Functional contributions of HCN channels in the primary auditory neurons of the mouse inner ear

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The hyperpolarization-activated current, Iₜᵢ₊ᵦ, is carried by members of the Hcn channel family and contributes to resting potential and firing properties in excitable cells of various systems, including the auditory system. Iₜᵢ₊ᵦ has been identified in spiral ganglion neurons (SGNs); however, its molecular correlates and their functional contributions have not been well characterized. To investigate the molecular composition of the channels that carry Iₜᵢ₊ᵦ in SGNs, we examined Hcn mRNA harvested from spiral ganglia of neonatal and adult mice using quantitative RT-PCR. The data indicate expression of Hcn1, Hcn2, and Hcn4 subunits in SGNs, with Hcn1 being the most highly expressed at both stages. To investigate the functional contributions of HCN subunits, we used the whole-cell, tight-seal technique to record from wild-type SGNs and those deficient in Hcn1, Hcn2, or both. We found that HCN1 is the most prominent subunit contributing to Iₜᵢ₊ᵦ in SGNs. Deletion of Hcn1 resulted in reduced conductance (Gᵢ₊ᵦ), slower activation kinetics (τᵢ₊ᵦ), and hyperpolarized half-activation (Vᵢ₊ᵦ/2) potentials. We demonstrate that Iₜᵢ₊ᵦ contributes to SGN function with depolarized resting potentials, depolarized sag and rebound potentials, accelerated rebound spikes after hyperpolarization, and minimized jitter in spike latency for small depolarizing stimuli. Auditory brainstem responses of Hcn1-deficient mice showed longer latencies, suggesting that HCN1-mediated Iₜᵢ₊ᵦ is critical for synchronized spike timing in SGNs. Together, our data indicate that Iₜᵢ₊ᵦ contributes to SGN membrane properties and plays a role in temporal aspects of signal transmission between the cochlea and the brain, which are critical for normal auditory function.

INTRODUCTION

Spiral ganglion neurons (SGNs) are the first-order sensory neurons of the auditory system, which provide afferent innervation of hair cells in the mammalian cochlea. SGNs convey signals from hair cells to the brain in a manner that preserves the amplitude, frequency, and temporal aspects of sound information (Geisler, 1998; Taberner and Liberman, 2005; Meyer and Moser, 2010). This high-fidelity signal transmission between the hair cells and the SGNs is critical for normal auditory processing and sound localization.

To perform these functions, SGNs express a variety of voltage-gated ion channels that allow efficient signal transfer from inner hair cells (IHCs; Davis, 2003). The expression of specific voltage-gated ion channels in SGNs allows them the ability to precisely encode graded membrane potentials of hair cells into trains of action potentials (APs). For example, SGNs express voltage-gated calcium and potassium channels along the cochlear tonotopic axis in a graded manner that reflects the tonotopic specialization of hair cells (Adamson et al., 2002; Davis, 2003; Chen et al., 2011; Lv et al., 2012).

The hyperpolarization-activated cationic current, Iₜᵢ₊ᵦ, has been identified in neurons of the auditory system (Banks et al., 1993; Bal and Oertel, 2000; Koch and Grothe, 2003), including SGNs (Chen, 1997; Mo and Davis, 1997; Yi et al., 2010), but the molecular composition of the channels that carry Iₜᵢ₊ᵦ and their precise function have not been clarified. Iₜᵢ₊ᵦ is carried by subunits of the HCN channel family, HCN1–4, which form homo- and heterotetrameric channels (Ludwig et al., 1998; Ishii et al., 1999). Unlike other channels that activate upon depolarization, Iₜᵢ₊ᵦ activates in response to membrane hyperpolarization negative to ~50 mV and passes inward current that depolarizes the cell membrane. Thus, the unusual biophysical properties of Iₜᵢ₊ᵦ contribute to resting membrane potential and firing properties in many different cell types (Robinson and Siegelbaum, 2003; Biel et al., 2009).

Recently, Yi et al. (2010) characterized Iₜᵢ₊ᵦ at the hair cell afferent synapse; however, the molecular correlates of Iₜᵢ₊ᵦ and their functional contributions in SGN cell bodies were not examined. To identify which Hcn genes are expressed in neonatal and adult SGNs, we harvested
to I_h, we recorded from SGNs of WT mice and mice that lacked Hcn1, Hcn2, or both. Our qPCR and electrophysiology data indicate expression of HCN1, HCN2, and HCN4 subunits in SGNs, with HCN1 being the most highly expressed subunit. Deletion of Hcn1 resulted in reduced conductance, hyperpolarized half-activation voltage, and slower activation kinetics. We recorded from SGNs in current-clamp mode to investigate the functional contributions of I_h to SGN membrane and firing properties. We show that I_h depolarizes SGN resting membrane potentials, regulates rebound spike timing, and synchronizes AP firing in response to small depolarizing stimuli. In addition, auditory brainstem responses (ABRs) indicate possible auditory deficits in Hcn1−/− animals. Hcn1−/− mice had delayed ABR latencies, suggesting a contribution to spike timing in the eighth cranial nerve. Collectively, the present study provides evidence that I_h, HCN1 in particular, contributes to the membrane properties of SGNs and plays a role in the temporal aspects of auditory signal transmission required for normal auditory function.

MATERIALS AND METHODS

Animals
All animal protocols used in this study were approved by the Institutional Animal Use and Care Committee at the University of Virginia (protocol #3123) and Boston Children’s Hospital (protocol #11-04-1959). Experiments were performed in strict accordance to the guidelines therein. Hcn1-deficient (Hcn1−/−) mice (strain: B6.129S-Hcn1tm4[Nak]/J; Nolan et al., 2003) were obtained from The Jackson Laboratory, and Hcn2-deficient (Hcn2−/−) mice were obtained originally from A. Ludwig (Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen, Germany; Ludwig et al., 2003). Mice deficient in Hcn1 and Hcn2 (Hcn1/2−/−) mice were generated by crossing Hcn1−/− and Hcn2−/− mice, as previously described (Horwitz et al., 2011). Both sexes of animals were used in nondiscriminatory fashion. WT control data were obtained from three different strains, Swiss Webster (Charles River; n = 11 SGNs), WT littermates (n = 5) obtained from Hcn1−/− and Hcn1−/− crosses, and WT littermates (n = 9) obtained from Hcn2−/− and Hcn2−/− crosses. We found no statistical difference in any of the analyzed parameters and have pooled the WT data.

Neonatal SGN preparation
SGNs were acutely dissected from neonatal mice, age ranging between postnatal day (P) 1 and P8. After rapid decapitation, cochlea from both sides of the inner ear were excised from the temporal bone and bathed in sterile MEM with GlutaMAX (Gibco) supplemented with 10 mM HEPES (Sigma-Aldrich) and 25 mg ampicillin (Sigma-Aldrich), pH 7.4. The bony labyrinth encapsulating the cochlea was chipped away to gain access to the organ of Corti facing down to gain better access to SGN cell bodies. The sections were secured in place under two thin glass fibers glued to the coverslip with Sylgard 184 (Dow Corning). SGN organotypic explants were incubated at 37°C in a humidified incubator (5% CO2) for 1–2 h, after which they were used for acute electrophysiological experiments. All SGN explants were used within 4–5 h after dissection.

Type 1 SGNs
SGNs can be categorized into type I and type II neurons based on their innervation pattern and morphology. Type I neurons, which constitute 90–95% of the entire SGN population, innervate IHCs and are responsible for sound information transfer (Liberman, 1982). Type I neurons are highly myelinated (Romand and Rand, 1987) and in general have relatively large cell body size compared with type II neurons (Rusznák and Szács, 2009). The myelin sheath tightly covers the axon fibers and loosely wraps around the cell bodies of SGNs, ensuring high velocity signal conduction to the central pathway. In contrast, type II neurons, which compose the remaining ~5% of SGNs, innervate the outer hair cells (Perkins and Morest, 1975). Type II neurons are unmyelinated and have smaller cell bodies than type I neurons (Rusznák and Szács, 2009). The different morphology and abundance in number allowed for easy detection of neonatal type I SGNs for electrophysiological recordings. All neonatal SGN data presented in this paper were obtained from type I cells.

