Association of the Kv1 Family of K⁺ Channels and their Functional Blueprint in the Properties of Auditory Neurons as Revealed by Genetic and Functional Analyses

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Abstract

Developmental plasticity in spiral ganglion neurons (SGNs) ensues from profound alterations in the functional properties of the developing hair cell (HC). For example, pre-hearing HCs are spontaneously active. However, at the post-hearing stage, HC membrane properties transition to graded receptor potentials. The dendrotoxin (DTX)-sensitive Kv1 channel subunits (Kv1.1, 1.2, 1.6) shape the firing properties and membrane potential of SGNs, and the expression of the channel undergoes developmental changes. Because of the stochastic nature of Kv subunit heteromultimerization, it has been difficult to determine physiologically relevant subunit-specific interactions and their functions in the underlying mechanisms of Kv1 channel plasticity in SGNs. Using Kcnal2 null mutant mice, we demonstrate a surprising paradox in changes in the membrane properties of SGNs. The resting membrane potential of Kcnal2−/− SGNs was significantly hyperpolarized compared to age-matched wild type (WT) SGNs. Analyses of outward currents in the mutant SGNs suggest an apparent ~2-fold increase in outward K⁺ currents. We show that in vivo and in vitro heteromultimerization of Kv1.2 and 1.4 α-subunits underlies the striking and unexpected alterations in the properties of SGNs. The results suggest that heteromeric interactions of Kv1.2 and 1.4 dominate the defining features of Kv1 channels in SGNs.
Introduction

Voltage-dependent potassium channels (Kv) sculpt neuronal excitability by regulating resting membrane potentials, spontaneous firing rates, neurotransmitter release and synaptic integration (Pongs, 1999; Schrader et al., 2002; Ishikawa et al., 2003). Indeed, Kv are the most diverse channels in the mammalian genome (Gutman et al., 2005). The functional assortment of Kv is further abounded by the heteromultimeric combination of pore forming (α) and auxiliary (β) subunits, demonstrated extensively in heterologous cells (Lai and Jan, 2006; Maffie and Rudy, 2008). However, in native neurons, because of the promiscuous features of Kv assembly, it is difficult to determine the precise contribution of each α-subunit to specific physiological processes in vivo or to predict the compensatory mechanisms that occur in response to perturbations in the activity of a given α- and/or β-subunit. To acquire this information, which is essential for a detailed understanding of multiple functions of Kv, in vivo analyses of gene deletion models are extremely powerful strategies (Brew et al., 2007).

The spiral ganglion of the cochlear nerve is the site where auditory information coding is initiated, transmitting precise and reliable information about the amplitude, duration and frequency of sound waves from hair cells (HCs) to the cochlear nucleus (CN). Ninety-five percent of the neurons (type I) innervate inner HCs (IHCs), whereas the remaining five percent (type II) innervate outer HCs (OHCs) (Morrison et al., 1975; Spoendlin, 1981). One of the Kv currents, the dendrotoxin (DTX)-sensitive K⁺ current (Mo et al., 2002; Schrader et al., 2002), serves as a key determinant of the resting membrane potential (rmp) (Ishikawa et al., 2003), rate of accommodation (Pongs, 1999), and adaptation of firing in spiral ganglion neurons (SGNs) information coding. The DTX-sensitive current contains Kv1.1, 1.2 and 1.6 subunits (Clark et al., 2008; Maffie and Rudy, 2008; Maffie et al., 2009). Analysis of the voltage-dependent
properties of DTX-sensitive currents in murine SGNs describes currents with at least two distinct half-activation voltages at -63 mV and 12 mV (Schrader et al., 2002; Rusznak and Szucs, 2009), raising the possibility that the current consists of heterotetrameric channels of the three Kv1 \( \alpha \)-subunits at different combinations. Meanwhile, among the Kv subunits tested for expression profiles in SGNs (Kv1.1, 1.2 and 1.6), only Kv1.2 has distinct differential expression in type I and II neurons (Amarillo et al., 2008), suggesting cell-type specific functions. To understand the roles of Kv1.2 subunits in SGNs, we examined the functional features of \( Kcna2 \) null mutant (\( Kcna2^{-/-} \)) neurons (Brew et al., 2007).

We report that null deletion of \( Kcna2 \) produced an unexpected result, which showed profound membrane hyperpolarization of SGNs. This effect resulted in increased membrane excitability. In accord with enhanced membrane hyperpolarization, further analyses of \( K^+ \) currents of \( Kcna2^{-/-} \) SGNs revealed an apparent ~2-fold increase in transient outward \( K^+ \) currents. We have demonstrated that \textit{in vivo} and \textit{in vitro} association of Kv1.2 and Kv1.4 \( \alpha \)-subunits is the mechanism underlying the unexpected results, providing insights into the complex interaction of \( K^+ \) channel subunits in SGNs.
Materials and Methods

Isolation of spiral ganglion neurons

We conducted these experiments in agreement with the guidelines of the Institutional Animal Care and Use Committee of the University of California, Davis. Spiral ganglion neurons (SGNs) were isolated from the inner ear of postnatal (P10-12) mice using a combination of enzymatic and mechanical procedures as described in detail (Lv et al., 2010). We restricted our studies to P10-12 because *Kcn2* null mutants (*Kcn2<sup>−/−</sup>*, hereafter referred to as Kv1.2<sup>−/−</sup>) do not survive past ~14 days due to seizures (Brew et al., 2007). Male and female mice were euthanized and the temporal bones were removed in a solution containing Minimum Essential Medium with Hank’s salt (Invitrogen), kynurenic acid (0.2 g/L), 10 mM MgCl<sub>2</sub>, 2% fetal bovine serum (FBS; v/v), and glucose (6 g/L). Additionally, we used *Kcn1* null mutants (*Kcn1<sup>−/−</sup>*, hereafter referred to as Kv1.1<sup>−/−</sup>) and their age-matched controls at P10-12 to conduct a set of experiments. The central spiral ganglion tissue was dissected out and split into three equal segments: apical, middle and basal, across the modiolar axis. We pooled three mice into each SGN culture. Since Kv1.2<sup>−/−</sup> mice do not live to be more than 2-weeks old, chances of obtaining mutant litters were less than the expected Mendelian ratio of 25%. Thus, for pragmatic reasons and paucity of null mutants, isolated SGNs were divided not by their location along the cochlear contour (Lv et al., 2010; Lv et al., 2012), but by their extent of excitability (see results).

