Optimized Tuning of Auditory Inner Hair Cells to Encode Complex Sound through Synergistic Activity of Six Independent K⁺ Current Entities

Highlights

- $K_v1.8$, $K_v7.4$, $K_v11.1$, $K_v12.1$, and $BK_{Ca}$ provide six K⁺ conductances in inner hair cells.
- These conductances shape receptor potentials during sound stimulation.
- Combined activity of all channels improves hair cell frequency and intensity coding.
- The channel repertoire is an evolutionary adaptation to encode complex sound precisely.

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In Brief

Dierich et al. show that $K_v1.8$, $K_v11.1$, $K_v12.1$, $K_v7.4$, and $BK_{Ca}$ channels give rise to six K⁺ conductances in auditory inner hair cells. The combined activity of all of the channels synergistically improves frequency and intensity coding. This channel repertoire constitutes an evolutionary adaptation to encode complex sound through multifaceted receptor potentials.

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Optimized Tuning of Auditory Inner Hair Cells to Encode Complex Sound through Synergistic Activity of Six Independent K⁺ Current Entities

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SUMMARY

Auditory inner hair cells (IHCs) convert sound vibrations into receptor potentials that drive synaptic transmission. For the precise encoding of sound qualities, receptor potentials are shaped by K⁺ conductances tuning the properties of the IHC membrane. Using patch-clamp and computational modeling, we unravel this membrane specialization showing that IHCs express an exclusive repertoire of six voltage-dependent K⁺ conductances mediated by Kv1.8, Kv7.4, Kv11.1, Kv12.1, and BKCa channels. All channels are active at rest but are triggered differentially during sound stimulation. This enables non-saturating tuning over a far larger potential range than in IHCs expressing fewer current entities. Each conductance contributes to optimizing responses, but the combined activity of all channels synergistically improves phase locking and the dynamic range of intensities that IHCs can encode. Conversely, hypothetical simpler IHCs appear limited to encode only certain aspects (frequency or intensity). The exclusive channel repertoire of IHCs thus constitutes an evolutionary adaptation to encode complex sound through multifaceted receptor potentials.

INTRODUCTION

The mammalian inner ear processes sound over a wide range of frequencies and intensities. This high performance is realized through the conversion of a tonotopic sound-frequency map along the basilar membrane into electrical signals in inner hair cells (IHCs) and outer hair cells (OHCs) (Robles and Ruggero, 2001; Schwander et al., 2010). In these cells, sound-induced deflection of actin-based stereocilia (hair bundle) controls the activity of mechanoelectrical transduction (MET) channels (Burg et al., 2009; Pan et al., 2018; Ricci et al., 2006). This gives rise to depolarizing and hyperpolarizing receptor potentials (Schwander et al., 2010) that in OHCs drive somatic length changes thought to provide cochlear amplification associated with high sensitivity and sharp frequency selectivity in mammals (Dallos et al., 2008; Liberman et al., 2002; Zheng et al., 2000). In IHCs, depolarization activates voltage-gated Ca²⁺ channels that control neurotransmitter release triggering action potential firing in auditory neurons (Platzer et al., 2000).

Receptor potential characteristics vary significantly along the cochlear axis. In apical (low-frequency) IHCs, they exhibit a phasic (AC) component following sound frequency precisely up to few kilohertz (Dallos, 1985a, 1985b; Johnson, 2015; Russell and Sellick, 1978). In high-frequency basal IHCs, the AC component is attenuated and receptor potentials show predominant sustained (DC) potentials proportional in size to sound intensity (Johnson, 2015; Russell and Sellick, 1978). In the gerbil, the IHC membrane is intrinsically tuned to efficiently encode sound phase (frequency following) or intensity (intensity following) in the cochlear apex and base, respectively (Johnson, 2015). This is achieved through tonotopically graded activity of MET channels and accordingly depolarized resting potentials, larger resting conductance, and faster membrane time constants, enabling more precise AC potentials in apical IHCs (Johnson, 2015). IHCs along the tonotopic axis are characterized by graded combinations of three basolateral
voltage-dependent K⁺ currents classified into fast-activating \( i_{K,s} \), slowly activating \( i_{K,s} \), and negatively activating \( i_{K,n} \), based on their characteristics (Kros and Crawford, 1990; Kros et al., 1998; Oliver et al., 2003). These currents determine electrical IHC membrane properties, thereby controlling the shape and amplitude of the receptor potentials (Johnson, 2015; Kros and Crawford, 1990; Kros et al., 1998; Marcotti et al., 2003). Amplitudes of the basolateral currents vary gradually along the tonotopic axis (Johnson, 2015; Marcotti et al., 2003), which may be critical for intrinsic tuning of the IHC membrane to encode complex sound (Johnson, 2015). It is unknown whether such gradual amplitude changes are a general mechanism for intrinsic membrane tuning across species, although there is evidence for such tonotopic gradients in mice (Marcotti et al., 2003).

\( i_{K,a} \) and \( i_{K,c} \) are mediated by \( K_7.4 \) and \( BK_{Ca} \) subunits, respectively (Kharkovets et al., 2006; Rüttiger et al., 2004), but the molecular identity of the channels mediating \( i_{K,c} \) is unknown. It remains elusive whether the expression of the different K⁺ currents provides physiological advantages to encode sound. We identified the K⁺ channels underlying \( i_{K,s} \) in mature IHCs and implemented all K⁺ channels into an extended biophysical model to unravel their physiological relevance. We show that mature IHCs express at least six independent K⁺ current entities with distinct biophysical properties that synergistically optimize the encoding of complex sound in the cochlea.

RESULTS

Pharmacological Isolation of \( i_{K,s} \)

In mice, IHC K⁺ conductances are fully developed at the end of the third postnatal week (Marcotti et al., 2003). We analyzed IHCs in the last cochlear turn of C57BL/6J mice (either sex) between 19 and 21 days after birth and refer to these as mature IHCs. As shown earlier (Kros and Crawford, 1990; Kros et al., 1998; Marcotti et al., 2003, 2004; Oliver et al., 2003), depolarizing voltage steps elicited large outwardly rectifying K⁺ currents in these IHCs (Figure 2A). To identify the underlying channels, we isolated \( i_{K,s} \) by inhibiting \( i_{K,a} \) and \( i_{K,c} \).

We inhibited \( i_{K,a} \) through the extracellular application of the \( BK_{Ca} \) antagonist iberiotoxin (ibTX; 100 nM) (Lingle et al., 2019; Marcotti et al., 2003, 2004; Oliver et al., 2003), which depolarized the resting membrane potential by \(-65 \text{ mV}\) (Figure 2E). We inhibited \( i_{K,c} \) through the application of the specific \( K_7.1 \) antagonist E-4031 (20 μM) (Kros et al., 1998; Thurm et al., 2005). Both inhibited K⁺ currents with the same characteristics and amplitudes (at 0 mV: ibTX sensitive \(-32\%\), TEA sensitive \(-34\%\) of control currents: Figures 1A, 1B, S1A, and S1B). IbinTX and TEA did not depolarize IHC resting membrane potentials (ibTX: \( n = 9 \); TEA: \( n = 10 \); Figure 1C; Oliver et al., 2003) and thus did not affect non-\( i_{K,s} \) currents (Kimitsuki et al., 2010; Oliver et al., 2003). Due to the faster time course (Figure S1A), we used TEA (5 mM) for \( i_{K,a} \) inhibition in further experiments.

We inhibited \( i_{K,c} \) with the specific \( K_7.4 \) antagonist XE991 (10 μM; Figures 1D and S1C) (Kharkovets et al., 2006; Oliver et al., 2003), which depolarized the resting membrane potential of untreated IHCs by \(-48 \text{ mV}\) (\( n = 8 \); Figure 1E, black). This was the same when \( XE991 \) was applied after TEA (5 mM; \( n = 6 \); Figure 1E, red), demonstrating TEA insensitivity of \( i_{K,c} \) (Kimitsuki et al., 2010; Oliver et al., 2003). We calculated XE991-sensitive currents by subtracting currents after the application of XE991 from controls (Figures 1F and 1G). The XE991-sensitive currents consisted of two fractions: a small component activated at negative membrane potentials (\( V_h = -85.5 \pm 5.1 \text{ mV} \); \( n = 5 \)) and a larger component activated at more depolarized potentials (\( V_h = -17.8 \pm 3.2 \text{ mV} \); \( n = 5 \); Figure 1F).

After the inhibition of \( i_{K,a} \) and \( i_{K,c} \), the IHC resting membrane potential was close to \(-65 \text{ mV}\) (Figure 1E), and large outward currents remained (Figures 1I and S1D) that are generally referred to as \( i_{K,s} \) (Johnson, 2015; Marcotti et al., 2003, 2004).