Adult SGN culture
Adult mouse SGN culture technique was performed in accordance to the method previously described (Lv et al., 2010). Adult mouse was sacrificed and decapitated rapidly. For each culture, three to four animals were pooled. Temporal bones were removed from the skull and bathed in ice-cold MEM with Hank’s salt (Gibco) supplemented with 0.2 mg/ml kynurenic acid (Sigma-Aldrich), 10 mM MgCl2, 2% (vol/vol) FBS (Gibco), and 6 g/liter d-glucose (Sigma-Aldrich). The bony shell encapsulating the cochlea was chipped off using a scalpel, and the organ of Corti was removed from the central core. The modiolus, which contained the SGNs, was isolated in the above dissecting media, in which the 2% (vol/vol) FBS was re-replaced with 2% (vol/vol) B27 (Gibco), and split into apical and basal sections. The sections were subsequently digested in enzyme solution containing 1 mg/ml collagenase type 1 (Sigma-Aldrich) and 1 mg/ml DNase (Sigma-Aldrich) at 37°C for 15 min. After enzyme treatment, 0.25% trypsin (Gibco) was added to the solution and incubated for an additional 10 min at 37°C. The reaction was quenched with equal volume FBS (vol/vol). After gentle trituration, the cell solution was transferred to Hank’s balanced salt solution (HBSS) containing 0.45 M sucrose (Sigma-Aldrich) and centrifuged at 2,000 rpm for 5 min. The cell pellets were reconstituted in Neurobasal A (Gibco) supplemented with 2% B27, 0.5 mM t-glutamine (Sigma-Aldrich), and 100 U/ml penicillin (Sigma-Aldrich). The freshly isolated SGN cells were filtered through a 40-µm cell strainer and plated onto glass coverslips, pretreated with 0.5 mg/ml poly-d-lysine (Sigma-Aldrich) and 1 mg/ml laminin (Sigma-Aldrich). The SGN culture was supplied with 10% FBS, 0.2 mg/ml kynurenic acid, 10 ng/ml NT3 (Sigma-Aldrich), and 10 ng/ml BDNF (Sigma-Aldrich) and incubated at 37°C in a humidified incubator (5% CO2) for 24–48 h before electrophysiological recordings. To ensure quality recording and minimize space-clamp errors, only spherical SGNs with one or two neurites were selected for voltage-clamp recordings.

Electrophysiology
The whole-cell, tight-seal patch clamp technique was used in voltage- and current-clamp modes to record I_h from SGN cell bodies. All electrophysiological recordings were performed at
room temperature (22–24°C). SGN explants were placed into a custom-made recording chamber and viewed under an Axioskop FS upright microscope (Carl Zeiss) equipped with 63× water-immersion lens and differential interface contrast optics. SGN explants were bathed in a standard external solution that contained (mM): 137 NaCl, 0.7 NaH2PO4, 5.8 KCl, 1.3 CaCl2, 0.9 MgCl2, 5.6 d-glucose, 10 HEPES, amino acids (1:50; Gibco), and vitamins (1:100; Gibco). The pH was adjusted to 7.4 with NaOH and the measured osmolarity was 303 mOsm/kg. Recording pipettes (3–5 MΩ) were pulled from R-6 soda lime capillaries (King Precision Glass) using a two-stage vertical pipette puller (PC-10; Narishige). To minimize pipette capacitance, tips were coated with ski wax. Recording pipettes were filled with standard internal solution that contained (mM): 135 KCl, 2.5 MgCl2, 2.5 K2-ATP, 5.0 HEPES, 5.0 EGTA, and 0.1 CaCl2, pH 7.4 (KOH), 283 mOsmol/kg. All reagents for electrophysiology solution were purchased from Sigma-Aldrich, unless otherwise noted. Ih was recorded immediately after the cell membrane was broken through at gigaohm (GΩ) seal. Series resistance (Rs) and membrane capacitance (Cm) were measured.

Electrophysiological data from SGNs was recorded using an Axopatch200B (Molecular Devices) amplifier. Signals were filtered at 1 kHz with a low pass Bessel filter and digitized at ±200 kHz using 12-bit acquisition system, Digidata 1332 (Axon Instruments), and pClamp 9.0 (Molecular Devices). All stimulus protocols were generated using pClamp 9.0, and data were stored on a PC.

**SGN microdissection and qPCR**

Neonatal SGN tissue was harvested from P3 Swiss Webster mice in ice-cold MEM with GlutaMAX, supplemented with 10 mM HEPES (Sigma-Aldrich) and 25 mg ampicillin (Sigma-Aldrich), pH 7.4. SGNs were gently isolated from cochlear modiolus by dissecting the central processes. To prevent cross-contamination from cochlear hair cells, organ of Corti was trimmed away along the cochlear turn using a microblade so that the SGN isolates only contained cell bodies. Microdissected SGNs were rinsed in fresh MEM, transferred into microcentrifuge tubes, and rapidly frozen on dry ice. Two preparations of SGN tissue were pooled from 10 (five mice) and 14 (seven mice) cochlear microdissection and qPCR experiments.

Adult SGNs were harvested from Swiss Webster mice 2–40. Temporal bones were removed from the skull and bathed in ice-cold MEM with Hank’s salt supplemented with 0.2 mg/ml kynurenic acid. The bony shell encapsulating the cochlea was chopped away using a scalpel. SGNs were excised from the central core, and processes were sectioned where the cochlear nerve joins the vestibular nerve to prevent contamination from vestibular neurons. Dissected SGN tissue was rapidly frozen in dry ice. Two samples of adult SGN tissue were pooled from 6 (three mice) and 10 (five mice) cochlear microdissection, respectively, and were processed in parallel for RNA isolation and subsequent qPCR experiments.

The frozen SGN microisolates were thawed, spun down in a centrifuge to remove excess media, and processed for RNA isolation. Total SGN RNA was prepared with an RNAqueous micro kit (Ambion) according to the manufacturer’s instruction. The isolated RNA was then purified with a DNAfree RNA kit (Zymo Research) and stored in −80°C. RNA concentration was measured on a spectrophotometer (Nanodrop ND-1000; Thermo Fisher Scientific). For RNA quality control, the samples were analyzed with a Bioanalyzer (Agilent Technologies) and found to have an RNA integrity number of >9.0. 100 ng RNA was reverse transcribed into cDNA using the QuantiTect Reverse Transcription kit (QIAGEN). qPCR was performed in triplicates using SYBR Green ER qPCR Supermix (Invitrogen) according to the manufacturer’s instructions and processed on a CFX Real-Time PCR Detection System (Bio-Rad Laboratories). Each reaction (25 µl) included primers at 0.2–0.4 µM and cDNA generated from 5 ng RNA. qPCR parameters were set for 40 cycles, and amplicon purity was confirmed with melt curve analysis. qPCR primers had melting temperatures ranging from 58 to 61°C, and the sequences were as follows: Hcn1, 5′-ACATGCTGGTGATTG-TTGCCG-3′ and 5′-AACAACTTCTGACAGCTTGCCG-3′; Hcn2, 5′-ACCTCCGCAACGCGATTGTATTG-3′ and 5′-TCGCCCTCTCCTCATAATGAGG-3′; Hcn3, 5′- CCTCACTGCCTACATACACCACTAG-3′ and 5′-GACACACGACGACTCATCTCC-3′; and Hcn4, 5′-ACTTATACCTGCGGAAAGCTTGTCG-3′ and 5′-GAAAGGCAACCTGTTCAGATGGA-3′. qPCR primer sets were designed to amplify specific regions of HCN amplicons produced by RT-PCR (Horwitz et al., 2010).

Expression levels and possible genomic DNA contamination were tested with ribosomal 28S as housekeeping gene by running reverse transcribed cDNA samples and no-reverse transcript controls simultaneously. Cochlear hair cell contamination was determined with cochlear hair cell-specific gene prestin as a control. The sequences of ribosomal 28S and prestin primers (Lelli et al., 2009) were as follows: 28S, 5′-GGAGTCACCCACGGAAGTTCG-3′ and 5′-GGAAAGGCACGGGACCATG-3′; and prestin, 5′-TTAAGCCTCAATTGGAGGTCTGGGA-3′ and 5′-GTGCAAGAGGCGCCGTGTTATGG-3′.