The inner ear tissues were digested separately in an enzyme mixture containing collagenase type I (1 mg/ml) and DNase (1 mg/ml) at 37°C for 20 minutes. After a series of gentle triturations and centrifugation in 0.45 M sucrose, the cell pellets were reconstituted in 900 ml of culture media (Neuralbasal-A<sup>™</sup>, supplemented with 2% B27 (v/v), 0.5 mM L-glutamine, 100 U/ml penicillin; Invitrogen), and filtered through a 40-μm cell strainer for cell culture and electrophysiological experiments. For adequate voltage-clamp and satisfactory electrophysiological experiments, we
cultured SGNs for ~24-48 hours to allow for the detachment of Schwann cells from neuronal membrane surfaces. Additionally, by recording from neurons ~24-48 hours after dissociation, we avoided neurons with extensive neurite outgrowth to reduce space clamp problems. Moreover, to minimize space clamp artifacts, we targeted spherical neurons with reduced neurite outgrowth for voltage-clamp experiments (Bar-Yehuda and Korngreen, 2008). All electrophysiological experiments were performed at room temperature (21-22°C). Reagents were obtained from Sigma Aldrich (St. Louis, MO) unless otherwise specified.

**Electrophysiology**

Action potentials were amplified (100X), filtered (bandpass 2-10 kHz), and digitized at 5-500 kHz. Extracellular solution for action potential recording experiments contained (in mM): NaCl 130, KCl 5, MgCl_2_ 1, CaCl_2_ 1, D-glucose 10, HEPES 10, pH 7.4, with NaOH. The recording electrodes contained (in mM): KCl 112, MgCl_2_ 2, CaCl_2_ 0.1, HEPES 10, EGTA 1, K_2_ATP 5, Na_2_GTP 0.5, pH 7.3, with KOH. The Ca^{2+} concentrations in solutions were measured with a Ca^{2+}-sensitive electrode as described (Yamoah et al., 1998).

Whole-cell voltage-clamp recordings of K^+ currents were performed on SGNs using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). Current traces were amplified, filtered (bandpass 2 kHz), and digitized at 5-500 kHz using an analog-to-digital converter, Digidata 1200 (Molecular Devices), as described earlier (Rodriguez-Contreras and Yamoah, 2001; Leivic et al., 2007). Fire-polished electrodes (2-3 MΩ) were pulled from borosilicate glass. Outward K^+ current traces were generated with depolarizing voltage steps from a holding potential of -90/-70 mV and stepped to varying positive potentials (ΔV = 2.5-10 mV). The cell capacitance was measured by fitting the current response elicited from the holding potential of -80 mV and stepped to -100 mV. The capacitative transients were used to estimate the capacitance of the cell as an indirect measure of cell size. The seal resistance was typically 10-20 GΩ. Currents were measured with capacitance and
series resistance compensation (>60-90%). The series resistance was monitored during the course of the experiments. The liquid junction potentials were measured (< 3 mV) and corrected. Besides these standard requirements for acceptance of data, several basic criteria were set to ensure optimum quality of recordings and acceptance of data. These include: 1) Stabilization of seals for at least 5 minutes before recordings; 2) Elimination of cells with current traces that exhibit signs of voltage inhomogeneities; and 3) Exclusion of cells with more than 20% change in the series resistance before and during recordings.

Whole-cell K⁺ current amplitudes at varying test potentials were measured at the peak and steady-state levels using a peak and steady-state detection routine. The current magnitude was divided by the cell capacitance (pF) to generate the current density-voltage relationship. Dendrotoxin-K (DTX-K) and α-dendrotoxin (α-DTX) were purchased from Alomone Labs (Israel). CP 339818 hydrochloride was purchased from Tocris Bioscience (Minneapolis, MN). Curve fits and data analyses were performed using Origin software (MicroCal Inc., Northampton, MA). Where appropriate, we presented data in the form of means ± S.D. (standard deviation). The mean values (n) listed represent data for each experimental group. Significant differences between groups were tested using paired/unpaired Student’s t test.

**Heterologous expression of Kv channels**

Kv1.2 (gene ID: NM_008417, MGC ID:170276) and Kv1.4 (gene ID: NM_021275, MGC ID:124445) cDNA clones were purchased from Open Biosystems and the open reading frames were subcloned into the pIRES2-EGFP expression vector for patch-clamp recordings. Chinese Hamster Ovarian (CHO) cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). CHO cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Invitrogen). Cell cultures were kept at 37°C in a 5% CO₂ incubator. The cells were trypsinized, plated at a concentration of $1.5 \times 10^5$ cells/ml in 2 ml of
culture medium in 35-mm dishes, and transfected with 1 μg of total DNA per dish. Transfections were performed using Lipofectamine, following the manufacturer's protocol (Invitrogen). The cells were rinsed in fresh culture medium and incubated for 24 hours before performing patch clamp recordings. Transfected cells were identified for recording by visualization of the EGFP co-expression (BD Bioscience, Clontech).

For the study of subcellular and membrane localization of Kv subunits, two different epitopes, modified c-Myc and HA tags, were inserted into pCMV-Kv1.2/1.4 constructs in which EGFP genes were eliminated. Modified HA and c-Myc epitopes were inserted at the end of the S1–S2 loop of Kv, as described previously for Kv7.4 channels (Kim et al., 2011). The S1–S2 loop amino acid sequences were changed to ETLPFRDENSEDMHGEQKLISEEDLGVTFTHTYSNSTIGYQQSTSFTDP for c-Myc tagged Kv1.2 and ETLPEFRRDDRLIMALSAGYPYDVPDYAGHSRLNDTSAPHLSENSGHTIFNPD for HA-tagged Kv1.4 constructs; whole S1-S2 loop sequences are shown with epitopes in bold. Epitope tagged constructs were generated by recombination polymerase chain reaction and verified by automated sequencing.

**Immunocytochemistry**

For histological cryosection experiments, sedated (Avertin (2,2,2,tribromoethanol); 300 μg/g body weight, i.p.) mice were transcardially perfused with 10 ml PBS, followed by 10 ml of 4% paraformaldehyde in 0.1 M PBS. The temporal bones were removed and the cochleae were perfused via the oval and round windows. The temporal bones were then immersed in fixative for 60 minutes. After fixation, the cochleae were decalcified (120 mM EDTA, pH 7.0; 24 hours; ~21°C). The cochleae were processed sequentially with 10% and 30% sucrose at 4°C overnight, and then embedded in OTC compound for cryosection. Sections were washed in PBS,
permeabilized in 0.1% Triton X-100 for 25 minutes, and then incubated for 30 minutes in a
blocking solution containing 1% bovine serum albumin and 1% goat serum. The 10-µm sections
were incubated with K⁺ channel antibodies against Kv1.2, residues 463-480, and Kv1.4, residues
14-35 (NeuroMab Facility, Davis, CA), at 1:100 to 1:500 dilutions. To identify neurons, samples
were counter-stained with an antibody against the neuronal marker TUJ1 as described (Lv et al.,
2010). Cells/tissues were then incubated with appropriate secondary antibodies for 2 hours,
washed and mounted using antifade mounting medium, and viewed with a Zeiss LSM 510
confocal microscope.
Results