Identification of \( K_7 \) Subunits Underlying \( i_{K,s} \)

We screened datasets provided by the SHIELD database for mRNA transcripts of voltage-gated K⁺ (\( K_7 \) ) channels (Liu et al., 2014; Scheffer et al., 2015; Shen et al., 2015). Taking \( K_7.4 \), which mediates \( i_{K,a} \) as a reasonable expression threshold (Kharkovets et al., 2006), we considered levels of \( K_7.1 \), \( K_7.3 \), \( K_7.11 \), and \( K_7.12 \) in a single-cell transcriptome analysis of mature IHCs as indicative of their presence in IHCs (Figure S2; Liu et al., 2014). We therefore asked whether these channels contribute to \( i_{K,s} \) in IHCs.

\( K_7.11 \) Channels Are Active at Rest in IHCs

We started with analyzing the expression of \( K_7.11 \) channels (\( K_7.11-1-K_7.11.3 \)) with reverse-transcriptase PCR (RT-PCR) and detected mRNA transcripts of \( K_7.11.1 \), \( K_7.11.2 \), and \( K_7.11.3 \) in cochlear lysates of wild-type (WT) mice (Figure 2A). Confocal microscopy demonstrated strong anti-\( K_7.11 \) immunosignals in the IHC membrane (Figure 2B) that were completely absent in mice carrying hair cell-specific deletion of the \( Kcnh2 \) gene encoding \( K_7.11 \) (\( Kcnh2^{Hc/-/-} \)). We did not detect any immunosignals against \( K_7.12 \) or \( K_7.13 \) in IHCs (Figure S3A), although the primary antibodies reliably detected recombinant subunits (Figure S3B). Supporting our initial screen (Figure S2), \( K_7.11 \) thus is the exclusive \( K_7.11 \) family member in IHCs. In untreated IHCs, the specific \( K_7.11 \) antagonist E-4031 (20 μM) (Ishi et al., 2003; Trudeau et al., 1995) inhibited substantial \( K_7.11 \) currents (\( n = 10 \); Figures 2C and S3C) and significantly depolarized the resting membrane potential by \(-4.7 \text{ mV}\) (\( n = 10 \); \( p < 0.05 \); Figure 2E). E-4301-sensitive currents and depolarization were completely absent in IHCs from \( Kcnh2^{Hc/-/-} \) mice, demonstrating that \( K_7.11 \) channels are functional in IHCs (Figures 2D, 2E, S3D, and S3F). To elucidate whether \( K_7.11 \) channels mediate \( i_{K,s} \), we applied E-4031 (20 μM) after the inhibition of \( i_{K,a} \) and \( i_{K,c} \). In these cells, E-4301 inhibited \(-20\% \) of the remaining currents at 0 mV (i.e., E-4301-sensitive currents accounted for one-fifth of the total \( i_{K,s} \); Figures 2F, 2G, and S2E). The E-4301-sensitive currents had a \( V_h \) and slope factors of activation of \(-12.8 \pm 1.4 \text{ mV} \) and \( 12.5 \pm 0.5 \text{ mV} \), respectively (\( n = 7 \); Figures 2H and 2I). These data showed that \( K_7.11 \) channels mediate a significant component of \( i_{K,s} \) in mature IHCs.

\( K_7.11.1 \) and \( K_7.1.8 \) Channels Mediate the 4-AP-Sensitive Component of \( i_{K,s} \)

Large outward currents remained in mature IHCs even after the inhibition of \( i_{K,a} (K_7.4) \), \( i_{K,c} (BK_{Ca}) \), and \( i_{K,c} (K_7.11) \), demonstrating that additional channels contribute to \( i_{K,s} \). In line, \( i_{K,s} \) comprises at least two components, of which only one is inhibited by 4-amino-pyridine (4-AP) (Marcotti et al., 2003). We thus hypothesized that the candidate channels \( K_7.11.1 \), \( K_7.1.8 \), and \( K_7.3.3 \) known to be
inhibited by 4-AP (Figure S2) (Dierich et al., 2018; Lang et al., 2000; Tian et al., 2002) mediate the 4-AP-sensitive IK_s component. Extracellular 4-AP (5 mM) rapidly inhibited outwardly rectifying currents in IHCs that accounted for 26% of controls (at 0 mV; Figures 3A, 3B, S4A, and S5A). Given the insensitivity of IK_f and IK_n (Kimitsuki et al., 2010; Marcotti et al., 2003; Oliver et al., 2003; Thurm et al., 2005), these currents represented 4-AP-sensitive IK_s. 4-AP blocked significantly fewer currents (19% of controls; p < 0.05) when applied after pharmacological inhibition of IK_s (Kv11.1) (together with IK_n and IK_f) (Figures 3C–3E and S4B).
Figure 2. Kv11.1 Channels Are Functional in Mature IHCs

(A) RT-PCR showed the expression of Kv11.1, Kv11.2, and Kv11.3 mRNA in cochlear lysates (M, nucleotide marker with size; K7.4 + glyceraldehyde 3-phosphate dehydrogenase [GAPDH], positive controls; neg., negative control without cDNA). The lower panel shows a second PCR with nested primers for Kv11.2 transcripts.

(B) Immunohistochemistry revealed strong anti-Kv11.1 immunosignals in WT IHCs (Kcnh2+/+; left) that were absent in Kcnh2+/- mice (right). Maximum intensity projections of confocal z stacks in apical turns of the organ of Corti (myosinVIIa, hair cell marker; all scale bars represent 10 µm). For the evaluation of antibodies and anti-Kv11.2/anti-Kv11.3 stainings, see Figures S3A and S3B.

(C and D) E-4031 (20 µM) inhibited K+ currents in IHCs in (C) Kcnh2+/+ (WT), but not (D) Kcnh2+/- mice.

(E) E-4031 (20 µM) did not depolarize the IHC resting potential in Kcnh2+/- mice (*p ≤ 0.05).

(F) Representative recordings of an untreated IHC and after application of the indicated substances.

(legend continued on next page)
These results showed that K_{11.1} channels contribute to the 4-AP-sensitive component of I_{K,S}.

4-AP still blocked significant currents even after the inhibition of K_{11.1}, demonstrating that other channels must contribute to 4-AP-sensitive I_{K,S}. To identify these channels, we isolated the remaining 4-AP-sensitive component by current subtraction early after application of the substance (Figures 3E and S5A). We found that these currents activated in a voltage-dependent manner (V_{th}: −2.2 ± 0.4 mV; slope: 13.3 ± 1.1 mV; n = 8) and did not inactivate (Figures 3E–3H). To evaluate whether these currents were mediated by Kv1.8, we used RT-PCR and indeed detected Kv1.8 mRNA transcripts in the cochlea (Figure 3I). After cloning cDNA-encoding Kv1.8 from the same cochlear lysates, we characterized recombinant Kv1.8 channels in Chinese hamster ovary (CHO) cells. Recombinant Kv1.8 channels did not inactivate, and their voltage dependence was indistinguishable from the respective 4-AP-sensitive IHC current (V_{th}: 0.9 ± 0.9 mV; slope: 11.1 ± 0.5 mV; n = 31; Figures 3F–3H). Of note, the native IHC currents activated significantly faster than the recombinant Kv1.8 channels (see scale bars in Figure 3E), just as the hair cell current recombinant K_{11.1} channels were completely inhibited by extracellular 4-AP (5 mM), but were largely insensitive to TEA (5 mM), E-4031 (20 μM), and XE991 (10 μM) (Figures S4C–S4I).

The properties of a second 4-AP-sensitive IHC component were consistent with recombinant K_{11.1} channels. As K_{11.1} mRNA transcripts are expressed in mature IHCs (Liu et al., 2014), K_{11.1} channels mediate 4-AP-sensitive I_{K,S} together with K_{11.1}.

4-AP Potentiates K^+ Currents in Mature IHCs

Extracellular 4-AP (5 mM) inhibited certain IHC currents very rapidly (Figure S5A), but it also induced a significant (and slow) hyperpolarization of the membrane potential (−7 mV) in the same IHCs (n = 6; p ≤ 0.001; Figure 4A). After the inhibition of I_{K,f}, I_{K,n}, and I_{K(S),K_{11.1}}, 4-AP (5 mM) still significantly hyperpolarized the IHC membrane potential (n = 7; p ≤ 0.001; Figure 4B), indicating that 4-AP activated K^+ currents. Whereas 4-AP inhibited IHC currents within seconds, it significantly potentiated outward currents at hyperpolarized potentials slowly (e.g., at −60 mV: n = 7; p ≤ 0.001; Figures 4C and S5A). These different time courses made possible pharmacological separation of two current components with differential 4-AP sensitivity (Figure S5A). Longer 4-AP (5 mM) application induced the activation of the remaining IHC currents at significantly more negative membrane potentials, as is evident by a change of V_{th} by −15 mV (n = 7; p ≤ 0.001; Figures 4D, 4E, and S5B) and by associated changes of the slope factors (n = 7; p ≤ 0.01; Figure 4F).