**ABRs**

Hcn1+/− mice (n = 7) and their WT littermates (n = 5) were used for the ABR recording. Because of the mixed genetic background (C57BL/6 and 129S) of the strain, mice were tested at 6–7 wk of age to minimize confounding factors from age-related hearing loss that these strains exhibit around 3 mo of age (Zheng et al., 1999; Kane et al., 2012).

ABRs were performed in a sound-proof chamber. Before ABR measurements, mice were anesthetized with 100 mg/kg ketamine and 20 mg/kg xylazine via intraperitoneal injection and placed on a heating pad to maintain body temperature. Needle electrodes were placed subdermally at the vertex and below the pinna, and a ground electrode was placed near the tail. Tone pips (5 ms, 40 Hz) were presented with alternating polarities, and the responses were recorded with increasing sound pressure levels, from 10 to 80 dB with 5-dB intervals. For each level, waveforms were averaged (gain 10,000), filtered (0.1–3 kHz), and averaged across 1,024 presentations. Threshold was determined with visual inspection of the waveforms. Amplitude and latencies of the ABR waveforms were analyzed using ABR Peak Analysis software.

**Data analysis**

Offline data analysis was performed using OriginPro 7.5 (Origin Laboratory). Liquid junction potentials (4 mV) were adjusted offline for all membrane potentials. Voltage-dependent Ih activation was analyzed by using the following Boltzmann equation:

\[ G(V_m) = G_{\min} + \frac{G_{\max} - G_{\min}}{1 + e^{-[V_m - V_{1/2}] / s}} \]  

where \(G_{\max}\) and \(G_{\min}\) are the maximum and minimum conductance, \(V_{1/2}\) is the voltage at half maximum activation and \(s\) is the slope factor.
Neonatal SGN samples were collected from a pool of 24 whole genes present in both neonatal (P3) and adult stages (≥P40). Hcn SGNs. qPCR was used to estimate the total mRNA copy number of Expression of scripts per cochlea. Error bars represent SEM.

RESULTS

Spatiotemporal development of Ih in SGNs

To examine the contributions of HCN subunits to Ih in SGNs, we began with a qPCR analysis using primers that were selective for Hcn1–4 and mRNA harvested from P3 WT SGN tissue. As shown in Fig. 1 A, the qPCR analysis indicated expression of Hcn1, Hcn2, and Hcn4 in early postnatal SGNs. The total mRNA expression for Hcn1, Hcn2, and Hcn4 per cochlea was 700,000:200,000:400,000, respectively, with little expression of Hcn3. When normalized to Hcn3 mRNA copy number, the relative expression was as follows: Hcn1-Hcn2-Hcn3-Hcn4 21:6:1:12. Based on the expression ratio, it is evident that Hcn1 was the most highly expressed in neonatal SGNs, followed by Hcn4 and Hcn2. We speculate that the Hcn3 expression in SGNs is minimal or nonexistent based on the low mRNA copy number; thus, its functional contributions in neonatal SGNs are likely to be negligible.

In adult SGNs our qPCR analysis of Hcn mRNA revealed expression of Hcn1, Hcn2, and Hcn4 (Fig. 1 B). Hcn1 was again the most highly expressed, with a significant increase in mRNA copy number relative to neonatal stages (Fig. 1, A and B). When normalized to Hcn3, the relative expression ratio was Hcn1-Hcn2-Hcn3-Hcn4 1,000:300:1:100. The data suggest that HCN1 is the principal subunit at both neonatal and adult stages.

To examine Ih in SGN cell bodies, we used the whole-cell, tight-seal technique in the voltage-clamp configuration to record from organotypic SGN explants acutely excised from neonatal mouse cochlea (P1–8). In response to 4-s hyperpolarizing voltage injections stepped from −74 to −144 mV in 10-mV increments from a holding potential of −84 mV, WT SGNs exhibited slowly activating inward currents. A representative family of current traces is shown in Fig. 2 A, in which the slowly activating inward current was detected at potentials negative to −84 mV and was fully activated at potentials negative to −124 mV. This current profile is characteristic of Ih in other neurons including SGNs (Chen, 1997; Mo and Davis, 1997; Yi et al., 2010) and was observed in every WT SGN we examined (n = 143).

To analyze the voltage dependence of Ih in WT SGNs, activation curves were generated by fitting a Boltzmann function (Eq. 1) to tail currents plotted as a function of step potential (Fig. 2 B). Tail currents were measured at the instant of a step to −84 mV, very close to potassium equilibrium potential (Ek). At −84 mV, contamination from potassium currents was minimal and other voltage-dependent currents were deactivated. WT SGNs (n = 25) had a mean maximum conductance (Gm) of 2.3 ± 0.8 nS and half-activation voltage (V1/2) of −97.1 ± 4.1 mV (range: −91.2 to −106.0 mV) with a slope factor (s) of 5.9 ± 0.6 mV (range: 5.0–7.2 mV). These activation parameters were similar to those previously reported for Ih activation in SGNs of various species (Chen, 1997; Mo and Davis, 1997; Szabó et al., 2002; Yi et al., 2010).

WT SGNs showed a developmental increase in the amplitude of Ih during the first postnatal week (Fig. 2 D). We measured Gm between P1 and P8 and found a gradual increase as a function of age. This developmental increase in Gm in WT SGNs also reflected graded expression of Ih along the cochlear tonotopic axis (Fig. 2, C and D). Comparison of Gm from apical and basal cells within the same age groups (P1–4) revealed significantly larger Gm in basal than apical SGNs (Fig. 2 D). By the end of the first postnatal week, the tonotopic gradient had diminished.

Ih in neonatal SGNs is carried by HCN1, HCN2, and HCN4 To verify that the hyperpolarization-activated inward currents in WT SGN cell bodies were carried by HCN channels, we applied the HCN blocker ZD7288 during

\[ I_h(t) = I_{ss} + I_f \exp(t/\tau_{slow}) + I_s \exp(t/\tau_{fast}), \]  

where \( I_{ss} \) is the steady-state current at a given potential, \( I_f \) and \( I_s \) denote the amplitude of the slow and fast current components, and \( \tau_{slow} \) and \( \tau_{fast} \) are the corresponding time constants for Ih activation.

Statistical analysis was performed using OriginPro 7.5. All results were reported as means ± SD unless otherwise specified. Statistical analysis for Ih conductance (Gm), half-activation voltage (V1/2), slope factor (s), and activation kinetics (\( \tau_{slow} \)) and \( \tau_{fast} \) were made using independent Student’s t test. If comparison involved multiple genotypes, ANOVA was used followed by Dunnett’s post-hoc test. S-Bicamp effect on Ih activation was analyzed using independent samples Student’s t test. The effects of ZD7288, NiCl2, and mibefradil on SGN firing properties were determined using paired Student’s t test. Difference in means was considered significant if P < 0.05. Degree of scatter in spike time variation in response to small depolarizing current injection was quantified using confidence interval (CI) at 95%.

Figure 1. Expression of Hcn mRNA in mouse SGNs. (A and B) Expression of Hcn1–4 in neonatal (A) and adult (B) WT mouse SGNs. qPCR was used to estimate the total mRNA copy number of Hcn genes present in both neonatal (P3) and adult stages (≥P40). Neonatal SGN samples were collected from a pool of 24 whole cochleas. Adult SGN samples were obtained from a pool of 16 whole cochleas. Each preparation was run in triplicate. Results are shown as mean total copies in thousands of Hcn mRNA transcripts per cochlea. Error bars represent SEM.
that the ZD7288-sensitive currents were nearly identical in amplitude and activation kinetics to the control currents. Importantly, the subtracted currents did not include properties of any other current, suggesting that 100 µM ZD7288 is specific for Ih in SGNs. Although we have shown ZD7288 to be an Ih-specific antagonist (BoSmith et al., 1993; Harris and Constanti, 1995), it does not distinguish among the four HCN subunits. Thus, we wondered which HCN subunits contribute to the biophysical properties of Ih in WT SGNs. A previous study indicated localization of HCN1 and HCN4 subunits in rat SGN cell bodies at P9–10 (Yi et al., 2010); however, functional evidence implicating specific HCN subunits was not reported. Another study reported the presence of all four HCN subunits in adult guinea pig SGN cell bodies (Bakondi et al., 2009), raising the possibility that all four HCN subunits may be present in WT mouse SGNs.

