sensitive subunits than VGNs without TTX-resistant current. Differences in β-subunit expression also affect properties of TTX-sensitive currents (Namadurai et al. 2015). A likely contributor to TTX-sensitive current in vestibular afferents is the NaV1.6 subunit, which is expressed at the afferent heminode adjacent to hair cell synapses (Hossain et al. 2005; Lyakowski et al. 2011).

Expression of NaV,1.8 in Peripheral Sensory Neurons

The properties of I_{Na1.8} are highly consistent with NaV1.8 channels but RT-PCR data also showed robust expression of NaV,1.9 (NaN) mRNA in vestibular ganglia. While we cannot rule out a contribution from NaV1.9 channels to I_{Na1.9}, the voltage and time dependence are better matched by known properties of NaV1.8 channels, and we have no clear indication of more than one TTX-resistant current. Perhaps in vivo or with further maturation, NaV1.9 channels in VGNs produce the persistent current for which they are known in nociceptors (Dibb-Hajj et al. 2015), which could support the high sustained firing of vestibular afferents.

Our data may be the first voltage-clamp characterizations of NaV1.8-like current in a ganglion without classic nociceptors. NaV1.8 channels were first isolated from rat DRG (Akopian et al. 1996; Sangameswaran et al. 1996) and were originally thought to be selectively expressed by unmyelinated small nociceptive neurons. It is now clear that NaV1.8 channels are also expressed by other sensory neurons. The SNS-Cre transgenic reporter mouse showed transgene expression in 28% of large-diameter DRG neurons in addition to the expected high percentage (93%) of small-diameter DRG neurons (Agarwal et al. 2004). In the NaV1.8-Cre reporter mouse line, many nonnociceptive DRG neurons are tdTomato-positive and have NaV1.8-like currents (Sheilds et al. 2012), consistent with other reports of TTX-resistant current in large DRG neurons (Renganathan et al. 2000; Ho and O'Leary 2011). Outside the DRG, the TTX-resistant current is found in nonnociceptive neurons of the nodose ganglion (Li and Schild 2007; Kwong et al. 2008). In addition, NaV1.8-like immunoreactivity has been reported in the retina (O'Brien et al. 2008). The apparently widespread distribution of NaV1.8 and TTX-resistant currents in sensory neurons argues for a broad sensory function not restricted to nociception. Thus candidate pain therapies targeting NaV1.8 channels (Liu and Wood 2011) should be tested for diverse sensory side effects, including vestibular symptoms. Potential side effects arising from drug action on NaV1.8 channels in the vestibular ganglion may be avoided by controlling the permeability of agents through the blood-labyrinth barrier (Soto and Vega 2010).

TTX-resistant current has been proposed to play a role in sensitization in inflammatory hyperalgesia and possibly neuropathic pain (Tanaka et al. 1998; Gold 1999; Cardenas et al. 2001; Coggeshall et al. 2004). If so, it might also contribute to the neuritis and vertigo that can follow infection and inflammation in the vestibular nerve (Strupp and Brandt 2013).

Functional Significance of NaV Channel Diversity in the Vestibular Ganglion

Afferents from central and peripheral zones of vestibular sensory epithelia show strong differences in spike timing (regular vs. irregular) and response dynamics (frequency-dependent gain and phase) (Goldberg 2000). Action potentials in isolated VGNs with regular and irregular firing have different current and voltage thresholds (Kalluri et al. 2010). The unique voltage-gated properties of both NaV1.5 and NaV1.8 channels could play a role in these systematic variations in spiking and stimulus encoding, as well as in development.

NaV1.5 channels. NaV1.5 protein may play roles in both vestibular development and mature vestibular function. In
cardiac myocytes, with resting potentials of −80 to −90 mV, NaV1.5 channels are largely responsible for the upstroke of the action potential. In isolated immature rat VGNs, resting potentials range from −45 to −75 mV (Kalluri et al. 2010), so that most NaV1.5 channels are inactivated at resting potential. Nevertheless, for those VGSCs with the most negative resting potentials and/or in the case of hyperpolarizations from rest, NaV1.5 channels may be critical for excitability because their activation range is also more negative than that of other NaV channels. In vivo, vestibular afferents may spend significant time at relatively negative potentials, as follows. First, they have high background firing rates (50–100 spikes/s in mammals) and each spike afterhyperpolarization can be 10 mV negative to resting potential (Schessel et al. 1991; Kalluri et al. 2010). Second, negatively directed hair bundle deflections reduce the excitatory glutamatergic input to afferent terminals. Third, resting potential in mature vestibular afferents in vivo may be more negative than we measured, for two reasons: external K+ concentration has been estimated at 3 mM (Anniko and Wroblewski 1986), about half the value in our external solutions, which would make ER and therefore resting potential more negative; and addition of KH channels to VGN cell bodies during the first two postnatal weeks (Kalluri et al. 2010) may drive resting potentials closer to ER.

NaV1.5 channels may also play a role in development, either through conducting or nonconducting functions (Kaczmarek 2006). NaV1.5-null mice die between embryonic day 10 and 11 (Papadatos et al. 2002), and knocking out NaV1.5 homologs in zebrafish causes severe morphological defects (Chopra et al. 2006). NaV channel β-subunits are part of the cell-adhesion molecule superfamily and regulate neurite outgrowth and axon pathfinding by mechanisms that depend on Na+ current (Davis et al. 2004; Brackenbury et al. 2008, 2010). In our sample, a high incidence of NaV1.5-like current at birth declined during the first postnatal week (Fig. 5), although NaV1.5-like immunoreactivity was seen at P21 (Fig. 6C). The incidence of NaV1.5 current in rodent DRG drops from ~80% at E15 to ~20% by P0 and ~3% by adulthood (Renganathan et al. 2002). The expression of NaV1.5 currents in hair cells of the rat utricle also declines with postnatal age (Woolorton et al. 2007).