IHC Currents Share Distinct Pharmacological Properties with K_{12.1}

The 4-AP-activated IHC current showed striking similarities to the I_{K,S} candidate channel K_{12.1}: 4-AP potentiated currents through recombinant K_{12.1} channels and induced channel activation at hyperpolarized potentials (Figures S5C–S5K) (Dierich et al., 2018). We thus probed the expression of K_{12.1} subunits in IHCs. Available antibodies did not detect recombinant K_{12.1} channels reliably (Figure S5L), precluding the direct analysis of channel abundance with immunohistochemistry. However, we detected mRNA transcripts for K_{12.1} and its close relative K_{12.3} with RT-PCR in cochlear lysates (Figure 4G). Considering the SHIELD datasets (Liu et al., 2014; Shen et al., 2015), we conclude that K_{12.1} is the exclusive family member expressed in IHCs.

Due to a lack of specific antagonists, we probed K_{12.1} activity in IHCs with NS1643, which inhibits recombinant K_{12.1} (Figures S5G and S5H; Dierich et al., 2018). As NS1643 is a known activator of K_{11} channels (Casisi et al., 2006), we applied NS1643 after inhibiting K_{11.1} with E-4031 (and after inhibition of I_{K,f} and I_{K,n}). Extracellular NS1643 (30 μM) substantially reduced the remaining I_{K,S} (Figures S6A–S6C). However, as NS1643 (30 μM) also inhibited recombinant K_{11.8} channels (Figures S4G and S4I), the substance was not suited for demonstrating the expression of K_{12.1} in IHCs unequivocally. It is noteworthy that NS1643-sensitive currents in IHCs displayed biphasic activating kinetics: the activation kinetics of the slower component (e.g., τ (−30 mV): 32 ± 0.2 ms, n = 9) were the same as for the K_{1.8} currents in IHCs (e.g., τ (−30 mV): 33 ± 0.4 ms, n = 8), and the kinetics of the faster component were comparable to the currents remaining after the inhibition of I_{K,f}, I_{K,n}, and I_{K(S),K_{11.1},K_{1.8}} (τ (−30 mV): ~24 ms). Thus, the kinetics of the latter component were similar to those potentially mediated by K_{12.1}. These data indicated that two current components in IHCs were sensitive to NS1643 in IHCs that were potentially mediated by K_{1.8} and K_{12.1} channels.

Voltage-Dependent Mode Shift of Activation in Mature IHCs

We then probed K_{12.1} currents in IHCs by means of the mode shift of activation (Villalba-Galea, 2017); this manifests through a shift of activating voltages to hyperpolarized potentials following conditioning depolarization of the membrane (Dai and Zagotta, 2017; Dierich et al., 2018; Dierich and Leitner, 2018; Li et al., 2015). Voltage protocols were designed to maximize mode shift as determined for recombinant K_{12.1} (Dierich et al., 2018, 2019b) and consisted of a 200-ms conditioning pre-pulse (hyperpolarized: −60 mV; depolarized: 0 mV), followed by 600-ms voltage steps for channel activation (Figure 5A). The depolarized conditioning potential (0 mV) induced a large shift in the voltage dependence of recombinant K_{12.1} channels by ~50 mV (Figures 5A–5C; n = 10; p ≤ 0.001; Dierich et al., 2018). To examine whether the currents in IHCs also exhibited mode shift, we applied the same voltage protocols after the pharmacological inhibition of I_{K,f}, I_{K,n}, and I_{K(S),K_{11.1},K_{1.8}} (Figure 5F).
significant mode shift, as is evident by an ~20-mV shift of the activation voltages after the depolarized conditioning potential (n = 5; p ≤ 0.01; Figures S4D and S5E).

IHC currents share striking properties with recombinant Kv12.1 channels (4-AP/NS1643 sensitivity; mode shift). Mode shift of the native currents was attenuated compared to recombinant Kv12.1 channels, which is consistent with the reduced mode shift of recombinant Kv12.1 channels after potentiation through 4-AP (Dierich et al., 2018) also present in the IHC recording solution. Based on these unique characteristics and expression of mRNA transcripts in IHCs, we conclude that Kv12.1 channels are functional in IHCs.
Mature IHCs Express Six Independent K⁺ Currents

Mature apical IHCs of mice express at least five K⁺ channel populations giving rise to six different K⁺ currents (Figures S6D and S6E). IK,f(BKCa) and Kv7.4 currents together account for ~49% (IK,f: 34%; Kv7.4: 15%) of controls at 0 mV. Kv7.4 subunits mediate two components: small negatively activating currents resemble IK,n found in IHCs earlier (Marcotti et al., 2003; Oliver et al., 2003), and a larger fraction activates at more positive potentials resembling the currents described in apical IHCs of the gerbil (Johnson, 2015). IK,s accounts for ~51% of control currents and is mediated by Kv1.8, Kv11.1, and Kv12.1. At 0 mV, Kv1.8 accounts for ~19% and Kv11.1 for ~10% of control currents. The component remaining after the inhibition of IK,f(BKCa), K,7.4, Kv1.8, and Kv11.1 is probably mediated by Kv12.1 channels and accounts for ~22% of controls (at 0 mV).

Assessing the Functional Relevance of the IHC K⁺ Currents

To elucidate the physiological relevance of the IHC K⁺ currents, we refined a recently published computational model of apical IHCs (Altoë et al., 2018) by implementing all six K⁺ conductances (native model; Figures 6A and S7A) (for the properties of IK,f, see Kros and Crawford, 1990; the properties of IK,s and of IK,n over the whole voltage range were implemented into the model, as recorded in our study). The model input is sinusoidal stereocilia deflection controlling the activity of the MET channels (Figures 6A and S7B) (Altoë et al., 2018). The model outputs are IHC K⁺ currents and conductances (Figure 6) and membrane (receptor) potentials (Figures 7, S7C, and S7D). We extrapolated from the recordings the individual activation time constants close to the resting membrane potential and included them in the model (Figure S7A; for calculation see Method Details). The kinetics of Kv1.8, Kv11.1, and Kv12.1 currents and positively activating Kv7.4 are significantly slower than those of IK,f (Figure S7A). Furthermore, due to full activation, IK,n provides a constant K⁺ conductance at rest in the simulations. Hence, following the established nomenclature of basolateral K⁺ conductances in IHCs (Marcotti et al., 2003), we discriminate between IK,f and IK,n and assign currents through Kv1.8, Kv11.1, Kv12.1, and K,7.4 (positive fraction) to IK,s (Figure 6A).
The native IHC model (all K+ conductances) predicts a resting potential of −53 mV and a membrane resistance-capacitance (RC) time constant ($t_m$) of 0.24 ms, in good agreement with published recordings (Johnson, 2015; Johnson et al., 2011). This model further shows that all K+ channels are active at rest and during (sound) stimulation (Figures 6B–6F). Whole-cell currents were made voltage insensitive by imposing constant current steps (Figures 6B and 6D), and I$_{K_{n}}$ is only small in apical IHCs (Figures 1F and 1G; Johnson, 2015; Marcotti et al., 2003; Oliver et al., 2003). At rest, of the I$_{K_{s}}$ components, K$_{1.8}$ and K$_{7.4}$ provide the most conductance followed by K$_{11.1}$ channels, whereas the contribution of K$_{12.1}$ is only small (K$_{1.8}$ > K$_{7.4}$ > K$_{11.1}$ > K$_{12.1}$; Figures 6B and 6E). Furthermore, the channels differentially activate during membrane potential depolarization (i.e., sound stimulation; Figures 6B, 6C, and 6F).

**The Six K+ Conductances Optimize IHC Responses to Encode Complex Sound**

To assess physiological relevance of the K+ channels, we compared the “native model” (see above) with other simulations. In the “voltage-independent (conductances)” model, all of the K+ currents were made voltage insensitive by imposing constant resting conductance. In others, only individual channels (or channel combinations) were implemented as voltage dependent (indicated in Figure 7), allowing for analysis of the relevance of the respective components. This in silico approach allows the evaluation of the physiological relevance of the K+ conductances (Altoe` et al., 2018) without affecting the IHC resting properties (resting potential, RC time constant). For comprehensive visualization, some panels of Figure 7 show I$_{K_{s}}$ as a single component, although the underlying channels were modeled independently in all of the simulations.