To determine whether the Hcn mRNA expression patterns (Fig. 1) were translated into physiological expression of Ih, we recorded from SGNs excised from mice deficient in Hcn1, Hcn2, or both. Because our data and several in situ hybridization studies indicate very low expression levels of Hcn3 mRNA (Ludwig et al., 1998; Moosmang et al., 1999), Hcn3−/− mice were not investigated. Hcn4 is expressed in SGNs; however, Hcn4−/− mice are embryonic lethal (Stieber et al., 2003), and conditional Hcn4−/− mice are not available. Representative families of Ih recorded from SGNs of WT, Hcn1−/−, Hcn2−/−, and Hcn1,2−/− mice are shown in Fig. 4 A. Inward currents indicative of Ih were present in SGNs harvested from mice of all four genotypes. Current amplitudes and activation kinetics were noticeably diminished in Hcn1−/− SGNs relative to WT. Hcn2−/− SGNs showed reduced current amplitudes; however, their current activation kinetics was not significantly different from WT. In Hcn1,2−/− SGNs, in which both Hcn1 and Hcn2 were deficient, Ih was significantly reduced.

Figure 2. Ih in SGNs. (A) Representative current traces recorded from a WT SGN cell body (P3, base) in response to 4-s voltage steps from a holding potential of −84 mV to potentials between −144 and −74 mV in 10-mV increments. The bottom panel shows the voltage-clamp protocol. (B) A representative activation curve for Gh generated from the tail currents shown in A. Tail currents were divided by driving force (40 mV) and fitted with a Boltzmann function (black line; Eq. 1): V1/2 = −98.3 mV, s = 5.3 mV, and Gh = 3.4 nS. (C) Representative Ih traces of from apical and basal SGNs (P3) from the same cochlear explant. The scale bar applies to both current families. (D) Mean maximal conductance (Gh) plotted as a function of postnatal age and cochlear location (apex vs. base). Statistically significant differences (**, P < 0.01; ***, P < 0.001) indicated a tonotopic gradient (apex vs. base) during the first postnatal week. There was also a gradual, yet significant (P < 0.001), increase in Gh during the first postnatal week (mean of P0–4 relative to P5–8). Number of samples for each group is shown at the bottom. Error bars equal +1 SD.

Figure 3. Ih in SGNs is carried by HCN channels. (A and B) Representative currents recorded from a WT, P2, basal SGN in response to hyperpolarizing voltage steps before (A) and after (B) 100 µM ZD7288 bath application. Voltage-clamp protocol is shown below. (C) Subtracted currents from datasets A and B show characteristics of Ih. The scale bar applies to all current families.
Representative activation curves are shown in Fig. 4 B. Relative to WT controls, the mean $G_h$ was significantly ($P < 0.01$) reduced in both $Hcn1^{-/-}$ and $Hcn2^{-/-}$ SGNs, indicating expression of both HCN1 and HCN2 subunits contributes to $I_h$ at early postnatal stages (Fig. 4 C). Reduction of $G_h$ was most prominent in $Hcn1,2^{-/-}$ SGNs ($P < 0.001$). We suggest the larger reduction of $G_h$ in $Hcn1,2^{-/-}$ SGNs is a consequence of simultaneous deletion of HCN1 and HCN2 subunits. The small residual $I_h$ in $Hcn1,2^{-/-}$ was blocked by ZD7288, indicating the presence of other HCN subunits, most likely HCN4 (Fig. 4 A). In summary, the data suggest a prominent role for HCN1 with minor contributions from HCN2 and perhaps HCN4.

Analysis of $G_h$ activation curves from $Hcn$ mutant mice revealed additional evidence suggesting involvement of HCN1, HCN2, and HCN4 subunits in neonatal SGNs (Fig. 4, C–E). The mean voltage of half-activation was shifted in the negative direction in both $Hcn1^{-/-}$ and $Hcn1,2^{-/-}$ SGNs relative to WT ($P < 0.001$). $Hcn2^{-/-}$ SGNs also showed a slight but significant ($P < 0.05$) negative shift in $V_{1/2}$, raising the possibility that HCN2 makes a minor functional contribution in neonatal SGNs (Fig. 4 D). There were significant differences in the mean slope factors of $Hcn1^{-/-}$, $Hcn2^{-/-}$, and $Hcn1,2^{-/-}$ SGNs relative to WT, further supporting involvement of both HCN1 and HCN2 in neonatal SGNs (Fig. 4 E).

The kinetics of $I_h$ activation was measured from double exponential fits to currents evoked by steps to $-144$ mV (Fig. 5 A). Fast time constants ($\tau_{fast}$) were significantly ($P < 0.001$) slower in both $Hcn1^{-/-}$ ($393 \pm 123$ ms; $n = 18$) and $Hcn1,2^{-/-}$ ($522 \pm 99$ ms; $n = 8$) SGNs compared with WT ($127 \pm 47$ ms; $n = 18$) and $Hcn2^{-/-}$ ($134 \pm 57$ ms; $n = 19$) SGNs (Fig. 5 B). Similarly, slow time constants ($\tau_{slow}$) were significantly ($P < 0.05$) slower in both $Hcn1^{-/-}$ ($2,922 \pm 2,168$ ms; $n = 18$) and $Hcn1,2^{-/-}$ ($2,993 \pm 1,970$ ms; $n = 8$) compared with WT ($799 \pm 901$ ms; $n = 7$) and $Hcn2^{-/-}$ ($951 \pm 670$ ms; $n = 19$).

In summary, the data from $Hcn$-deficient mice revealed $I_h$ had significantly different biophysical properties in SGNs that lacked $Hcn1$. The differences included reduced current and conductance amplitudes, more negative $V_{1/2}$ values, and slower activation kinetics. These differences suggest that HCN1 subunits contribute to the
properties of \( I_h \) in SGNs. Interestingly, we found some differences in the properties of \( I_h \) in \( \text{Hen}2^{-/-} \) SGNs compared with WT, including reduction in \( G_h \), a small negative shift in \( V_{1/2} \), and a slight broadening of the activation curve, but no difference in kinetics, suggesting that the functional contributions of HCN2 to \( I_h \) in SGNs during early postnatal stages are minor relative to HCN1. Lastly, we suggest that small residual \( I_h \) in \( \text{Hen}1,2^{-/-} \) SGNs implies the presence of another HCN subunit. As \( \text{Hen}3 \) expression was shown to be minimal (Fig. 1 A), the small residual \( I_h \) is mostly likely caused by expression of \( \text{Hen}4 \).

\( I_h \) is modulated by cAMP

HCN channel activity can be modulated by intracellular cyclic nucleotides (DiFrancesco, 1986; DiFrancesco and Tortora, 1991). Previous work in auditory brainstem neurons has shown that cAMP shifts \( I_h \) activation toward more depolarized potentials and speeds up activation kinetics (Banks et al., 1993; Yamada et al., 2005). To examine cAMP modulation of \( I_h \) in SGNs, we measured \( I_h \) activation after application of a membrane-permeable cAMP analogue, 8-Br-cAMP (200 µM), to the external bath (Fig. 6 A). In the presence of 8-Br-cAMP, the \( I_h \) activation range was shifted (\( P < 0.001 \)) toward more depolarized potentials (\( V_{1/2} = -88.5 \pm 0.6 \text{ mV}; n = 4 \)) compared with control (\( V_{1/2} = -95.3 \pm 0.5 \text{ mV}; n = 4 \); Fig. 6 B), with no increase in the maximal conductance. The 7-mV shift in \( V_{1/2} \) in the presence of cAMP accounted for a 20% increase in \( I_h \) active at rest (approximately −80 mV). Similar results were found in rat SGN afferent dendrites, in which application of 200 µM 8-Br-cAMP resulted in a 12-mV positive shift in \( V_{1/2} \) (Yi et al., 2010).