The voltage-dependent K⁺ channel Kv1.2 is recognized as a component of the dendrotoxin (DTX)-sensitive K⁺ currents in spiral ganglion neurons (SGNs), clamping the cells close to the K⁺ reversal potential (E_K) and controlling adaptation of spike activity (Adamson et al., 2002; Mo et al., 2002). To examine the specific roles of Kv1.2 in SGN membrane properties, we determined the resting input resistance of Kv1.2⁻/⁻ compared to their age-matched control wild types (WT; Kv1.2⁺/⁺). Despite removal of a prominent K⁺ current in SGNs, the input resistance of Kv1.2⁻/⁻ neurons was reduced significantly from 89.9 ± 3.2 MΩ to 58.2 ± 3.0 MΩ (p < 0.05, n = 15) (Fig. 1A). Consequently, the current required to elicit spike activity was raised (Fig. 1B). Isolated SGNs were divided not by their location along the cochlear contour (Lv et al., 2010; Lv et al., 2012), but by their extent of excitability. SGNs were divided into four classes according to the number of spikes/stimulus: 1) One spike, 2) Two-to-Six spikes, 3) > Six spikes, and lastly 4) Spontaneously active neurons (Fig. 1C-E). Further examination of the resting membrane potential (rmp) showed that not only were the Kv1.2⁻/⁻ neurons more hyperpolarized than the Kv1.2⁺/⁺, the mutants were paradoxically more excitable (Fig. 1-2; Table 1). In spontaneously active neurons, the null mutants portrayed characteristic burst activity punctuated by shorter interspike intervals with robust afterhyperpolarization that were less prominent in Kv1.2⁺/⁺ neurons (Fig. 1G-H). The distribution of the membrane properties of neurons favored an increased number of excitable cells in the null mutants (Fig. 1H). Figure 2 and Table 1 show the summary data of the spike properties of Kv1.2⁺/⁺ and Kv1.2⁻/⁻ SGNs.

Next, we examined the features of individual spikes in the Kv1.2⁺/⁺ and Kv1.2⁻/⁻ SGNs. The threshold of activation of action potentials was derived from the voltage at which the second derivative turned positive, as shown in the inset with an arrow in Figure 3A. To avoid the masking effects of input stimulus artifacts, action potentials were elicited using very brief pulses
There were significant differences in these properties of action potentials between the Kv1.2+/+ and Kv1.2-/- SGNs: $\text{rmp}$, threshold of activation, duration, and the rise and fall phases of action potentials recorded from one spike and two-to-six spike SGNs (Fig. 3B-D; Table 1). For neurons that elicited more than six spikes/stimulus, action potential properties remained statistically unchanged between the Kv1.2+/+ and Kv1.2-/- SGNs (Fig. 3D-E; Table 1). The results raise the possibility that fast adapting SGNs may express larger Kv1.2 currents than their slowly adapting counterparts. Additionally, it can be inferred from alterations in action potential properties that after null deletion of Kv1.2, outward currents were enhanced. To identify subtle differences that may explain the functions of Kv1.2 channels, we recorded from SGNs isolated from Kv1.2 +/-, which carries only one copy of the gene (heterozygous), and compared their spike features with the Kv1.2+/+ neurons (Fig. 4A-G; Table 2). Deletion of one copy of Kv1.2 was sufficient to produce significant changes in the rmp and the negative slope of action potentials, but not the threshold of activation, duration, and positive slope of spike waveforms (Table 2).

By virtue of their sensitivity to DTX, one of the ascribed functions of Kv1.2 channels is to regulate the rate of accommodation and spike timing in SGNs, and thus regulating information coding to the cochlear nucleus (Pongs, 1999; Mo et al., 2002). In Figure 5, we illustrate examples of spike activity in slowly adapting SGNs from Kv1.2+/+, Kv1.2+/− and Kv1.2−/− mice. Upon deletion of one copy of Kv1.2, the number of spikes increased, which was enhanced further in the null mutant SGNs (Fig. 5B). Moreover, spike activity and timing, represented as inter-spike intervals were altered (Fig. 5C). Whereas the WT neurons produced trains of spikes that are irregular, showing a wide range of inter-stimulus intervals (~1-80 ms), removal of Kv1.2 channel currents resulted in firing patterns with regular inter-spike intervals (~15 ms). The inter-
spike interval of the heterozygous mutants was fairly regular (~15-30 ms).

At least three Kv1 channels, namely Kv1.1, 1.2 and 1.6 subtypes and currents have been identified biochemically and pharmacologically (sensitivity towards $\alpha$-DTX and DTX-K) in SGNs (Mo et al., 2002; Rusznak and Szucs, 2009). Thus, it is expected that in the Kv1.2 null mutant neurons, Kv1.1 and 1.6 are fully operational. To determine the roles of Kv1 channel subtypes in SGNs, we examined the effects of $\alpha$-DTX and DTX-K on $\text{Kv1.2}^{+/+}$ and $\text{Kv1.1}^{-/-}$ neurons (Fig. 6-7). The application of $\alpha$-DTX resulted in an increase in spike frequency in $\text{Kv1.2}^{+/+}$ neurons. For fast adapting SGNs that elicit a single action potential upon stimulation, increasing the magnitude of injected current was not sufficient to evoke subsequent spikes. However, application of 100 nM $\alpha$-DTX was sufficient to render fast to slowly adapting neurons (Fig. 6A-B). Indeed, a similar concentration of the toxin transformed quiescent SGNs to spontaneously active cells, and for neurons that were spontaneously active to begin with, $\alpha$-DTX further enhanced the spike frequency (Fig. 6C). Moreover, analyses of spike timing revealed a transformation of neurons with irregular inter-spike intervals to those with virtually uniform spiking pattern (Fig. 6D-E), consistent with data described in Figure 5 for the $\text{Kv1.2}^{+/+}$ SGNs.