First, we found that in the native IHC model the simulated receptor potentials as reported (Johnson, 2015) show characteristic onset and offset relaxations (e.g., depolarization and hyperpolarization sags) and are characterized by distinct AC and DC components (Figure 7A). Due to the slow activation kinetics, the I$_{K_{s}}$ components (K$_{1.8}$, K$_{7.4}$, K$_{11.1}$, and K$_{12.1}$) largely determine these onset and offset responses.

Second, we analyzed the AC component of the IHC receptor potential (Figure 7A, insert) by simulating the amplitudes of the oscillating receptor potentials as a function of stimulus frequency. These data are normalized to maximal receptor potential amplitudes at low frequencies to visualize membrane low-pass filtering (Figures 7B and 7C). The simulations show that...
the native K+ channel repertoire dramatically increases the upper cutoff frequency of the AC response. The cutoff frequency of the IHC membrane is approximately one octave higher in the native model (black) than in the voltage-independent model (gray; Figure 7B). More importantly, an increase in the cutoff frequency may be achieved by the expression of either IK,f (blue) or IK,s (red) (Figure 7B). Concerning the IK,s components, the improvement in AC responses is mainly mediated by the voltage-dependent activity of Kv1.8 or Kv7.4, with smaller contributions of Kv11.1 and Kv12.1 (Figure 7C). Voltage-dependent activation of the channels mediating IK,s produces an effective increase in basolateral conductance over stimulus presentation, hence leading to a significant decrease in the effective membrane time constant. Fast voltage-dependent activation of IK,f produces “phase-locked” fluctuation of the IHC conductance, thus increasing the membrane cutoff frequency beyond that predicted by the effective membrane time constant (see also Altoé et al., 2018). The increase in the cutoff frequency is always most pronounced in the native model.

We then analyzed the relevance of the K+ channels for the DC response of IHCs and plotted the DC component of receptor potentials in the different models as a function of the amplitude of stereocilia vibrations (i.e., stimulus intensity; Figures 7D and 7E). This showed that the exclusive presence of all of the IK,s components (red) dramatically compresses the DC potential (Figure 7D). IK,f (blue) alone also considerably compresses the DC component of the receptor potentials, but sole inclusion of IK,n (Kv7.4) does not affect the DC response (Figure 7D). The combination of all of the currents causes an even stronger compression of the DC component, leading to a dramatic reduction in the steepest slope of the curve from 0.21 mV/nm without voltage-activated channels (gray) to 0.08 mV/nm in the native model (black) (slope with all IK,s = 0.09 mV/nm, red; slope with IK,f = 0.16 mV/nm, blue) (Figure 7D). This curve slope difference highlights that in native IHCs, the largest range of stereocilia vibration amplitudes (greater than a factor of 2) can be encoded within the same receptor potential range. Assuming that mechanical responses of the cochlea increase by $\frac{1}{C^2}$ for a 1-dB sound level increase (compressive growth of mechanical responses at a rate of 0.3 dB/dB sound level; Robles and Ruggero, 2001), a factor 2 of deflection amplitudes corresponds to 20 dB for sound pressure levels. Our simulations hence point out that the

Figure 6. All K+ Current Entities Are Active at Rest and during Sound Stimulation
(A) Extended computational model of IHCs implementing all K+ currents. (B and C) At rest and during sound stimulation, all identified K+ currents are active (B) and whole-cell currents are dominated by IK,f (BKCa) (C) (4-kHz and 40-nm hair bundle deflections, gray inset). (B2) shows currents from (B1) on an expanded timescale. (D–F) Simulated conductance-voltage curves for IHC K+ currents. (D) At rest (dashed line; Vm), the whole-cell conductance is dominated by IK,f (followed by the sum of the IK,s components), and (E) all channels mediating IK,s are active (Kv1.8, Kv7.4, and Kv11.1 provide the largest conductance). (F) shows the fractional (normalized) voltage-dependent activation of IK,s components. For simulated conductance-stereocilia vibration (i.e., intensity) curves, see Figure 7F.
native K⁺ conductance repertoire extends by ~20 dB the range of sound levels that an individual IHC can encode within the same range of receptor potentials. This significant effect of the K⁺ currents on the dynamic range is dominated by the activation of the channels mediating I_{K,s} (Figure 7D), with most substantial contribution from either K₇,4, K₃,3, or K₁₁,1.1 (Figure 7E). More important, due to their heterogenous voltage dependence, these channels activate and saturate at different membrane potentials, with K₇,4 providing the most conductance at the resting membrane potential, followed by K₇,4, K₃,3, and K₁₁,1.1 (Figure 6E). Accordingly, they activate and saturate at different levels of stereocilia vibrations (i.e., depolarization) and thus stimulus level (Figure 7F). The individual components of I_{K,s} compete with the IHC DC response over an individually exclusive range of stereocilia displacements (Figure 7E). The individual activation curves of the channels sum up to an overall conductance (Figure 7F, red) that increases over a wide range of stereocilia vibration amplitudes (red; Figures 6E, 6F, and 7E); in other words, the combination of all “slow” components allows for sensitive conductance changes with stereocilia deflection over a far larger range of intensities. This overall I_{K,s} conductance does not saturate completely for stereocilia vibrations up to 1 μm (Figure 7F), which enables compressive growth of the DC potential over a larger range of stimulus intensities in IHCs expressing all I_{K,s} channel components (Figures 7D and 7E).

We further plotted simulated AC:DC ratios as a function of stimulus frequency (Figure 7G), which are a metric tailored for continuous signals conceptually equivalent to vector strength (or synchronization index) used to characterize phase locking in spiking neurons (Palmer and Russell, 1986). Thus, higher AC:DC ratios indicate more efficient phase locking. Compared to the voltage-independent conductances model, the frequency limit for phase locking increases dramatically with active I_{K,n} or I_{K,s} (Figure 7G). The native combination increases AC:DC ratios at all frequencies and intensities more significantly than the individual contributions. This improvement in AC:DC ratios is achieved mainly by the pronounced compression of the DC component through the channels mediating I_{K,s} (Figures 7D and 7E) and, to a lesser extent, by the increase of the cutoff frequency associated with the IHC low-pass filtering (Figures 7B and 7C). In particular, the compression of the DC potential through the slow currents accounts for the significant increase in AC:DC ratios at low frequencies (Figure 7G).

The I_{K,n}-to-I_{K,s} Relation Largely Determines the Tonotopic Response Properties of IHCs

In the models, I_{K,n} as the exclusive Kᵦ current does not significantly alter AC and DC responses of IHCs compared to the voltage-independent conductance model (Figures 7B, 7D, and 7G). This is caused by the fact that the I_{K,n} current is too small at the resting membrane potential. However, we wondered about the physiological relevance of I_{K,n} in IHCs. Recently, it was shown in the gerbil that I_{K,n} amplitudes were large in basal IHCs, whereas, in line with our results, apical IHCs express larger K₇,4-type currents activating at more positive potentials (Figures 1F and 1G; Johnson, 2015). Apical and basal IHCs are specialized for the precise encoding of sound frequency and intensity, respectively (Johnson, 2015). We thus hypothesized that the I_{K,n}-to-I_{K,s} relation determines the tonotopic response characteristics of IHCs. We compared two models (implementing I_{K,n} and I_{K,s}), in which the entire K₇,4-mediated conductance was assigned either to I_{K,n} (extreme basal IHC) or to the K₇,4-like fraction (extreme apical IHC), thereby varying the relative contribution of I_{K,n} to the whole-cell conductance. In these simulations, apical IHCs exhibit more depolarized resting membrane potentials than basal IHCs in full agreement with recordings (Johnson, 2015). However, in contrast to the recordings (Johnson, 2015), modeled apical IHCs display a slower membrane time constant, as the depolarization of the membrane potential is caused by a decrease in the total K⁺ conductance at rest (i.e., increased membrane resistance). This automatically increases the membrane time constant and highlights the importance of tonotopically graded activity of the MET channels for intrinsic tuning of the time constants in living IHCs (Johnson, 2015). Of note, graded MET channel activity was not included in the model, as this hampers analysis of the relevance of basolateral K⁺ channels. Furthermore, we found that apical IHCs exhibit more pronounced compression of DC responses, as the depolarized resting potential renders the K⁺ channels more sensitive to voltage changes (i.e., the channels retain an effectively larger activation range over stimulus presentation). This results in strikingly higher AC:DC ratios (especially at low frequencies) for apical than for basal IHCs (Figure 7H). Accordingly, this apical IHC model shows a more compressed DC response and more precise phase locking than the basal, just as shown for living IHCs (Johnson, 2015). Accordingly, these simulations suggest that the relative contribution of I_{K,n} to the whole-cell conductance (i.e., I_{K,n}/I_{K,s} ratio) largely determines the tonotopic response characteristics of IHCs.