\( I_h \) in adult SGNs

We found a significant increase in the amplitude of \( I_h \) at adult stages, indicating developmental regulation (Fig. 7 A). Cultured WT SGNs (\( n = 11 \); apex, \( n = 5 \); base, \( n = 6 \)) from adult mice P40 or older had a mean conductance of 13.4 ± 4.2 nS, which represented a sixfold increase in \( G_h \) compared with neonatal SGNs (P1–4, \( G_h = 2.3 \pm 0.8 \text{ nS}; n = 25 \)). The tonotopic gradient in \( G_h \) expression during the first postnatal week (Fig. 2 D) was absent at adult stages (apex, 15.1 ± 5.8 nS; base, 12.0 ± 1.9 nS; \( P = 0.24 \)), \( V_{1/2} \) (apex, −94.9 ± 3.8 mV; base, −92.3 ± 6.3 mV; \( P = 0.44 \)), and \( s \) (apex, 7.8 ± 1.8 mV; base, 7.8 ± 2.8 mV; \( P = 0.93 \)).

Representative activation curves for adult WT and \( \text{Hen} \)-deficient SGNs are shown in Fig. 7 B. Relative to adult WT SGNs, adult \( \text{Hen}1^{-/-} \) SGNs had significantly (\( P < 0.001 \)) reduced mean \( G_h \) (Fig. 7 C), whereas \( \text{Hen}2^{-/-} \) SGNs had \( G_h \) values similar to WT. The \( V_{1/2} \) values of adult \( \text{Hen}1^{-/-} \) were also significantly (\( P < 0.001 \)) hyperpolarized compared with WT, whereas \( \text{Hen}2^{-/-} \) SGNs were not (Fig. 7, B and D). The slope factors were not significantly different between the three genotypes (not depicted). Activation kinetics of adult \( I_h \) was analyzed as described for neonatal \( I_h \). Mean fast time constants (\( \tau_{\text{fast}} \)) for WT, \( \text{Hen}1^{-/-} \), and \( \text{Hen}2^{-/-} \) SGNs are presented in Fig. 7 E. Interestingly, the activation kinetics (\( \tau_{\text{act}} \)) was significantly (\( P < 0.01 \)) faster in adult WT neurons (57.3 ± 17.7 ms; \( n = 11 \)) than in early postnatal neurons (127 ± 47 ms; \( n = 18 \)). Yet, like the early postnatal cells, deletion of HCN1 subunits resulted in a significant slowing of \( I_h \) activation in adult SGNs (255.6 ± 67.3 ms; \( n = 6 \); \( P < 0.01 \)).

Figure 5. \( I_h \) activation kinetics (\( \tau_{\text{act}} \)) comparison in WT versus \( \text{Hen} \)-deficient SGNs (P1–3). (A) Representative currents in WT, \( \text{Hen}1^{-/-} \), \( \text{Hen}2^{-/-} \), and \( \text{Hen}1,2^{-/-} \) in response to −144-mV voltage steps. \( I_h \) activation kinetics was measured by fitting a double exponential equation (black line; Eq. 2). (B) Summary of mean fast activation time constants. Number of samples and genotype are indicated below. Error bars equal ±1 SD. ***, \( P < 0.001 \).

Figure 6. Modulation of \( I_h \) voltage dependence by cAMP. (A) Representative \( I_h \) traces from WT SGNs (P4, apex) in control (left) and after bath application of 200 µM 8-Br-cAMP (right). Recordings were made in two different cells from the same SGN explant tissue. Currents were evoked using the same voltage-clamp protocol shown in Fig. 2. (B) Mean voltage-dependent \( G_h \) activation curve from P3–4 SGNs. 200 µM 8-Br-cAMP was used. Data were fitted by a Boltzmann function. Note the shift in \( V_{1/2} \) toward depolarized potentials with 8-Br-cAMP. Error bars equal ±1 SD.
whereas deletion of HCN2 subunits did not have a significant effect on fast activation kinetics (43.2 ± 16.4; n = 11) compared with WT. Likewise, mean slow activation time constants (τslow) were significantly (P < 0.001) prolonged in Hcn1−/− (2,209 ± 1,408 ms; n = 6) compared with WT (870 ± 699 ms; n = 11) and Hcn2−/− (611 ± 335 ms; n = 11). Hcn1,2−/− mice do not survive into adulthood and thus were not examined. The adult qPCR data (Fig. 1 B) are consistent with the voltage-clamp data (Fig. 7), which showed significant reduction (approximately sixfold) of Gh in Hcn1−/− SGNs, supporting the notion that HCN1 is the principal subunit contributing to Ih function in WT adult SGNs.

In summary, genetic deletion of Hen1 had qualitatively similar effects in neonatal and adult SGNs, indicating that HCN1 subunits are major components of Ih channels in both early postnatal and adult SGNs. Deletion of Hen2 was without significant effect at adult stages, raising the possibility that HCN2 contributions to SGN cell bodies may be transient. Doubly deficient Hen1,2−/− mice did not live to adult stages, yet the similarity between residual Ih in neonatal Hen1,2−/− SGNs and the small residual Ih in adult Hen1−/− SGNs suggests the presence of additional HCN subunits, mostly likely HCN4 based on the qPCR analysis. The faster Ih activation kinetics at adult stages suggests that additional factors, perhaps intrinsic differences in endogenous cAMP levels, may also contribute to Ih function in adult SGNs.

Ih contributes to resting potential

To examine the functional contributions of Ih to SGN membrane responses, we recorded from neonatal SGNs (P1–8) in current-clamp mode. Application of ZD7288 resulted in significant (P < 0.05) hyperpolarization of WT SGN resting membrane potentials (Fig. 8, A and B). The mean resting membrane potentials before and after bath application of 100 µM ZD7288 revealed a 6-mV hyperpolarization. The hyperpolarized resting membrane potential after ZD7288 treatment was similar to that of Hen1−/− SGN resting membrane potentials (Fig. 8 A), whereas there was no significant difference in Hen2−/− or Hen1,2−/− SGNs. The data suggest that HCN1 subunits contribute depolarizing current that shifts the WT SGN resting membrane potential in the excitatory direction. The lack of resting potential effect in the Hen1,2−/− SGNs was unexpected but may be a result of compensatory up-regulation of HCN4 subunits or some other depolarizing current as a consequence of genetic deletion of both Hen1 and Hen2.

Ih contributes to sag and rebound potentials

To examine the contributions of Ih to membrane responses evoked by small input currents, we injected 5-pA hyperpolarizing currents for 2 s in WT SGNs. The membrane responded with a small fast hyperpolarization followed by a depolarizing “sag,” which returned the membrane potential toward the resting level (Fig. 8 B). The 5-pA current injections were sufficient to produce prominent sag, while ensuring that the cell did not hyperpolarize negative to the physiological range, approximately −100 mV. The depolarization sag developed over ~150 ms, which reflected the slow activation kinetics (τslow) of WT Ih (Fig. 5). At the termination of the hyperpolarizing current step, a prominent rebound potential (10–15 mV; n = 11) was evident (Fig. 8 B, black trace).

To confirm whether these membrane responses were the result of Ih, we blocked Ih with bath application of 100 µM ZD7288 (Fig. 8 B). ZD7288 hyperpolarized the resting potential and abolished the depolarization sag (Fig. 8 B, red trace) with a difference of 11.6 ± 2.8 mV (n = 10) relative to control (Fig. 8 C). The sag was measured as the difference between the peak hyperpolarization and the steady-state potential near the end of the

Figure 7. Ih expression in cultured adult SGNs. (A) Family of representative Ih traces from adult (≥P40, base) WT, Hen1−/−, and Hen2−/− SGNs. (B) Normalized Ih activation curves generated by fitting a Boltzmann function to tail currents shown in A. Note the hyperpolarized half-activation voltage shift in Hen1−/− SGNs compared with WT and Hen2−/−. (C and D) Summary of conductances (C) and half-activation voltages (D) for 11 WT, 6 Hen1−/−, and 11 Hen2−/− SGNs. (E) Summary of Ih fast activation time constants. Error bars equal +1 SD. **, P < 0.01; ***, P < 0.001.
channels are maximally activated within the physiological range.