The decreasing incidence of INa in VGNs over the first postnatal week could, alternatively or additionally, reflect trafficking of the channels to afferent terminals. Unlike central neurons, vestibular afferent neurons are bipolar neurons that initiate spikes near their distal terminals on hair cells, as indicated by nodal markers (Lysakowski et al. 2011). NaV1.5 mRNA was present in the vestibular ganglion at both P1 and P21 (Fig. 1). NaV1.5-like immunoreactivity in afferent cytocaudal terminals increases in intensity during postnatal development (Woolorton et al. 2007) and is present in adult (Lysakowski et al. 2011).

NaV1.8 channels. Varying the proportions and types of TTX-sensitive channels in conjunction with TTX-resistant channels could alter firing threshold and sensitivity while ensuring overshooting action potentials. The depolarized activation range of NaV1.8 may help reduce depolarization block and maintain action potential height during intense stimulation. As in other peripheral sensory neurons, the spike initiation zones of vestibular afferents lie close to the afferent synapse (Lysakowski et al. 2011) and may therefore be particularly prone to depolarization block from sustained synaptic input. VGNs with TTX-resistant current maintained spike height in the presence of a significant sustained depolarization (Fig. 9B2).

The percentage of DRG neurons expressing NaV1.8 current increases dramatically around the time of birth and persists (Renganathan et al. 2002). We saw an increase in INa prevalence just after birth (Fig. 5) but do not know whether the current persists into adulthood. The Cre reporter mouse indicates whether a cell expressed NaV1.8 at some point in the past but does not indicate the level of expression or whether expression continues into adulthood. Fewer VGNs were labeled in the knockin NaV1.8-Cre reporter mouse than in the transgenic SNS-cre reporter mouse, despite similar fractions of labeled DRG neurons in the two lines (Chiu et al. 2014). Expression may be lower in VGNs than in highly expressing DRG subpopulations; expression of NaV1.8 may be regulated differently in neurons originating from the neural crest (DRG) and placodes (nodose and vestibular ganglia) (Lu et al. 2015). The transgenic mouse may have a higher copy number, while the knockin has a regulatory context closer to native conditions. In the same NaV1.8-Cre reporter mouse line, extensive tdTomato staining was reported in “the vestibular-geniculate ganglion mass” of adult mice (Gautron et al. 2011).

Function depends on whether the NaV channel location is somatic, at the initial heminode, or at nodes of Ranvier. In the immature VGNs that we studied, myelination is not complete and the distribution of ion channels may be immature. NaV1.8 channels cluster at nodes of Ranvier in a subpopulation of myelinated axons in radicular tooth pulp (Henry et al. 2005), consistent with a role in action potential propagation. Even when ion channels are normally restricted to nodes, the retraction of neurites in the process of dissociation may relocate ion channels to the soma.

Relationship to afferent populations. Unlike DRG neurons, all vestibular afferents receive input from a single kind of mechanoreceptor, the hair cell, and are myelinated. Vestibular afferents are systematically heterogeneous, however, forming parallel streams with different spike timing, spatial and temporal integration, frequency dependence, sensitivity, and dynamic range (reviewed in Goldberg 2000 and Eatock and Songer 2011). Here we speculate about how NaV1.5, NaV1.8, and TTX-sensitive subunits of different voltage dependence might complement the expression of low-voltage-activated (KLV) channels to permit different vestibular afferent types to spike in different voltage regimes.

In vestibular ganglia from SNS-cre reporter mice, costaining for calretinin suggests that NaV1.8 is most strongly expressed by calretinin-negative afferents (Fig. 10A), and therefore may include the regular afferents that innervate peripheral epithelial zones. The ability of NaV1.8 channels, with their depolarized inactivation ranges, to counter depolarization block (Patrick Harty and Waxman 2007; Choi and Waxman 2011) could help regular vestibular afferents sustain high tonic firing. Extrapolating from the association of NaV1.8 currents with TTX-sensitive currents of relatively depolarized voltage dependence (Fig. 8), suggests a combination that would give (some) regular afferents more depolarized spike thresholds than irregular afferents. Isolated VGNs that fire trains in response to current steps, which may correspond to regular afferents, do have less negative spike thresholds than single-spiking VGNs, which...
may correspond to irregular afferents (Kalluri et al. 2010). Lower expression of Kᵥ_channels in regular afferents (Kalluri et al. 2010; Lysakowski et al. 2011; Schuth et al. 2014) may produce less negative resting potentials, which would complement the less negative voltage ranges of their Naᵥ_channels. For central-zone irregular afferents, the more negative resting potentials could reduce inactivation of the Naᵥ1.5 channels on their calyceal terminals (Wooltorton et al. 2007; their Fig. 12). Whether these specific possibilities are borne out, we can add diversity of sodium channels to intrinsic properties of afferents with potential to influence analysis of head motions by the inner ear.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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TTX-INSSENSITIVE Na⁺ CURRENTS IN THE VESTIBULAR GANGLION