Physiological Advantages of the Native K⁺ Current Repertoire

Our simulations showed that the native channel repertoire clearly optimizes IHC receptor potentials to encode complex sound. However, we wondered whether a similar improvement in receptor potentials may also be achieved through the expression of fewer K₇ population. We therefore performed additional simulations in which the entire conductance size provided by the native K₇ ensemble (Figures 6D and 6E: I_{K,n} + I_{K,s}) is assigned to either of the channels mediating I_{K,n} or I_{K,n}(K₇,4) exclusively: These simulations considered only I_{K,n} and one K₇ isoform and thus only two K⁺ current entities. As quality measures, IHC phase locking (i.e., AC:DC ratios versus frequency or intensity; Figures 7I and 7J) and characteristics of receptor potential DC components (compression, dynamic range, sensitivity) were assessed. These simulations show that assigning all of the K₇ conductance to I_{K,n}(K₇,4), I_{K,n}(K₇,11.1), or I_{K,n}(K₇,12.1) substantially reduces the efficacy of phase locking (i.e., frequency coding) compared to native IHCs (Figures 7I and 7J). Whereas sensitivity is not altered for I_{K,n}(K₇,4) or I_{K,n}(K₇,11.1), assigning all of the K₇ conductance to I_{K,n}(K₇,12.1) renders IHC receptor potentials more sensitive to low sound intensities by 5.5 dB (of stereocilia vibration) as compared to native IHCs (Figures 7K, S7C and S7D). Thus, expressing K₇,12.1 as the only K₇ subunit optimizes receptor potentials to detect faint sounds, but deteriorates phase locking. Conversely, assigning all of the K₇ conductance to either
Figure 7. Six K⁺ Current Entities Optimize IHC Receptor Potentials to Encode Complex Sound

(A) Simulated IHC receptor potentials during stereocilia deflections exhibit sustained DC and oscillating AC potentials (stereocilia vibration 40 nm at 4 kHz; gray inset).

(B–G) Comparison of different IHC models that consider only voltage–dependent activation of the conductances highlighted in the graph (all others are voltage insensitive; see text and Method Details for description). Differences represent the contributions of the individual components.

(B and C) Amplitude of the oscillating AC component as a function of stimulus frequency (normalized to maximum values at low frequencies) in the different models. (C1) Simulated AC responses for frequencies between 100 and 10 kHz. (C2) highlights a smaller frequency sector (close to 2 kHz) of simulations shown in (C1).

(D and E) Simulated DC response in IHCs presented as a function of stereocilia deflection amplitude (stimulation at 1 kHz).

(F) Channel conductances as function of stereocilia vibration amplitude: IHC K⁺ channels activate at different levels of vibrations, and their maximal conductances saturate at different sound intensities.

(G) AC:DC ratios (versus frequency) as measure of phase-locking efficiency (60 nm stereocilia deflection, 300 ms) in different models.

(H) AC:DC ratios in two models in which all of the Kv7.4-mediated conductance is either assigned to IK,n (extreme basal model) or to the current fraction that activated at more positive membrane potentials (extreme apical model). All of the other conductances were implemented with their native properties.

(I–K) Simulations in which all of the native Kv conductance (IK,s and IK,n) is assigned only to the one Kv subunit indicated in the panels.

(I and J) AC:DC ratios as function of (I) frequency or (J) stereocilia vibration amplitude.

(K) DC response as function of stereocilia vibration amplitude (stimulation at 1 kHz; see also Figure S7C for a logarithmic presentation of the same data). See text for details.
IK,s (K₁,7.4) or Ikᵦₖ(K₁,8) improves the phase locking of IHC potentials but reduces the sensitivity at low sound intensity by ~3 dB (Figures 7K, S7C, and STD). Thus, scaling-up Ikᵦₖ(K₁,7.4) or Ikᵦₖ(K₁,8) currents improves temporal precision at the expense of sensitivity. These simulations show that the combined activity of all of the Kᵥ channels is beneficial for IHCs to fine-tune the membrane electrical properties in terms of sensitivity to low-level sounds, temporal precision, and dynamic range.

**DISCUSSION**

*Kᵥ1.8, Kᵥ11.1, and Kᵥ12.1 Contribute to Ikᵦₖ in IHCs*

Our study was motivated by a single-cell transcriptome analysis of mature IHCs (Liu et al., 2014; Shen et al., 2015). Taking the Kᵥ7.4 expression as the threshold, Kᵥ1.8, Kᵥ3.3, Kᵥ11.1, and Kᵥ12.1 were the only Kᵥ subunits in IHCs. Experiments with a specific Kᵥ11 antagonist and in mice with hair cell-specific Kcnh2 deletion (Gutman et al., 2005; Ishii et al., 2003) demonstrated the functional expression of Kᵥ11.1 in IHCs, and 4-AP inhibited a current with properties consistent with recombinant Kᵥ1.8. As no other Kᵥ1 and Kᵥ11 subunits are expressed in IHCs, this demonstrated the expression of Kᵥ1.8 and Kᵥ11.1. Recent studies indicated the expression of Kᵥ1.8 and Kᵥ11.1 protein in hair cells (Lee et al., 2013; Nie et al., 2005), and Kcna10 (encoding Kᵥ1.8) knockout mice displayed mild auditory deficits (Lee et al., 2013).

Due to the lack of specific tools, the identification of native Kᵥ12.1 currents depends on unique channel properties. Similarly, closely related Kᵥ10 and Kᵥ11 were functionally identified in tissues by exploiting their distinct characteristics (see, for example, Hardman and Forsythe, 2009; Hirdes et al., 2005, 2009; Meyer and Heinemann, 1998). Recently, we identified 4-AP (a known Kᵥ channel inhibitor) as a potent activator (Dierich et al., 2018) and NS1643 (a Kᵥ11 agonist) as an inhibitor of Kᵥ12.1 (Casis et al., 2006; Dierich et al., 2018; Hansen et al., 2006). Kᵥ12.1 channels exhibit a mode shift of activation that manifests as a shift of activation to hyperpolarized potentials after membrane depolarization (Dai and Zagotta, 2017; Dierich et al., 2018; Dierich and Leitner, 2018; Li et al., 2015). Here, we identified IHC Kᵥ Kᵥ currents that reproduce the exclusive combination of these distinct Kᵥ12.1 characteristics, providing very strong evidence for the abundance of Kᵥ12.1. With the same strategy, we recently identified Kᵥ12.1 activity in cells heterologously expressing Kᵥ combinations, including Kᵥ11.1 and Kᵥ7 (Dierich et al., 2018). Hence, Kᵥ1.8, Kᵥ11.1, and Kᵥ12.1 channels are functional in IHCs and collectively mediate the IHC current Ikᵦₖ (Kros and Crawford, 1990; Marcotti et al., 2003). This is supported by their pharmacological characteristics completely reproducing these of Ikᵦₖ (Marcotti et al., 2003).

**Are Kᵥ3.3 Channels Expressed in IHCs?**

Although it was suggested by our database screen, we did not find any evidence for Kᵥ3.3 currents in IHCs: (1) we did not detect currents with fast voltage-dependent inactivation, a hallmark of Kᵥ3.3 (Fernandez et al., 2003; Vega-Saenz de Miera et al., 1992; Zhang et al., 2016); (2) Kᵥ3.3 channels are highly TEA sensitive (Fernandez et al., 2003; Vega-Saenz de Miera et al., 1992), but TEA and BKCa inhibitor IbTX blocked exactly the same IHC currents; and (3) Kᵥ3.3 channels are sensitive to 4-AP, but 4-AP-sensitive IHC currents were TEA insensitive and activated more negatively than Kᵥ3.3 (Vega-Saenz de Miera et al., 1992; Zhang et al., 2016). We thus consider the expression of Kᵥ3.3 channels in IHCs unlikely.

**Kᵥ7.4 Mediates Two Components in IHCs**

Kᵥ7.4 subunits mediate the current Ikᵦₖ in both hair cell types, which is small in IHCs but predominant in OHCs (Housley and Ashmore, 1992; Kharkovets et al., 2006; Leitner et al., 2011; Marcotti and Kros, 1999; Oliver et al., 2003). However, recombinant Kᵥ7.4 channels activate at 60 mV more positive potentials (Vᵦ ~ −20 mV) than Ikᵦₖ (Vᵦ ~ −80 mV) (Kubisch et al., 1999; Leitner et al., 2011, 2012), suggesting that unknown hair cell-specific interaction partners determine the properties of Kᵥ7.4 in these cells. In mature IHCs of mice, Kᵥ7.4 channels mediate a small Ikᵦₖ-like and a larger Kᵥ7.4-like component (Kubisch et al., 1999; Leitner et al., 2011, 2012). As amplitudes of these two fractions vary reciprocally along the cochlear axis (e.g., in the gerbil; Johnson, 2015), this hair cell-specific modulation could be regulated tonotopically. As the proportion of Ikᵦₖ to the whole-cell conductance determines the tonotopic differences in receptor potentials, this may constitute a physiological adaptation of IHCs to encode complex sound.