As shown in Fig. 9 (A and B), an initial spike (at the onset of a depolarizing step) and a rebound spike (at the offset of a hyperpolarizing step) were evoked both before and after ZD7288 treatment. We found no significant difference in threshold for either the initial spike or the rebound spike, although the current required to evoke rebound spikes was less than that required for the initial spike (not depicted).

However, spike latency was significantly delayed for the rebound spike after treatment with ZD7288. Latency was measured as the time from the current step to the peak of first spike. There was a slight delay in the initial spike after ZD7288 exposure, but the latency difference after ZD7288 treatment (16.5 ± 7.7 ms; n = 9) was not statistically significant compared with control (15.2 ± 7.6 ms; n = 9; P = 0.08; Fig. 9, A [inset] and C). In contrast, there was a significant delay in the rebound spike in ZD7288-treated cells (P = 0.02) compared with control (Fig. 9, B [inset] and C). Blockage of Ih delayed the rebound spikes, on average, by 9.1 ± 9.6 ms.
To investigate the possibility that the delay in the rebound spike may have been affected by activation of low-voltage-activated calcium channels, we used bath application of the calcium channel blockers, nickel (100 µM) or mibefradil (2 µM), either alone or together with 100 µM ZD7288. Neither nickel nor mibefradil alone affected rebound spike latency, as no difference was found before (control) and after treatment (Fig. 10, A–C). However, when ZD7288 was added subsequently, there was a significant (P < 0.01) delay in the rebound spike, again indicating that Ih and not low-voltage-activated calcium channels is a significant contributor for rebound spike timing in SGNs (Fig. 10, A–C).

To investigate whether the Ih effects on resting potential, sag potential, and spike latency were caused by HCN1 or HCN2, we examined adult SGNs of WT, Hcn1−/−, and Hcn2−/− mice. SGNs from adult Hcn1−/− mice had resting potentials that were significantly more hyperpolarized than both WT and Hcn2−/− SGNs (Fig. 11 A). Hyperpolarizing current steps evoked a prominent sag (Fig. 11 B) that decayed more quickly than the sag recorded from neonatal SGNs (Fig. 8 B), consistent with the faster Ih activation kinetics in adult SGNs (Fig. 7 E). The amplitude of the sag potential was also significantly reduced in adult Hcn1−/− SGNs relative to WT and Hcn2−/− SGNs (Fig. 11 C). Lastly, we noted a delay in rebound spike latency in adult Hcn1−/− SGNs (Fig. 11, B and D), which confirms a prominent role for HCN1 in spike timing in adult SGNs.

Ih contributes to synchronized firing
To address the extent to which tonically active Ih contributes to AP firing, we delivered a series of 20 AP threshold current steps in WT SGN cell bodies before and after bath application of 100 µM ZD7288. Before ZD7288 treatment, SGNs exhibited little variation in spike latency for a threshold stimulus (Fig. 12 A, black traces). After application of ZD7288, we found both an AP delay and a larger distribution in spike latency (Fig. 12 A, red traces), suggesting that Ih active at rest contributes to the regularity of spike timing. To examine the contributions of Ih to the AP wave form, the spikes shown in Fig. 12 A were aligned with their peaks at time 0 and averaged. The averaged spikes before and after application of ZD7288 (Fig. 12 B) revealed that Ih functions to speed the depolarization to threshold and depolarize the membrane during the after hyperpolarization but does not affect the peak or rising/falling phases of the AP. Spike latency was also delayed and with greater distribution for small super-threshold stimuli of 30–40 pA (Fig. 12 C), but the effect was absent for larger stimuli (≥50 pA). The data support the notion that Ih helps shape the membrane response to small current steps, has significant impact on AP latency, and functions to enhance synchronized firing in response to small depolarizing stimuli.

Ih contributes ABR latency
Given that the results presented in the previous sections show HCN1 subunits carry a significant fraction of Ih in both neonatal and adult SGNs and that Ih contributes to resting membrane potential, spike latency, and synchrony, we wondered whether Ih may contribute to auditory processing. Behavioral deficits in auditory function have not been reported in previous studies of Hcn-deficient mice (Ludwig et al., 2003; Nolan et al., 2003, 2004; Herrmann et al., 2007). As none of these prior studies focused on auditory function, we decided to measure ABRs to sound stimuli in adult Hcn1−/− mice and their WT littermate controls. We restricted our analysis to the first peak of the ABR waveform, which represents the summed activity of the eighth cranial nerve. Relative to WT littermate controls, Hcn1−/− mice showed no difference in threshold (Fig. 13 A) or amplitude (Fig. 13 B), indicating that

**Figure 10.** Ih regulates rebound spike latency. (A) Representative traces of rebound spikes recorded from a WT SGN (P3, base) in three different conditions, control, 100 µM nickel, and both nickel and 100 µM ZD7288. Note that calcium channel blocker, nickel, had little effect on resting membrane potential, sag potential, and rebound spike latency (inset). However, subsequent block of Ih by ZD7288 abolished the depolarizing sag and delayed the rebound spike (inset). (B) Representative traces of rebound spikes recorded in WT control conditions, with 2 µM mibefradil, and with mibefradil and 100 µM ZD7288. Here again, the T-type calcium channel blocker had little effect on resting membrane potential, sag potential, and rebound spike latency (inset), whereas ZD7288 hyperpolarized the cell, blocked the sag potential, and delayed the rebound spike. (C) Spike delay was measured as the difference between spike peaks in WT control conditions and after application of the drugs indicated below. Number of cells is indicated on the bar graph. Error bars equal ±1 SD. **, P < 0.01.
auditory sensitivity and the number of responsive SGNs was unaffected. However, we did notice that *Hen1*−/− mice showed longer compound AP latencies at several stimulus intensities (Fig. 13 C). As temporal fidelity is a critical parameter for auditory processing, we suggest that differences in latency at the level of the single SGN AP and in the ABR waveform may have significant consequences for normal sound processing.

**Discussion**

We characterized *Ih* in SGN cell bodies harvested from neonatal (P1–8) and adult (≥P40) mice using the whole-cell, tight-seal technique. Biophysical analysis of *Ih* in WT and *Hcn*-deficient mice indicated a prominent role for HCN1 subunits as major carriers of *Ih* in SGNs. *Hen1*−/− SGNs exhibited significantly reduced conductances (*Gh*), hyperpolarized half-activation voltages (*V1/2*), and slow activation kinetics (*τa*). qPCR analysis of *Hen* mRNA revealed strong expression of *Hen1* in both neonatal and adult SGNs, strengthening the notion that HCN1 is a major functional subunit underlying *Ih* in SGNs. *Ih* in SGNs is partially active at the cell’s resting membrane potential and its activation increases with hyperpolarization. Here we present evidence that *Ih* contributes to SGN resting membrane potentials and firing properties, as blockage of *Ih* hyperpolarized the resting potential, abolished depolarization sag potentials, delayed rebound spikes, and increased variance in spike latencies. Furthermore, *Hen1*−/− mice showed longer ABR latencies, further supporting the view that HCN1 subunits contribute to AP firing patterns and high-fidelity auditory information transfer between the periphery and the brain.

SGN *Ih* is developmentally regulated

SGNs express various voltage-dependent currents (Adamson et al., 2002; Chen et al., 2011) in a manner that may serve to enhance developmental mechanisms throughout the first postnatal week until the onset of hearing, P10–12 (Romand, 1983). Consistent with this notion, we found that *Ih* in SGNs is developmentally...
regulated. Whole-cell voltage-clamp recordings from WT SGN cell bodies revealed a gradual increase in $G_h$ over the first postnatal week. Because of myelination of SGN cell bodies (Romand and Romand, 1987; Echteler, 1992), which presents a technical limitation for whole-cell electrophysiological recordings, we did not attempt to record from SGNs beyond P8, a time point after which myelination becomes prohibitively thick and the bony labyrinth encapsulating SGNs becomes fully calcified. Although our recording time frame (P1–8) does not provide the full view of SGN $I_h$ development, it did reveal a clear pattern leading up to the onset of hearing. Because synaptogenesis and mature innervation patterns between IHCs and SGNs are nearly complete by the end of the first postnatal week (Simmons et al., 1991; Pujol et al., 1998), it is likely that early postnatal development of $I_h$ in SGNs impacts maturation of the auditory system before the onset of hearing.