Because DTX-K blocks both Kv1.1 and 1.2, we surmised that by applying the toxin on SGNs isolated from the $\text{Kv1.1}^{-/-}$ mice, the distinct function of Kv1.2 could be unmasked. DTX-K did not produce substantial or significant changes in evoked action potential (Fig. 7A), and neither did it alter the spike frequency, nor timing in spontaneously active SGNs in $\text{Kv1.1}^{-/-}$ mice (Fig. 7C-D). It can be inferred from these results that upon null deletion of Kv1.1, Kv1.2 association with other subtypes of Kv1 channels renders the heteromeric channels insensitive to DTX-K. Although not mutually exclusive, the other alternative is that the purported association of Kv1.2 and 1.6 (Rusznak and Szucs, 2009) may not be prominent in SGNs.
The functional paradox of apparent increase in the magnitude of outward currents after deletion of Kv1.2 in SGNs remains unresolved. To address the mechanism, we performed a voltage-clamp experiment on isolated SGNs to examine outward K⁺ currents in Kv1.2⁻/⁻ and their age-matched Kv1.2⁺/⁺ controls. Not surprisingly, the whole-cell outward current density was enhanced in Kv1.2⁻/⁻ compared to the Kv1.2⁺/⁺ controls. Figure 8 describes the whole-cell outward current profile in Kv1.2⁻/⁻ SGNs and Kv1.2⁺/⁺ SGNs, which suggests that there is an increase in the magnitude of a transient K⁺ current following null deletion of Kv1.2. For example, at 0 mV step voltage, the magnitude of the whole-cell K⁺ current density was ~2-fold greater in the Kv1.2⁻/⁻ than in the Kv1.2⁺/⁺ controls (0 mV; (in pA/pF) Kv1.2⁺/⁺ = 77.1 ± 6.5; and Kv1.2⁻/⁻ = 41.0 ± 6.2; p < 0.05; n = 18: Fig. 8A-B). The difference between the WT and null mutant currents revealed a transient current with a faster activation profile (Fig. 8C-D). Analyses of the voltage-dependence of the tail current indicated that the apparent increase in the whole-cell current amplitude can be seen as a leftward shift in the sensitivity of the whole-cell K⁺ current by ~20 mV in Kv1.2⁻/⁻ neurons (Fig. 8E). The kinetics of the voltage-dependent activation were also altered, as the mutant neurons expressed K⁺ currents that had ~2-fold faster time constant (τ) of activation than the Kv1.2⁺/⁺ neurons (Fig. 8F). To identify the nature of the current that was up-regulated in the null mutant SGNs, we tested the sensitivity of the whole-cell current in the presence of low concentrations of 4-AP (100 μM). The difference current was virtually abolished in the presence of low concentrations of 4-AP (data not shown). Next, we streamlined on the potential current that was up-regulated by testing the sensitivity of the current to CP 339818 hydrochloride, a non-peptide specific blocker of Kv1.3 and 1.4 channels. As shown in Figure 8G, the drug inhibited a sizable portion of the transient current, which is further depicted in the form of normalized current in the inset. The fractional block versus the
concentration of CP 339818 yielded an IC₅₀ of ~440 nM (Fig. 8H). Since the CP 339818-sensitive current was a transient current, it could be inferred that it was derived from Kv1.4 channel activity.

To further ascertain the identity of the difference current, we invoked the reductionist assertion that Kv1 channel subtypes form heteromeric complexes with members of the same family of channels. Since the resultant outward current after null deletion of Kv1.2 showed prominent inactivation and the difference current had a rapid onset and decay, we further surmised that the current may be derived from the activity of the Kv1.4 channel subtype. To test whether the findings from our analyses could be explained, we expressed mouse Kv1.2 and 1.4 singly or in combination at different ratios in CHO cell lines. In Figure 9, we show exemplary current profiles recorded from CHO cells 24 hours after transfection of Kv1.2, Kv1.4 alone and in combination (Fig. 9A). We examined the voltage dependence of the steady-state inactivation as depicted with representative current traces when Kv1.4 was expressed alone and in combination with Kv1.2 at a 1:1 ratio (Fig. 9B). The voltage-dependence of the resulting currents revealed several marked differences: 1) When expressed alone, Kv1.4 currents activate at a more negative potential than in combination with Kv1.2 such that the activation voltage for Kv1.4 alone was ~-75 mV and V₁/₂(act) was -7.1 ± 0.6 mV (n = 14). Meanwhile, the combined (Kv1.2/Kv1.4) current activation voltage was ~40 mV and the estimated V₁/₂(act) was 13.3 ± 1.8 mV (n = 15); 2) The steady-state inactivation curves for Kv1.4 current alone had V₁/₂(inact) = -33.7 ± 1.2 mV (n = 17) when compared to the combined (Kv1.2/Kv1.4) current, which had V₁/₂(inact) = -29.1 ± 2.2 (n = 17); 3) Consequently, a marked window current ensued between ~-75 to -40 mV for the homomeric Kv1.4 current in contrast to the heteromeric Kv1.2/1.4 current (Fig. 9C). The kinetics of activation and inactivation also revealed fast onset and decay for the
Kv1.4 currents as compared to the Kv1.2/Kv1.4 combined currents. 4) Lastly, the magnitude of the homomeric Kv1.4 current was comparable to the heteromeric Kv1.2/1.4 current. At a step potential of 40 mV the current density (in pA/pF) for Kv1.4, and Kv1.2/1.4 currents were; 75.9 ± 10.3 and 77.1 ± 15.8 (n = 13, p = 0.82). Recent studies have demonstrated that functional heteromeric channels can express distinct pharmacology compared to the homomeric channels (Chen et al., 2010). We examined the sensitivity of homomeric Kv1.2 and heteromeric Kv1.2/1.4 to α-DTX. Whereas homomeric Kv1.2 channel currents showed marked sensitivity towards α-DTX with an IC$_{50}$ of ~ 50 nM (49.3 ± 1.8 nM; n = 8), currents derived from heteromeric Kv1.2/1.4 were impervious to α-DTX (Fig. 9D).

Using epitope-tagged Kv1.2 and Kv1.4 in non-permeabilized conditions, we demonstrate in Figure 10 that the two channel subtypes co-localized in the plasma membrane. Finally, we analyzed the expression profiles of Kv1.2 and 1.4 in SGNs. Since variations in the densities of different subtypes of K$^+$ channels along the cochlear contour have been demonstrated, we examined the expression levels of the two Kv1 channels at the apical, middle and basal turns (Fig. 11). Whereas positive immunoreactivity of Kv1.2 was uniform across the three turns of the cochlea, the intensity of Kv1.4 labeling was consistently (in 7 different preparations) higher at the apical and middle turns than at the base (Fig. 11).
Discussion