**The Kᵥ Channel Repertoire Optimizes the Encoding of Complex Sound**

Exquisite sensitivity, dynamic range, and temporal precision of hearing impose certain IHC specifications; detection of faint sounds requires sensitivity to small stereocilia vibrations, whereas encoding of a large range of sound intensities necessitates low sensitivity at high levels. Especially at low frequencies (<2 kHz), temporal precision needs to be high to encode temporal sound information in the auditory nerve precisely. These contrasting demands require the nonlinear regulation of IHC sensitivity. This level-dependent sensitivity of IHCs is greatly determined by asymmetric Boltzmann-type nonlinearity of MET channel activation (Cheatham and Dallos, 2000). However, if this was the exclusive mechanism, temporal precision would rapidly degrade with increasing sound levels due to the faster growth of DC components compared to AC components (see, for example, Shamma et al., 1986). We found that the Kᵥ channel repertoire plays a fundamental role in regulating the nonlinear sensitivity of IHCs and in improving temporal precision. As discussed earlier (Altoe et al., 2018), we found that nonlinear activation of the Kᵥ and BKCa channels produces a nonlinear increase in outward membrane conductance over continuous stimulus presentation, simultaneously reducing effective membrane time constant and electrical excitability. Thus, IHC Kᵥ currents act as compressive nonlinearity for receptor potentials and significantly increase the dynamic range of sound levels that a single IHC and consequently nerve fibers can encode (Altoe et al., 2018; Kros and Crawford, 1990; Lopez-Poveda and Eustaquio-Martin, 2006). The slow kinetics of the Kᵥ channels prevent that their conductance fluctuates at stimulus frequency, thereby producing a larger compression of the DC than of the AC component of receptor potentials. This increases temporal precision at all frequencies and opposes the degradation of AC:DC ratios.
with increasing stimulus levels. In contrast, fast BKCa channels allow for phase-locked fluctuations of the membrane conductance, which substantially increases the membrane cutoff frequency, rendering membrane potential oscillations effectively faster than those determined by the effective RC time constant (Figure 7B; Altoé et al., 2019). The combined activity of BKCa and K+ channels synergistically optimizes AC and DC responses more significantly than predicted from individual contributions. We conclude that the expression of K+ channels constitutes an important evolutionary adaptation to improve the dynamic range and temporal precision of IHC responses.

Expression of Various K+ Channels Is an Evolutionary Advantage

Our models show how the expression of various K+ channels is advantageous to IHCs: (1) Combined activity of the K+ channels leads to more pronounced improvement in the dynamic range and phase locking than expected from individual contributions. This is an advantage of multi-component systems. (2) Activation of the K+ channels (with limited individual sensitivity) sums up into a high-order Boltzmann nonlinearity, with steep sensitivity near rest that does not saturate over the entire range of physiological potentials. (3) As suggested earlier (Johnson, 2015), the k_ws/k_ws ratio essentially determines and optimizes tonotopic responses, which is clearly beneficial for sound encoding. (4) Expression of the channel repertoire optimizes receptor potentials to encode multiple modalities of complex sound, as each conductance contributes to the improvement of a different aspect. They individually optimize frequency responses (k_ws and k_ws(Kv1.8, Kv7.4)) or improve phase locking (k_ws-to-k_ws relation). At the same time, k_ws(Kv1.8, Kv7.4, Kv11.1) dramatically increases the range of sound levels that can be encoded. Supporting this, a study in gerbils showed that receptor potentials in apical IHCs with relatively large k_ws and k_ws more precisely follow sound frequency (Johnson, 2015). Our data demonstrate that the expression of multiple K+ channel entities provides clear functional advantages for IHCs. Could comparably precise encoding of complex sound be achieved by expressing fewer K+ channel entities? We addressed this in silico in cells in which all of the native K+ conductance was assigned to a single K+ entity. These simulations revealed that the native channel repertoire allows for an effective trade-off between the requirements of (1) high IHC sensitivity at low stimulus levels, (2) dynamic range compression, and (3) high temporal precision. Assigning all K+ conductance to individual K+ subunits either deteriorates the encoding of sound frequency and intensity or improves the encoding of one sound quality at the expense of another. Thus, expressing the native channel repertoire and the observed separation of physiological functions of the components constitutes an evolutionary adaptation to encode complex sound through multifaceted receptor potentials. “Simpler” IHCs may be limited to encoding only certain aspects (frequency or intensity) of sound.

Physiological Roles of Kv1.8, Kv11.1, and Kv12.1

We provided evidence for the relevance of Kv12.1 and Kv1.8 channels, as well as for homeric Kv11.1 channels that have been shown mainly in cardiac muscle. We found that Kv12.1 currents in IHCs exhibited a significant mode shift, but our results cannot provide evidence for the relevance of this phenomenon for sound encoding. It is tempting to speculate that biophysical properties of IHCs may be adjusted to continuous sound stimulation or pathological depolarization through such a mode shift, adding supplementary specialization to intrinsic IHC membrane tuning. However, further work is needed to unravel whether mode shift can be important for sound processing in hair cells.

STAR METHODS

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

M.D., A.A., S.V., D.O., and M.G.L. designed the research; M.D., J.K., S.E., V.R., M.K.S., R.N., and M.G.L. conducted the experiments; A.A. did the modeling; R.N. generated the mouse line; M.D., J.K., S.E., V.R., and M.G.L. performed the analysis; and M.D., A.A., D.O., and M.G.L. wrote the manuscript.
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### STAR METHODS

#### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Rabbit polyclonal anti-Kv11.1 | Alomone Labs | Cat #: APC-016 |
| Rabbit polyclonal anti-Kv11.2 | Alomone Labs | Cat #: APC-114 |
| Rabbit polyclonal anti-Kv11.3 | Enzo Life Sciences | Cat #: ALX-215-053-R100 |
| Rabbit polyclonal anti-Kv12.1 | Alomone Labs | Cat #: APC-113 |
| Mouse monoclonal anti-MyosinVIIa | Santa Cruz Biotecnology | Cat #: sc-74516 |
| Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 | Thermo Fischer | Cat #: A11008 |
| Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 633 | Thermo Fischer | Cat #: A21050 |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| Tetraethylammonium chloride | Tocris | Cat #: 3068 |
| Iberiotoxin | Tocris | Cat #: 1086 |
| XE991 dihydrochloride | Tocris | Cat #: 2000 |
| E-4031 dihydrochloride | Tocris | Cat #: 1808 |
| 4-AP | Tocris | Cat #: 0940 |
| NS1643 | Tocris | Cat #: 3062 |
| **Experimental Models: Cell Lines** |        |            |
| Chinese hamster ovary (CHO) dhFR- cells | ATCC | CRL-9096 |
| **Experimental Models: Organisms/Strains** |        |            |
| C57BL/6NCrl mice | Charles River | Strain Code 027 |
| C57BL/6NJ mice | The Jackson Laboratory | Stock No: 005304 |
| Kcnh2<sup>tm1a(EUCOMM)Wtsi</sup> KO first allele ES cells | European Conditional Mouse Mutagenesis Program (EUCOMM) | MGI ID |
| Prestin-Cre (BAC transgenic) mice | Tian et al., 2004 | N/A |
| **Oligonucleotides** |        |            |
| Primers for RT-PCR, see Table S1 (last page of supplementary pdf) | This Paper | N/A |
| **Recombinant DNA** |        |            |
| K<sub>0</sub>,1.8-pRFP-N1 | This paper | Uniprot: B2RQA1 |
| K<sub>0</sub>,1.1a-pcDNA3.1 | Dr. CK Bauer | Uniprot: O08962 |
| K<sub>0</sub>,1.2-pcDNA3.1 | Dr. CK Bauer | Uniprot: O54853 |
| K<sub>0</sub>,1.3-pcDNA3.1 | Dr. CK Bauer | Uniprot: O54852 |
| K<sub>0</sub>,12.1-pcDNA3.1-IRESeGFP | Dr. T Jegla | Uniprot: Q96L42 |
| Lyn11-GFP-pcDNA3.1 | Dr. D. Oliver | Uniprot: P07948 |
| **Software and Algorithms** |        |            |
| PatchMaster (HEKA) | HEKA Elektronik | https://www.heka.com/downloads/ |
| IgorPro | Wavemetrics | https://www.wavemetrics.com/downloads/current |
| **Other** |        |            |
| Inner hair cell biophysical model codes | This Paper | https://www.mechanicsofhearing.org/apg/ |
| DAPI | Sigma-Aldrich | Cat #: D9542 |
RESOURCE AVAILABILITY

Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Michael G. Leitner (Michael.Leitner@i-med.ac.at).