The lack of $I_h$ data around the onset of hearing raises the possibility that the developmental trend we noted is a transient phenomenon. However, our data from cultured adult SGNs indicate that $I_h$ expression is sustained into adulthood. Previous studies that showed the presence of $I_h$ in dissociated adult guinea pig SGN culture (Chen, 1997; Szabó et al., 2002) support this view.

**Tonotopic expression of $I_h$ in SGNs**

An important task of the auditory system is decomposing complex sound into constituent frequencies along the tonotopic axis of the organ of Corti. IHCs located in the basal part of the cochlea encode high-frequency sound, whereas those located in the apical region encode low-frequency sound. The tonotopic specialization is retained at each level along the ascending auditory pathway, including SGNs which are organized to reflect the tonotopic arrangement of IHCs (Rubel and Fritzsch, 2002).

To enable this tonotopic arrangement, SGNs are equipped with morphological and electrophysiological specializations, including graded expression of voltage-gated channels (Adamson et al., 2002; Lv et al., 2010, 2012; Chen et al., 2011).

In the present study, we found that $I_h$ in neonatal WT SGNs is also graded along the cochlear tonotopic axis, with greater $I_h$ amplitudes in neurons from the basal end of the cochlea. Considering the graded expression of other voltage-gated channels in neonatal SGNs (Adamson et al., 2002; Chen et al., 2011), it seems $I_h$ follows this trend. However, because the cochlea develops from base to apex, we cannot exclude the possibility that the graded $I_h$ expression we report here for neonatal SGNs might be a transient phenomenon reflecting the developmental lag between the cochlear base and apex. Indeed, at adult stages, we noted little difference between $I_h$ of basal and apical SGNs. Nonetheless, if larger $I_h$ in SGNs from the basal, high-frequency end persists in the interval between P8 and more mature stages, we predict it will contribute to enhanced spike timing. Similar trends have been shown in neurons of the medial nucleus of trapezoid body, higher-order auditory neurons in the brainstem, in which graded $I_h$ expression with high to low expression along the mediolateral (high to low frequency) axis results in a gradient of time delays in neuronal firing (Leao et al., 2006).

**Characterization of $I_h$ in mouse SGNs**

The biophysical properties of $I_h$ in WT SGNs in the present study are consistent with those previously reported for $I_h$ in the auditory system, including SGNs (Chen, 1997; Mo and Davis, 1997; Szabó et al., 2002; Yi et al., 2010) and auditory brainstem neurons (Banks et al., 1993; Bal and Oertel, 2000). WT SGN $I_h$ was a ZD7288-sensitive, slowly activating, noninactivating inward current evoked by hyperpolarizing voltage steps. $I_h$ was active at the cell’s resting potential, and its activation range was well fit by Boltzmann functions with parameters ($V_{1/2} = -97$ mV and $s = 6$ mV) similar those reported for SGN $I_h$ in previous studies of various rodent species (guinea pig: $V_{1/2} = -101$ mV and $s = 9$ mV [Chen, 1997]; and $V_{1/2} = -104$ mV and $s = 6$ mV [Szabó...]

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**Figure 13.** $Hcn1^{-/-}$ animals have delayed ABR latency. (A) Mean ABR thresholds for control and $Hcn1^{-/-}$ animals at 6–7 wk of age. (B and C) Mean wave 1 (P1) amplitude (B) and mean wave 1 (P1) latency responses (C) evoked by 16-kHz tone pips delivered across increasing sound stimulus intensity. Note that $Hcn1^{-/-}$ mice show delayed P1 latency relative to controls, whereas no significant difference was found in threshold (A) or amplitude (B). Error bars equal ±1 SEM. *, P < 0.05.
and examination of biophysical properties of Ih in vivo of their functional relevance. Through qPCR analysis of mRNA expression in neonatal SGNs indicating the presence of HCN1, HCN2, and HCN4 subunits in rat SGNs (Yi et al., 2010), none presented definitive physiological evidence identifying the precise HCN subunits involved.

Expression of HCN1, HCN2, and HCN4 in neonatal SGNs Given their complex heterogeneous biophysical properties, identification of HCN subunits mediating SGN Ih is challenging, yet critical for a comprehensive understanding of their functional relevance. Through qPCR analysis and examination of biophysical properties of Ih in Hcn1−/− deficient animals, we demonstrated that Ih in neonatal SGNs is carried by HCN1, HCN2, and HCN4 subunits, with HCN1 being the most prominent subunit. Deletion of Hcn1 resulted in significant reduction in Ih, hyperpolarized half-activation voltage, and slower activation kinetics. The biophysical differences we noted in neonatal Hcn1−/− deficient SGNs are consistent with those of HCNs expressed in various heterologous systems. Homomeric HCN1 channels have the fastest activation kinetics and most depolarized half-activation range (Santoro et al., 2000; Ishii et al., 2001; Altomare et al., 2003), whereas homomeric HCN4 channels show the slowest activation kinetics (Ludwig et al., 1999; Ishii et al., 2001). Homomeric HCN2 channels exhibit activation kinetics intermediate to HCN1 and HCN4 (Ludwig et al., 1999). In our study, Ih in Hcn2−/− SGNs had reduced amplitudes and a negative shift in the half-activation voltage; however, the differences were not as striking as those between Hcn1−/− and WT SGNs, suggesting that the functional contributions of HCN2 subunits are minor. The low expression levels of Hcn2 mRNA in neonatal SGNs also suggested a small contribution from Hcn2. Previous immunolocalization work (Yi et al., 2010) demonstrated HCN2 immunoreactivity in P20 rat SGNs, but earlier time points were not examined. Thus, it is unclear whether there are actual differences in the temporal expression pattern of HCN2 or differences between rodent species. Yi et al. (2010) also reported robust expression of HCN1 and HCN4 in rat SGN cell bodies at P9–10. Their HCN1 and HCN4 localization data are consistent with our qPCR and electrophysiological data, and both studies suggest contributions from HCN1 and HCN4 before the onset of hearing at P10–12.

HCN1 contributes to Ih in adult SGNs Analysis of mRNA expression in neonatal SGNs indicated that Hcn1 is the most abundantly expressed subunit, followed by Hcn4 and Hcn2. Strong Hcn1 expression persisted into adult stages, suggesting HCN1 as the major subunit in adult SGNs. Our qPCR data are in agreement with Hcn1 gene expression results from the SHIELD database (Shared Harvard Inner-Ear Laboratory Database; Lu et al., 2011). This database incorporates RNA gene chip sequencing information from SGNs harvested from mice expressing GFP under an SGN-specific MafB promoter at several developmental time points (embryonic day 16, P0, P4, P7, and P16). Among the three Hcn genes (Hcn1, Hcn2, and Hcn3) examined, Hcn1 showed the strongest signal with a gradual developmental increase, consistent with our findings. The strongest evidence supporting a prominent role for Hcn1 in adult SGNs is derived from our voltage-clamp data, which showed a ~80% reduction in Ih in SGNs from adult Hcn1−/− mice relative to WT. There was also significant difference in half-activation voltage and activation kinetics (τact) relative to WT, strengthening the notion that Hcn1 is indeed a major subunit contributing to Ih function in adult SGNs. There was no difference in Ih of adult SGNs from Hcn2−/− mice relative to WT, suggesting Hcn2 does not contribute at adult stages. The qPCR data suggested expression of Hcn4 at adult stages; however, in the absence of electrophysiological data supporting this result, we are reluctant to draw conclusions about functional contributions of Hcn4 in adult SGNs.

Ih contributes to SGN membrane potential Ih has been shown to contribute to resting membrane potentials by providing inward current at rest. We demonstrated Ih has a similar role in resting membrane potentials in SGN cell bodies, as acute block of Ih with ZD7288 resulted in significant resting potential hyperpolarization (~6 mV) in WT SGNs. Similar results were reported for postnatal rat SGN dendrites, which had a ~4-mV hyperpolarization after ZD7288 treatment (Yi et al., 2010). Ih contribution to resting potential has also been observed in higher-order auditory neurons, such as cochlear nucleus and inferior colliculus, where inhibition of Ih resulted in similar resting membrane potential hyperpolarization (Bal and Oertel, 2000; Koch and Grothe, 2003; Nagtegaal and Bosr, 2010).