The mechanisms that regulate K\(^+\) channel subunit-specific heteromultimerization in native neurons remain unresolved. Because of the promiscuous association of Kv channel subtype families in neurons, it has been exceedingly difficult to predict and resolve the exact subunit assemblies that confer the native currents. Yet, Kv channel subtypes from the same family can have distinct current phenotypes and a cocktail of the channels assemble to yield more diverse current properties and functions (Vacher et al., 2008; Jenkins et al., 2011). For example, Kv1.1, 1.2 and 1.6 produce a depolarization-activated K\(^+\) current with a slow onset and sustained time course (Smart et al., 1997; Mo et al., 2002). Moreover, Kv1.4 current is characterized by a rapid onset and reliably fast inactivation (Patel and Campbell, 2005). As demonstrated in this study, co-expression of Kv1.2 and 1.4 and the resulting stochastic interaction between the two channels resulted in whole-cell current that was not only kinetically different from currents derived from the individual channels, but also pharmacologically distinct. Whereas successful identification of Kv subtype-specific blockers has been a powerful pharmacological strategy to disentangle channel-specific functions and properties, the approach loses its robustness in native cells, where Kv channel assembly is predictively and demonstrably heteromultimeric (Scott et al., 1994; Rhodes et al., 1997; Hopkins, 1998; Pongs, 1999; Harvey, 2001; Dodson et al., 2002).

Selective null deletion of Kv subtypes may be the prevailing scheme to determine the in \textit{vivo} function of a channel, but the use of this approach in understanding the underlying cellular mechanism of Kv channel has not been exploited extensively. Previously, multiple studies have used heterologous cell lines to demonstrate that surface expression of Kv1 channel subunits occurs among members of the same family (Isacoff et al., 1990; Hopkins et al., 1994). Besides,
some of the latent confines for channel trafficking resides in the ER (Li et al., 2000), which is dependent on the channels’ ER retention signals, assembly and folding, as well as their state of phosphorylation (Isacoff et al., 1990; Yang et al., 2007).

Here we investigated the role of Kv1.2 channels in regulating the functions of SGNs by identifying the physiologically relevant in vivo interaction partners that define the channel as one of the determinants in information coding of auditory neurons (Davis and Liu, 2011). The findings revealed a surprising puzzle that could not have been possible using only pharmacological schemes. First, genetic ablation of Kv1.2 in SGNs resulted in unanticipated increases in membrane hyperpolarization and apparent enhancement of transient outward current. Although it was tempting to invoke potential up-regulation of the transient outward current as the mechanism for the results, our analyses suggest that the voltage-dependent activation of the remaining transient outward current after null deletion of Kv1.2 shifted by ~20 mV in the negative direction, generating a window current at potentials around the rmp of SGNs (~50-60 mV). Consequently, the rmp moved towards Ek. The resulting 5 mV change in the rmp (Table 1) is significant, since Na⁺ currents in SGNs have been estimated to have a gating charge (z) of ~4.6 (Santos-Sacchi, 1993) and given one elementary charge to be ~24 mV, the Na⁺ channel open probability is expected to change e-fold for a ~5.2 change in voltage. The low-voltage activated transient Ca²⁺ current in SGNs (Lv et al., 2012) is expected to become readily available for activation as the rmp shifts to hyperpolarized potentials. Increased availability of inward Na⁺ and Ca²⁺ channels ready for activation at the set rmp in the Kv1.2⁻/⁻ SGNs may explain not only the increased excitability, but also the significant increase in the rate of rise of the action potentials. Conceivably, the apparent shift in the activation of the transient K⁺ current is likely to shorten the width of the action potentials and induce a rapid decline in the falling
phase of the spikes.

Second, a potential cause for the apparent increase in transient outward $K^+$ current in 
$Kv1.2^{−/−}$ SGNs derived from $Kv1.4$ channels may rely on the findings that suggest $Kv1$ $α$-subunits, e.g. $Kv1.1$ and $Kv1.2$, contain ER retention signals that may be transferable to heteromeric $Kv1$ complexes (Manganas and Trimmer, 2000). Thus, ablation of $Kv1.2$ is likely to increase surface expression of $Kv1.4$ channels. Additionally, in contrast to $Kv1.1$ which is invariably destined for ER retention, $Kv1.2$ is more plastic and its translocation to the plasma membrane is not constrained by a predetermined ER localization sequence. In agreement with this assertion, we found that: 1) Co-expression of $Kv1.2$ and 1.4 did not alter the surface expression of $Kv1.4$; 2) There is no evidence to indicate that $Kv1.2$ promotes ER retention of $Kv1.4$; finally, 3) There were no differences in the estimated current density when $Kv1.2$ and $Kv1.4$ were expressed singly or when combined. Data from estimates of the number of functional channels also rule out the possibility that surface expression of $Kv1.2$ impacted $Kv1.4$ expression (not shown).

Moreover, the third most significant finding was that the combined expression of $Kv1.2$ and $Kv1.4$ yielded whole-cell $K^+$ currents with steady-state activation properties that were shifted rightward by ~15 mV compared to current derived from $Kv1.4$ alone. In contrast, currents derived from homomeric $Kv1.2$ channels had half-activation parameters that were ~5 mV more positive than the heteromeric $Kv1.2/1.4$ channels. Additionally, the time constants of activation were starkly different between the homomeric and heteromeric channels. Heteromeric association of $Kv1.2$ and 1.4 resulted in currents with distinct insensitivity to $α$-DTX, a drug that is used to define $Kv1.1$, 1.2 and 1.6 currents in SGNs (Adamson et al., 2002; Rusznak and Szucs, 2009). These findings are in line with a recent report that demonstrated heteromeric $Kv1.x$
complexes of different configurations produce distinct sensitivity towards the Conus snail venom, κM-conotoxin (Chen et al., 2010). The implications of altered sensitivity of heteromeric channels to specific drugs as compared to their effects on homomeric channels underpin the need to exercise caution in assigning specific pharmacology to any Kv1 channel subunit in native cells. Conversely, it raises the possibility that pharmacological agents can be designed to target specific heteromeric channel blends. Of further relevance to neuronal function is the finding that a specific Kv1 subunit, e.g. Kv1.2, can control the localization of heteromeric channels of a specific combination at axonal compartments and synaptic sites that are not seen at the cell body (Jenkins et al., 2011). Another cautionary note from the conclusions from the findings of this report is that any number of other transient K⁺ currents could be operational in the Kv1.2⁻/⁻ mouse model. Only one specific blocker was used in this study and a more exhaustive testing could be done in future work. Lastly, P12 is the onset of hearing in the mouse, and at P11-12 BK channels are strikingly up-regulated in inner hair cells within approximately one day. The transition from spontaneous activity to graded receptor potential in inner hair cells may undoubtedly alter the response properties of SGNs. However, in this study we did not examine the properties of SGNs between pre- and post-hearing stages.