Materials Availability
Plasmids encoding cloned Kᵥ1.8 channel subunits will be provided upon request by the Lead contact, Michael G. Leitner (Michael.Leitner@i-med.ac.at).

Data and Code Availability
The code generated during this study is available at: https://www.mechanicsofhearing.org/apg/

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animal handling and animal experimentation
Mice were kept and treated according to German law and institutional guidelines at Philipps-University Marburg (Germany). All procedures complied to 3R principles and were approved by the research ethics committee (Tierschutzkommission) at Regierungspräsidium Gießen (Germany).

Mouse lines analyzed in this study
This study was performed on wild-type C57BL/6 mice and on mice with hair cell-specific deletion of the Kcnh2 gene (see below) of either sex between 18 and 23 days after birth.

Hair cell-specific Kcnh2 (Kᵥ11.1) knock-out mice
Kcnh2^tm1a(EUCOMM)Wtsi KO first allele (reporter-tagged insertion with conditional potential) embryonic stem (ES) cells were obtained from the European Conditional Mouse Mutagenesis Program (EUCOMM) (Dickinson et al., 2016; Skarnes et al., 2011) and were injected into C57BL/6NClr 8-cell embryos (Poueymirou et al., 2007). The resulting chimeras were mated to C57BL/6NCrl mice, and progeny were screened to confirm germline transmission. Following the establishment of germline transmitting progeny, heterozygotes were crossed with C57BL/6NCrl wild-type (WT) mice to produce a heterozygous mouse population. Mice heterozygous for the desired insert were then mated with Flp-O(N) deleter mice for removal of the neomycin-reporter cassette (Kranz et al., 2010), and resulting heterozygous mouse progeny were intercrossed to produce homozygous mice. Kcnh2^tm1a(EUCOMM)Wtsi mice were crossed with Prestin-Cre (BAC transgenic) mice (Cox et al., 2012; Tian et al., 2004) to obtain mice carrying loxP insertions together with Prestin-Cre (BAC transgenic). These mice were then crossed with Kcnh2^loxP/loxP mice to obtain Kcnh2^loxP/loxP mice with Prestin-Cre (BAC transgenic) that were then used for the experiments. These hair-cell specific knock-out mice are denoted as Kcnh2^HC/0 to differentiate from WT (Kcnh2^+/+) mice.

Cell line
Chinese hamster ovary (CHO) dhFR- cells (ATCC: CRL-9096) were utilized for transient overexpression of proteins (see below). Chinese hamster ovary (CHO) dhFR- cells were maintained as previously reported (Leitner et al., 2016). In brief, cells were kept in MEM Alpha Medium supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (pen/strep) (Invitrogen GmbH, Darmstadt, Germany) in a humidified atmosphere at 5% CO₂ and 37 °C.

METHOD DETAILS

Transient transfection
CHO cells were transiently transfected with jetPEI transfection reagent (Polyplus Transfection, Illkirch, France). All experiments were performed 48 h after transfection at room temperature (22°C-25°C). The expression vectors used were: Kᵥ11.1a-pcDNA3.1 (rat Kcnh2; isofrom 1a; Uniprot: O08962), Kᵥ11.2-pcDNA3.1 (rat Kcnh6; O54853), Kᵥ11.3-pcDNA3.1 (rat Kcnh7; O54852), Kᵥ12.1-pcDNA3.1-IRESeGFP (human KCNH8; Q96L42); Kᵥ1.8-pRFP-N1 (mouse Kcna10; B2RQA1), and Lyn11-GFP-pcDNA3.1 (transfection control; P07948).

RNA isolation and reverse transcription PCR
For isolation of RNA, the two inner ears were isolated from a C57BL/6J mouse, as previously reported (Leitner et al., 2011) and the cochleae were separated from the vestibular organ. The two cochleae were homogenized in 400 μl lysis buffer on ice three times for 10 s, and total RNA was isolated using thepeqGOLD Total RNA Kit (peqlab/ VWR Life Science, Radnor/USA). RNA purity was evaluated by NanoDrop One® (Thermo Fisher Scientific, Waltham, USA). For RT-PCR, 1 μg total RNA was reverse transcribed using random hexamer primers for Superscript II Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA) or gene-specific primers for
GmbH, Gottingen, Germany). For identification of Kv11.2 transcripts in cochlear lysates nested PCR was performed. A11008 and A21050) were used for detection. Nuclei were stained with 2e3 Cell Reports

Cloning of K\textsubscript{1.8} channels

Total RNA was extracted from the cochlea of P20-P23 mice usingpeqGOLD Total RNA Kit (peqlab/ VWR Life Science, Radnor/ USA), and cDNA synthesis (QIAGEN OneStep RT-PCR Kit) was performed with gene-specific primers for K\textsubscript{1.8}. The full-length RT-PCR product was separated with standard gel-electrophoreses and purified using QiAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). This 1826 bp fragment was cloned into a pGEM®-T Easy Vector (Promega, USA) by taking advantage of 3’ terminal deoxyadenosine overhangs of the PCR product and the 3’-deoxythymidine terminals of the vector. The coding region was excised with a single restriction digestion (NotI), and was inserted into a pRFP-N1 (Takarabio Europe) expression vector dephosphorylated with FastAP Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific). For the ligation reactions, we used a T4 DNA Ligase (Thermo Fisher Scientific, Waltham, USA) and incubated vector and insert in a ratio of 1:8 overnight at 4°C. The transformation was done in XL-10 gold E. coli cells.

Immunohistochemistry

Immunohistochemistry was performed on formaldehyde-fixed whole-mount preparations of the apical turn of the organ of Corti of C57BL/6J mice (between P19 and P21), as previously reported (Vogl et al., 2017). In brief, cochleae were removed from the temporal bone and after introduction of a small hole were placed in 2% paraformaldehyde for 2 h at 4°C. The apical turn of the organ of Corti was then dissected from the cochlea and separated from modiolus, stria vascularis and tectorial membrane. Samples were blocked and permeabilized for 1 h at room temperature in a buffer containing 10% normal goat serum (NGS), 0.3% Triton X-100, 20 mM PB, and 450 mM NaCl. Immunostaining was performed overnight at 4°C with commercially available primary antibodies detecting K\textsubscript{1.1} (1:400; Alomone labs: #APC-016; for K\textsubscript{1.1} and K\textsubscript{1.2} see Key Resources Table) and Myosin VIIa (1:200; Santa Cruz: sc-74516) was then dissected from the cochlea and separated from modiolus, stria vascularis and tectorial membrane. Samples were blocked and permeabilized for 1 h at room temperature in a buffer containing 10% normal goat serum (NGS), 0.3% Triton X-100, 20 mM PB, and 450 mM NaCl. Immunostaining was performed overnight at 4°C with commercially available primary antibodies detecting K\textsubscript{1.1} (1:400; Alomone labs: #APC-016; for K\textsubscript{1.1} and K\textsubscript{1.2} see Key Resources Table) and Myosin VIIa (1:200; Santa Cruz: sc-74516) and K\textsubscript{1.2} (1:400; Alomone labs: #APC-113). Alexa 488- and Alexa-633-conjugated secondary IgG antibodies (1:200; life technologies: A11008 and A21050) were used for detection. Nuclei were stained with 2 μg/ml 4’,6-Diamidine-2’-phenylindole dihydrochloride (DAPI, Sigma-Aldrich, Hamburg, Germany, D9542). For imaging, tissue was mounted on Superfrost microscope slides (Thermo Scientific; 4951PLUS4). Specificity of all primary antibodies was assessed on not transfected CHO cells and on CHO cells transiently expressing K\textsuperscript{+} channel subunits, and for the anti-K\textsubscript{1.1} antibody also on organ of Corti preparations isolated from hair cell-specific Kcnrh2 knock-out mice (see above). As commercially available antibody against K\textsubscript{12.1} (available at Alomone labs; #APC-113) produced unspecific immunosignals in not transfected CHO cells, we did not utilize this antibody for expression analyses in cochlear tissue. For K\textsubscript{1.8} channels there are no commercial antibodies available.

Confocal Imaging

Confocal imaging was performed with an upright LSM 710 Axio Examiner microscope using W-Plan-Apochromat 63x 1.0 VIS-IR water immersion objective (Carl Zeiss, Jena, Germany) (Leitner et al., 2018; Mavrantoni et al., 2015). Alexa 488 was excited at 488 nm with an argon laser and fluorescence emission was recorded at 493-597 nm, and Alexa-633 was excited at 633 nm with a HeNe633 laser (Zeiss) and fluorescence emission was sampled at 640-740 nm. All Images are presented as maximum intensity projections of confocal z stacks (step size 1 μm).