In WT neurons, depolarization of the resting membrane potential is caused by tonic activation of Ih at rest. Based on the SGN Ih activation curve, we estimate that ~5% of Ih is active at rest at neonatal stages and ~20% at adult stages, suggesting an Ih contribution may be greater at adult stages. Because the reversal potential of Ih in SGNs is more positive than the cell’s resting membrane potential (Chen [1997]: −36 mV; Mo and Davis [1997]: −41 mV; and Yi et al. [2010]: −45 mV), the resulting inward current drives the membrane toward more depolarized voltages. The tonic inward current produced by Ih in SGNs is most likely mediated through HCN1 subunits, as Hcn1−/− SGNs exhibited hyperpolarized resting membrane potential similar to WT cells treated with ZD7288.
Given that SGNs are critical for high-fidelity information transfer between IHC and central neurons in the auditory pathway, the functional significance of Ih in SGNs is clear. Because APs are generated at the spike initiation zone close to the IHC-SGN afferent synapse, it could be argued that the role of the cell body may be minor for auditory signal propagation. Recently, Yi et al. (2010) showed the presence of Ih in SGN afferents and its contribution to shortening the EPSP (excitatory postsynaptic potential) waveform at the IHC-SGN synapse. We suggest Ih in SGN cell bodies may also contribute to signal propagation from afferent terminals to central axons. SGNs are bipolar with large cell bodies positioned between dendrites and axons, which may present a problem for signal propagation (Hossain et al., 2005). The large SGN cell body may behave as an electrical sink, where the electric load may cause impedance mismatch, which in turn could interfere with rapid and efficient signal propagation from dendrite to axon. One mechanism for overcoming this electrical challenge in SGNs is expression of ion channels at peripheral and central initial segments flanking SGN cell bodies (Hossain et al., 2005). Indeed, SGNs express several varieties of voltage-gated channels (Santos-Sacchi, 1993; Szabó et al., 2002) that may help enhance current density in the cell bodies. Given that HCN channels are also expressed in SGN cell bodies, it is plausible that the tonic activation of Ih at rest may provide an extra “boost” by depolarizing the resting membrane potential and thereby enhancing auditory signal transmission.

**Ih contributes to spike timing**

We found that Ih activation modulates SGN firing properties. Here we show that activation of Ih by physiologically relevant hyperpolarizing currents induced depolarizing sag potentials and enhanced rebound potentials in SGNs. The depolarization sag, with a time course that reflects Ih activation kinetics, slowly restored membrane potentials toward the resting potential. The rebound potentials at the end of hyperpolarizing steps enhanced membrane depolarization, bringing the cell closer to threshold for AP generation. During ZD7288 inhibition of Ih, the depolarizing sag and rebound potentials were completely abolished, suggesting that activation of Ih provides subthreshold inward currents that enhance SGN excitability. We have extended this observation in adult SGNs to show that HCN1, in particular, contributes to depolarizing sag potentials, enhanced rebound potentials, and shorter rebound spike latencies.

Previous studies indicated that additional factors may contribute to rebound spikes such as low-voltage-activated T-type calcium channels (Aizenman and Linden, 1999, Lüthi and McCormick, 1998). Upon hyperpolarization, T-type calcium channels can recover from inactivation, making them available to open and enhance rebound potentials. Recent studies demonstrated that Ih and low-voltage–activated T-type calcium channels act in concert to enhance rebound spike timing in auditory brainstem neurons (Felix II et al., 2011; Koppscheinpfug et al., 2011). As T-type calcium channels have been identified in murine SGNs (Chen et al., 2011; Lv et al., 2012), we wondered whether rebound spike timing in SGNs is caused by Ih alone or functions in concert with T-type calcium channels. We found that neither nickel, a broad spectrum calcium channel inhibitor, nor mibebradil, a T-type channel blocker, affected the depolarization sag or rebound spike timing. Thus, we conclude that Ih is a prominent contributor to rebound spike timing in SGNs with little contribution from calcium channels.

Ih also contributes to synchronized AP firing in response to small depolarizing stimuli. In the presence of ZD7288, the scatter in AP spike timing increased significantly for small depolarizing stimuli. The effect became less pronounced for larger depolarizing stimuli. We reason that synchronous firing in response to small depolarization is a direct consequence of tonic Ih activation at rest, which provides additional inward current, thereby driving membrane potentials toward the AP threshold more quickly. For larger depolarizing stimuli, Ih is reduced as membrane potential is driven closer to the Ih reversal potential and the relative contribution of Ih is diminished as a result of enhanced activation of voltage-gated sodium channels. Thus, Ih is most influential for small membrane potential deviations around the SGN resting potential.

Given the slow activation kinetics, it is unlikely that Ih can follow rapid membrane potential changes at auditory frequencies. Rather than following hair cell receptor potentials on a cycle by cycle basis, it is more likely that the tonic activation of Ih helps shape the direct current component of auditory signal processing by depolarizing the resting SGN membrane potential, leading to synchronized AP firing.

During development, however, the slow activation kinetics of Ih may suit the system. Before the onset of hearing (P12), IHCs fire recurrent Ca$^{2+}$ APs at a very low discharge rates (2–4 Hz; Kros et al., 1998), which are hypothesized to drive spontaneous activity in SGNs during development (Tritsch et al., 2007; Tritsch and Bergles, 2010). Considering the tonotopic and developmental regulation of Ih in SGNs as shown in the present study, it is possible that Ih primes SGNs to faithfully follow the recurrent Ca$^{2+}$ spikes from IHCs during development (Tritsch et al., 2007; Tritsch and Bergles, 2010; Johnson et al., 2011).

**Contributions of SGN Ih to auditory function**

Our results indicate that Ih contributes to SGN firing properties by regulating rebound spike timing and enhancing synchronized AP firing in response to small
depolarizing stimuli. If so, what is the functional significance of \(I_h\) for auditory processing? Because SGNs relay auditory information from the IHCs to higher-order auditory neurons via the eighth cranial nerve, our ABR data from *Hen1-/-* mice, which indicate measurable delays in the latency of auditory signals, suggest that HCN1 helps preserve temporal fidelity in the eighth cranial nerve.

Accurate and precise transmission of IHC signals with high temporal criticality is crucial for normal auditory information processing. Thus, we propose \(I_h\) has a role in ensuring temporal precision, particularly for faint auditory signals. Although we did not detect differences in ABR threshold in *Hen1-/-* mice, \(I_h\) may serve to boost sensitivity to faint auditory stimuli. In the mature auditory system, SGNs innervate IHCs in a 1:1 ratio (i.e., each SGN contacts only one IHC), whereas each IHC is innervated by groups of 20–30 SGNs (Liberman, 1982). This IHC-SSG innervation pattern ensures the activity of each SGN is strictly dependent on the activity of just one presynaptic hair cell and that each hair cell can drive the activity of multiple SGNs. Our findings suggest that \(I_h\) enhances synchronized AP firing to small depolarizing stimuli both within a single neuron and across a population of neurons. Thus, in response to slight changes in the membrane potential of a single IHC, SGN \(I_h\) enhances the likelihood of triggering synchronous APs in populations of SGNs. Synchronous firing, with reduced temporal jitter, across a population of neurons can help preserve temporal fidelity and enhance signal to noise ratios, particularly for small stimuli. Preservation of these signals may allow higher-order auditory centers to extract relevant faint signals from a noisy background. Thus, \(I_h\) in SGNs may serve to enhance temporal fidelity and boost sensitivity to soft sounds.

Yi et al. (2010) showed that \(I_h\) in the postsynaptic SGN dendrites can boost EPSP speed, particularly for small signals. Here we extend those observations and show that \(I_h\) carried mostly by HCN1, contributes to processing and transmission of auditory information in SGN cell bodies as well. Whether preservation of \(I_h\)-dependent temporal information is critical for behaviorally relevant auditory tasks such as sound localization remains to be determined.

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