Heterogeneity of auditory and vestibular afferent neurons responses to rectangular pulse and synthetic excitatory postsynaptic current injection yields a broad range of response dynamics and spike timing in information coding (Iwasaki et al., 2008; Kalluri et al., 2010; Eatock and Songer, 2011). In broad strokes, these afferent neurons can be classified according to their regularity of firing, namely regular and irregular neurons (Fernandez et al., 1990; Goldberg et al., 1990b, a). Whereas the firing patterns of vestibular afferents have been well studied and demarcated into morphologically and functionally distinct regular and irregular neurons
pre-hearing auditory afferents have been assumed to consist of mainly regular neurons. However, the striking resemblance between fast and slow adapting auditory, irregular and regular vestibular afferents, respectively, is apparent (Adamson et al., 2002; Mo et al., 2002; Risner and Holt, 2006; Kalluri et al., 2010). The presence of intrinsically generated spontaneously active SGNs may add further complexity in the dynamic range of auditory afferents (Lin and Chen, 2000; Lv et al., 2012). Nonetheless, among the ionic conductances underlying the firing pattern of afferent neurons in the inner ear are the low voltage-activated K\(^+\) currents, of which Kv1 (DTX-sensitive) and Kv7 channels are prominent components (Adamson et al., 2002; Iwasaki et al., 2008). Indeed, Kalluri et al. (2010) have demonstrated that fast adapting vestibular afferent neurons could be transformed into slowly adapting ones, by blocking DTX-sensitive and Kv7 conductances. Thus, the intricacies of dynamic range of auditory neurons is further abound, given that Kv1.2 and Kv1.4 channels can undergo heteromultimers with distinct pharmacological and electrophysiological properties.

Plasticity in hippocampal neurons can be expressed in the form of changes in dendritic excitability by activity-dependent trafficking of the K\(^+\) channel subunit Kv4.2 (Kim et al., 2007). Because changes in Kv subunit composition alter functional channel properties, the effect of selective association of Kv1.2 and 1.4 in SGNs can have a robust impact on their functions. Indeed, Kv1.2 subunits can act in a dominant fashion to control membrane localization of synaptic proteins (Poliak et al., 1999; Inda et al., 2006). Thus, depending on the subcellular localization of Kv1.2 and other Kv channel subunits in SGNs, it is conceivable that Kv1.2 can be used as a bait to determine other components of SGN functions. Nonetheless, the present findings have revealed several previously unknowns. In SGNs, Kv1.2 associates with Kv1.4 to
confer the functional properties as a conduit for coding auditory information. The high voltage-dependent activation of Kv1.2 appears to dominate, such that heteromeric Kv1.2/Kv1.4 channels in SGNs set the rmp to a relatively depolarized voltage compared to Kv1.4-dominant neurons. Lastly, the sensitivity of Kv1.2 to α-DTX may not be a reliable indicator for their roles in SGNs in which heteromeric association of other DTX-insensitive subunits is most certain.


Acknowledgements

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Null deletion of Kv1.2 results in altered membrane properties of spiral ganglion neurons (SGNs)

A. Postnatal twelve-day old (P12) spiral ganglion neurons (SGNs) from Kv1.2<sup>+/+</sup> and Kv1.2<sup>−/−</sup> mice were isolated and cultured for 48 hours. The steady state input resistance was measured from membrane potentials as a function of injected currents. The inset is of a family of traces obtained from Kv1.2<sup>+/+</sup> (left) and Kv1.2<sup>−/−</sup> (right) neurons. Null deletion of Kv1.2 decreased the steady state input resistance from 88.9 ± 3.2 MΩ in the wild type neurons to 58.2 ± 3.0 MΩ (n = 15; p < 0.05). Data consist of neurons with 1 spike and 2-6 spikes/stimuli (see Fig. 1C).

B. Summary data of current thresholds required to generate action potentials in Kv1.2<sup>+/+</sup> (○) and Kv1.2<sup>−/−</sup> (●) neurons.

C. Representative action potentials were recorded by injecting a 0.2-nA current for a 200-ms duration. Quiescent neurons at rest were classified into three groups: neurons which generate one (upper panel), two-to-six (middle panel) and more than six (lower panel) spikes/stimulus. For Kv1.2<sup>+/+</sup>, the number of neurons used for data analyses are as follows: one spike = 29, two-to-six spikes = 18, and >six spikes = 12. For Kv1.2<sup>−/−</sup>, one spike = 22, two-to-six spikes = 18, and >six spikes = 17.

D. The histogram depicts proportions of SGNs in Kv1.2<sup>+/+</sup> (solid lined columns) and Kv1.2<sup>−/−</sup> (dashed lined columns) neurons representing the different firing patterns.

E. For SGNs, which display spontaneous activity, differences in the firing patterns were not discernible between Kv1.2<sup>+/+</sup> (E) and Kv1.2<sup>−/−</sup> (G) neurons. We found ~10% of Kv1.2<sup>+/+</sup> and 18% of Kv1.2<sup>−/−</sup> SGNs (total of 62 cells) to be spontaneously active.

F. However, analyses of the inter-spike intervals showed that spike activity in Kv1.2<sup>+/+</sup> was less than that of Kv1.2<sup>−/−</sup> (H) neurons. The insets in (F and H) portray the differences in the spike burst patterns between Kv1.2<sup>+/+</sup> and Kv1.2<sup>−/−</sup> neurons.
Properties of action potentials in SGNs from Kv1.2\(^{+/+}\) and Kv1.2\(^{-/-}\) mice.

Action potentials were recorded by injecting a 0.2-nA current for a 200-ms duration. We used SGNs from 12-day-old Kv1.2\(^{+/+}\) and Kv1.2\(^{-/-}\) mice. A. Summary data describe alterations in the resting membrane potential (rmp) in Kv1.2\(^{+/+}\) and Kv1.2\(^{-/-}\) SGNs. B. Changes in the afterhyperpolarization potential (AHP), and duration of the action potentials. C. For Kv1.2\(^{+/+}\), the number of neurons used for analyses were: one spike = 29, two-to-six spikes = 18, and >six spikes = 12. For Kv1.2\(^{-/-}\) the number of neurons were: one spike = 22, two-to-six spikes = 18, and >six spikes = 17, * = \(p < 0.05\); ** = \(p < 0.01\). Other parameters of action potential properties that remained statistically unchanged are presented in Tables 1 and 2.

Alterations in spike properties in SGNs between Kv1.2\(^{+/+}\) and Kv1.2\(^{-/-}\).