Electrophysiological Recordings

Whole-cell recordings were performed on acutely isolated IHCs (in a whole mount organ of Corti preparation) and transfected CHO cells in culture, as previously reported (Leitner et al., 2011, 2018). In brief, apical turns of the organ of Corti of C57BL/6J mice (P19-P21) were isolated in extracellular solution containing (in mM): 144 NaCl, 5.8 KCl, 1.3 CaCl\textsubscript{2}, 0.7 Na\textsubscript{2}HPO\textsubscript{4}, 0.9 MgCl\textsubscript{2}, 5.6 glucose, 10 HEPES, pH adjusted to 7.4 (NaOH) (305-310 mOsm/kg). For recordings, the tissue was transferred into an experimental chamber and continuously perfused with the same extracellular solution. Outer hair cells and supporting cells were removed carefully by gentle suction through a cleaning pipette to obtain access to the basal cell pole of IHCs (Dierich et al., 2019a). Whole-cell patch clamp recordings were performed at room temperature (22-24°C) with an HEKA EPC10 USB patch clamp amplifier controlled by PatchMaster software (HEKA, Lambrecht, Germany). Voltage clamp recordings were low-pass filtered at 2.5 kHz and sampled at 5 kHz. Recordings were excluded from analyses, when the series resistance (Rs) was ≥ 6 MΩ, and Rs was compensated through-out the recordings (80%-90%). Rs typically was ~4 MΩ and consequently remaining Rs was about 0.4 MΩ (90% compensation) to 0.8 MΩ (80% compensation) in the majority of recordings. We did not correct for voltage errors (according max. 3 – 6 mV at largest control currents of about 7.5 nA recorded at +30 mV) caused by this remaining Rs. Steady-state current amplitudes are presented as normalized to cell capacitance (current density: pA/pF; main text) and as absolute current amplitudes (Supplemental Figures). Membrane potentials shown were not corrected for liquid junction potentials (approx. –4 mV). Patch pipettes were pulled from borosilicate glass (Sutter Instrument Company, Novato, CA, USA) and had a resistance of 2-3.5 MΩ after filling with intracellular solution containing (mM): 135 KCl, 3.5 MgCl\textsubscript{2}, 2.4 CaCl\textsubscript{2} (0.1 free Ca\textsuperscript{2+}), 5 EGTA, 5 HEPES and 2.5 Na\textsubscript{2}ATP (pH adjusted with KOH to 7.3; 290-295 mOsm/kg).
Reagents
Tetraethylammonium (TEA; Tocris: Cat. 3068), iberiotoxin (ibTX; Tocris: Cat. 1086), 10,10-bis(4-Pyridinylmethyl)-9(10H)-anthracenone dihydrochloride (XE991; Tocris: Cat. 2000), N-[4-[[1-[(6-Methyl-2-pyridinyl)ethyl]-4-piperidinyl]carbonyl]phenyl]methanesulfonyamide dihydrochloride (E-4031, Tocris: Cat. 1808), 4-aminopyridine (4-AP; Tocris: Cat. 0940), 1,3-Bis-(2-hydroxy-5-trifluoromethyl-phenyl)-urea (NS1643, Tocris: Cat. 3062), were diluted in extracellular solution to concentrations indicated in Results. All substances were applied locally to the cell under investigation via a glass capillary through a custom-made application system. “Control currents” signify currents recorded in untreated IHCs through-out the manuscript.

Computational Model of Receptor Potentials
We extended a previously published computational model of IHCs (Altoè et al., 2018) by implementing six voltage-dependent K+ current components in IHCs (Ik,t, Ik;c, and the components of Ik,s in this study: Ik,s(Kv1.8), Ik,s(Kv7.4), Ik,s(Kv11.1), Ik,s(Kv12.1)) together with a shunt IHC membrane capacitance (set to 9.8 pF in good agreement to our recordings, 9.8 ± 1.2 pF, n = 38 IHCs) and activity of mechano-electrical transduction (MET) channels (see Figure 7A for model details). Note that the individual current (conductance) voltage curves for the K+ current components Ik,s and Ik,c were implemented into the models over the whole membrane potential range as recorded. The model input is the sinusoidal deflection of the IHC hair bundle, which controls the activation of the MET channels (detailed description in Appendix A of Altoè et al., 2018; for activation curves of the MET channels see Figure S7B). The outputs of the model were the IHC receptor potentials and whole cell K+ currents modeled at 37°C. Voltage-dependent activation of the K+ channels is expressed by the following equations:

\[ I(t) = g \ast (V_m - E_K), \quad g(t) = m \ast G_{max}, \quad m + \tau \frac{dm}{dt} = m_i, \quad m_i = \left[ 1 + \exp \left( -\frac{V_m - V_{0.5}}{s} \right) \right]^{-1}. \]

where \( V_m \) is the IHC membrane potential, \( E_K \) the reversal potential for K+, \( G_{max} \) the maximal conductance, \( V_{0.5} \) and \( s \) voltages of half-activation and sensitivity of the K+ conductance, respectively. \( m \) describes the steady fraction of open channels at a specific membrane potential, and \( \tau \), the activation time constant, describes the kinetics of the variation in open versus close channels with membrane potential changes (\( \tau \) does not model the kinetics of single channels). Thus, \( m \) and \( \tau \) are determined by the opening versus closing rates of the individual channels at a given membrane potential (see Chapter 6 in Koch, 2004). \( G_{max}, V_{0.5} \) and \( s \) were extrapolated from tail current recordings using least-square fit in MATLAB (MathWorks, Natick, MA) while imposing \( E_K \) to match recorded reversal potentials. The simulated currents and activation curves of the inner hair cell conductances are shown in Figures 6B–6D.

For the numerical simulations, \( E_K \) was set to −70 mV in line with our recordings and an earlier report (Marcotti et al., 2003). For simplicity, \( \tau \) was considered voltage-independent and set to values obtained near the IHC resting potential of about −53 mV in the model. Reliable recording of \( \tau \) close to the resting membrane potential was virtually impossible due to small current amplitudes. We thus extrapolated the value of \( \tau \) at the resting potential from Boltzmann fits to the time constants (derived from mono- or double exponential fits) recorded at more depolarised membrane potentials (more depolarised than −40 mV) for Ik,c, and the individual components of Ik,s. Note that the NS1643-sensitive current consisted of two components, of which the faster component was considered to be mediated by Kv12.1 channels (see Results for details). All \( \tau \) values were corrected for temperature by utilizing a Q10 factor of 2, following a previous report on temperature sensitivity of IHC K+ currents (Q10 between 1.83–3 in Kimitsuki and Komune, 2013). The time constant of activation of Ik,c was set to 0.3 ms based on Figure 12 of Kros and Crawford (1990). By means of various manipulations of the model parameters, computational simulations were utilized to elucidate the physiological relevance of the IHC currents. These manipulations (i.e., the different employed models) are comprehensively described in the Results section.

Quantification and Statistical Analysis
Patch clamp recordings were analyzed with PatchMaster (HEKA) and IgorPro (Wavemetrics, Lake Oswego, OR). Voltage dependence of activation was derived from tail current amplitudes using voltage protocols indicated: Maximal amplitudes of tail currents (quantified approx. 0.2–0.4 ms after the test potential) were fit with a two-state Boltzmann function with \( I = I_{min} + (I_{max} - I_{min})/(1 + \exp((V-V_i)/s)), \) where \( I \) is current, \( V \) is the membrane voltage, \( V_i \) is the voltage at half maximal activation, and \( s \) describes the steepness of the curve. Tail currents of XE991-sensitive currents were fitted with a double (sum of two) Boltzmann functions considering two \( V_i \) and \( s \) parameters (Figures 1F and 1G) \( (I_{min} + I_{max}/(1 + \exp((V-V_{i1})/s1)) + I_{max}/(1 + \exp((V-V_{i2})/s2))) \). \( s \) was presented as positive values to describe the slope of voltage-dependent channel activation. Results on voltage dependence are shown as normalized tail current (conductance)-voltage curves, obtained by normalizing to \( (I_{max} - I_{min}) \), obtained from fits to data of individual experiments. Time constants of activation were derived from mono- or double-exponential fits to activating current components at indicated potentials (Dierich et al., 2018). Statistical analysis was performed with two-tailed Student’s, Wilcoxon signed, Dunnett or Scheffé test. Statistical significance was assigned at \( p \leq 0.05 \) (\(* p \leq 0.05, ** p \leq 0.01, *** p \leq 0.001\)). All data are presented as mean ± SEM. In electrophysiological experiments, \( n \) represents the number of individual cells recorded from at least three independent experiments (independent transfections or independent tissue, i.e., biological replicates).