A. Representative action potentials were elicited by a brief (< 0.05 ms) current injection into 12-day-old SGNs from Kv1.2\(^{+/+}\) (in solid line) and Kv1.2\(^{-/-}\) (in dashed lines). The voltage at which the first and second differential turned positive was considered the threshold voltage (inset, arrow). B. Injection of a 0.25-nA current for a 0.05-ms duration produced a single spike in SGNs in Kv1.2\(^{+/+}\) (left panel), and Kv1.2\(^{-/-}\) (middle panel) neurons show similar changes in spike properties. In the right panel, we have superimposed the wild type and mutant action potentials. C. and D. Summary data show alteration in rise slope 10-90% and decay slope 10-90% in SGNs between Kv1.2\(^{+/+}\) and Kv1.2\(^{-/-}\) mice. For Kv1.2\(^{+/+}\), the number of neurons used for analyses were: one spike = 29, two-to-six spikes = 18, and >six spikes = 12. For Kv1.2\(^{-/-}\), the numbers were: one spike = 22, two-to-six spikes = 18, and >six spikes = 17, * = \(p < 0.05\); ** = \(p < 0.01\).
Figure 4

Characteristics of action potentials in 12-day-old SGNs

A. Representative action potentials were recorded by injecting a 0.2-nA current for a 200-ms duration in SGNs isolated from Kv1.2+/+ (black) and Kv1.2+/- (blue) mice. The upper, middle and lower panels illustrate examples of neurons with one, two-to-six, and >six spikes/stimuli, respectively. B. The histogram depicts the percentages of SGNs with different firing patterns. C. Single action potentials from Kv1.2+/+ (black) and Kv1.2+/- (blue) are superimposed for comparison. Data are from one spike/stimulus neurons. D. Changes in resting membrane potential (rmp) for Kv1.2+/+ (solid histogram) and Kv1.2+/- (dotted histogram). E. Summary data of the action potential decay slope 10-90%. Data were amassed from Kv1.2+/+: one-spike = 29, two-to-six spikes = 18, and >six spikes = 12, and from Kv1.2+/-: one-spike = 13, two-to-six spikes = 11, and >six spikes = 10, * = p < 0.05; ** = p < 0.01.

Figure 5

Alterations in firing pattern in SGNs after null and partial deletion of Kv1.2

A. Action potentials were recorded from SGNs isolated from Kv1.2+/+, Kv1.2+/- and Kv1.2-/- mice by injecting a 0.2-nA current for a 5-s duration. The dashed lines indicate 0 mV. The upper, middle and lower panels correspond to trains of spikes from Kv1.2+/+ (in black), Kv1.2+/- (in green) and Kv1.2-/- (in blue), respectively. B. A magnified segment of the action potential profiles in the Kv1.2+/+ (in black), Kv1.2+/- (in green) and Kv1.2-/- (in blue). Note the prominent afterhyperpolarization after null deletion of Kv1.2. C. A plot of inter-spike intervals revealed unambiguous transformation of irregular to regular inter-spike interval between Kv1.2+/+, Kv1.2+/- and Kv1.2-/- SGNs.
**Figure 6**

α-DTX increases the excitability of 12-day-old SGNs in Kv1.2\(^{+ +}\) mice.

A. Action potentials were evoked by a brief pulse (0.05 ms) to elicit spikes in fast (upper panel) and slow adapting (two lower panels) SGNs. Bath application of external solution containing 100 nM α-DTX resulted in increased spike activity. Shown in (B) are two superimposed traces before (in black) and after (in blue) application of α-DTX. C. Effects of α-DTX on two different types of SGNs: 1) Quiescent neurons at baseline (upper panel); and 2) Spontaneously active at baseline (lower panel). The arrows indicate the time at which the external solution containing 100-nM α-DTX was perfused. α-DTX transformed quiescent neurons into spontaneously active neurons, and accentuated the spike frequency of neurons which were spontaneously active at baseline. D. Comparison of the spike activity in spontaneously active neuron and after application of α-DTX. E. Analyses of the inter-spike intervals with respect to the number of events. α-DTX modified the irregular activity to a quasi-regular activity.

**Figure 7**

DTX-K does not produce alterations in membrane excitability in SGNs from Kv1.1\(^{-/-}\) mice

A. DTX-K is known to block Kv1.1 and 1.2 currents. By using DTX-K on Kv1.1\(^{-/-}\) SGNs, we surmised that the bona fide effects of Kv1.2 on membrane properties of SGNs could be unraveled pharmacologically. Action potentials were evoked by injecting a 0.2-nA depolarizing current for 200 ms in Kv1.1\(^{-/-}\) SGNs. Bath perfusion of 100 nM DTX-K produced no visible effects on the membrane properties. The left panel shows evoked spikes from Kv1.1\(^{-/-}\) SGNs, and
the middle panel (in blue) illustrates evoked spikes after bath perfusion of external solution containing 100 nM DTX-K. The right panel superimposes the left and middle traces for comparison. B. Membrane properties of two Kv1.1−/− SGNs: 1) Fairly quiet neuron (upper panel); and 2) Spontaneously active neuron (lower panel). The dashed lines indicate 0 mV. The arrows show the time of application of DTX-K (100 nM). There were no noticeable effects on the membrane properties after application of DTX-K. Similar data were obtained from 19 different neurons. C. Segments of traces in a spontaneously active SGN from Kv1.1−/−, for control (upper panel, black) and after application of DTX-K (lower panel, blue). D. Shows analysis of inter-spike intervals in a spontaneously active Kv1.1−/− SGN (●), and after application of DTX-K (○). DTX-K had no measurable effects on Kv1.1−/− SGNs (n = 17).

Figure 8

Whole-cell K+ currents recorded from SGNs from Kv1.2+/+ and Kv1.2−/− mice

Before switching to voltage-clamp configuration, the membrane properties of the SGN were determined and classified as one-spike, two-to-six-spikes or more than six-spikes. Representative K+ current traces were recorded from SGNs from the one-spike group of Kv1.2+/+ mice (n = 18). A. Family of current traces obtained from a holding potential of -90 mV and stepped from -110 to + 40 mV using ΔV of 10 mV. B. SGNs from the one-spike group from Kv1.2−/− mice (n = 18) showed similar results. C. Illustrates the difference-K+-current traces between K+ currents from Kv1.2+/+ and Kv1.2−/− SGNs. D. Current density-voltage curves obtained from Kv1.2+/+ (n = 15) and Kv1.2−/− (n = 14) SGNs. The peak and steady-state current densities are shown as mean ± S.D. E. Steady-state activation curves were generated from tail currents following a 50-ms family of voltage steps. The activation curves were fitted to the Boltzmann function in the form:

\[ I/I_{\text{max}} = \frac{1}{1+\exp((V_{1/2} - V)/k)} \]

where \( V_{1/2} \) is the half activation voltage and \( k \) is a slope factor.
$V_{1/2}$ for the K$^+$ current recorded from Kv1.2$^{+/+}$ neurons (open circles, $\bigcirc$) was 13.6 ± 2.1 mV and $k = 23 \pm 1$ mV ($n = 11$). For Kv1.2$^{-/-}$ neurons (solid circles, $\bullet$), the $V_{1/2}$ was -1.1 ± 1.2 mV and $k = 19.8 \pm 3.2$ mV ($n = 9$). F. The time constant ($\tau$) of inactivation of currents in Kv1.2$^{+/+}$ (square symbols) and Kv1.2$^{-/-}$ (circle symbols). Two $\tau$s, fast and slow, were obtained from the inactivation time courses. ** $p < 0.01$ ($n = 12$). G. Representative traces showing the effect of CP 339818 hydrochloride (1 $\mu$M) of outward currents recorded from Kv1.2$^{-/-}$ SGNs. To determine the drug-sensitive component, we normalized the current against the control and examined the difference current plotted with a dotted line (inset). H. The dose-response relation yielded an IC$_{50}$ of 438 ± 50 nM. The numbers of cells tested are indicated in parenthesis.

**Figure 9**

**Properties of mouse (m) mKv1.2 and mKv1.4 channel currents**

Whole-cell K$^+$ currents were recorded upon expressing the mKv1.2 and mKv1.4 channels singly and after co-expression of mKv1.2 and mKv1.4 at a 1:1 ratio. A. Representative current traces for a family of K$^+$ currents obtained from a holding potential of -90 mV and stepped from -90 to 30 mV using $\Delta V = 10$ mV (upper panel). c-Myc-tagged mKv1.2 and HA-tagged mKv1.4 show similar current profiles (middle panel), suggesting that membrane expression and functions of the K$^+$ channels remained intact despite introduction of HA- and c-Myc-tags. CHO cells transfected with mKv1.2:Kv1.4 at a ratio of 1:1. Cells were held at a holding potential of -90 mV and stepped from -90 to 30 mV using $\Delta V = 10$ mV (lower left panel, in blue). The same cell was held at a holding potential of -30 mV and stepped to similar varying voltages (lower right panel, in blue). At a holding potential of -30 mV, the transient current was inactivated. B. Activation
curves of normalized peak tail current of mKv1.2/Kv1.4 (in blue, open circles; n = 15) and mKv1.4 (in black, solid circles; n = 14) are shown for comparison. Using the Boltzmann function (see legend Fig. 8), the $V_{1/2}$ of activation ($V_{1/2\text{(act)}}$) for mKv1.4 alone was $-7.1 \pm 0.6$ mV and $k = 22.5 \pm 3.8$ mV (n = 14). Moreover, for mKv1.2/1.4 (1:1), the $V_{1/2\text{(act)}}$ was $13.3 \pm 1.8$ mV and $k = 19.6 \pm 4.3$ mV (n = 15). A standard steady-state inactivation protocol was used by holding the cells at -90 mV and stepped to varying pre-pulses from -90 to 30 mV ($\Delta V = 10$ mV).

After a brief (2-ms) gap at -90 mV to allow for deactivation, the steady-state inactivation was tested at 10 mV. The insets are the representative traces. (Kv1.4 in black; Kv1.2:Kv1.4 1:1 in blue). The graph shows voltage-dependence of inactivation of mKv1.4 (in black, solid squares) and mKv1.2/Kv1.4 currents (in blue, open squares). C. Using the activation ($m$) and inactivation ($h$) variables determined from the fits derived from the Boltzmann functions, we employed the HH formalism to determine the “window” current. The black solid line = $m^2h$ represents currents derived from mKv1.4 alone, and it shows a window current that is active at the rmp of SGNs. The dashed black line = $m^3h$ and the dotted black line = $m^4h$. The fits for mKv1.2/1.4 (1:1) showed a current that only turns on at voltages positive to -40 mV using $m^2h$ (plotted in blue solid line). The dashed blue line = $m^3h$ and the dotted blue line = $m^4h$ turn on at voltages positive to -10 mV. Thus, for extrapolation purposes using $m^2h$ as the product of the state variables, the Kv1.4 homomeric channels are more likely to be active at the rmp of SGNs than the Kv1.2/1.4 heteromeric channels. D. Dose-response curve generated using different concentrations of $\alpha$-DTX on CHO cells expressing homomeric Kv1.2 channels (●). The half (50%) inhibitory concentration IC$_{50}$ was $49.3 \pm 1.8$ nM (n = 8). The heteromeric Kv1.2/1.4 channel currents were insensitive to $\alpha$-DTX (○). Shown in the insets are representative traces (control in black solid
lines) and the effects of α-DTX (in red lines) in homomeric Kv1.2 currents (upper panel) and heteromeric Kv1.2/1.4 currents (lower panel).

**Figure 10**

**Detection of epitope-tagged wild type mKv1.2 and mKv1.4 channels in CHO cells**

c-Myc and HA-epitopes were inserted into wild type mKv1.2 and mKv1.4 channels extracellular domains, respectively. Farnesylated green fluorescent protein (GFP) was used as a reporter that binds to the plasma membrane. Expression of mKv1.2 and mKv1.4 was detected using anti-c-Myc or anti-HA antibody, and anti-mKv1.2 or anti-mKv1.4 antibody in non-permeabilized (NP) conditions. Anti-c-Myc and anti-HA antibody stained only the channels expressed in the cell surface in NP cells. Detection of cell surface expression of c-Myc-tagged mKv1.2 and HA-tagged mKv1.4 channels in co-expression. The overlap of cyan and red signals in the histogram in NP cells indicates their co-localization on the cell surface. The scale bar is 10 μm.

**Figure 11**

**Expression of Kv1.2 and Kv1.4 in mouse cochlear sections**

A and B. Immunolabeling sections of the cochlea from 12-day-old mice. SGNs (apical, middle and basal) were labeled with the neuronal marker TUJ1 (red). C-D. Photomicrographs of a magnified view of the apical, middle and basal region (large square) in the samples. SGNs were labeled with antibodies against mKv1.2 and mKv1.4 (green). The nuclei (blue) were stained with DAPI. Images were merged (right). E-F. Magnified view of SGNs (small square). Expression pattern of Kv1.2
channel (E) and Kv1.4 channel (F) in apical, middle and basal neurons. We plotted fluorescent intensity with respect to distance across the neurons. Scale bars for (A) and (B) = 100 μm, and for (C) and (D) = 50 μm.
## Table 1

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Properties of action potentials in spiral ganglion neurons
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Properties of action potentials in spiral ganglion neurons in wild type and mutant mice