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A critical period of prehearing spontaneous Ca\(^{2+}\) spiking is required for hair-bundle maintenance in inner hair cells

Adam J Carlton\(^1\), Jing-Yi Jeng\(^1\), Fiorella C Grandi\(^2\), Francesca De Faveri\(^1\), Federico Ceriani\(^1\), Lara De Tomasi\(^1\), Anna Underhill\(^1\), Stuart L Johnson\(^1,3\), Kevin P Legan\(^4\), Corné J Kros\(^4\), Guy P Richardson\(^4\), Mirna Mustapha\(^1,3\)* & Walter Marcotti\(^1,3,4\)

**Abstract**

Sensory-independent Ca\(^{2+}\) spiking regulates the development of mammalian sensory systems. In the immature cochlea, inner hair cells (IHCs) fire spontaneous Ca\(^{2+}\) action potentials (APs) that are generated either intrinsically or by intercellular Ca\(^{2+}\) waves in the nonsensory cells. The extent to which either or both of these Ca\(^{2+}\) signalling mechanisms are required for IHC maturation is unknown. We find that intrinsic Ca\(^{2+}\) APs in IHCs, but not those elicited by Ca\(^{2+}\) waves, regulate the maturation and maintenance of the stereociliary hair bundles. Using a mouse model in which the potassium channel Kir2.1 is reversibly overexpressed in IHCs (Kir2.1-OE), we find that IHC membrane hyperpolarization prevents IHCs from generating intrinsic Ca\(^{2+}\) APs but not APs induced by Ca\(^{2+}\) waves. Absence of intrinsic Ca\(^{2+}\) APs leads to the loss of mechanoelectrical transduction in IHCs prior to hearing onset due to progressive loss or fusion of stereocilia. RNA-sequencing data show that pathways involved in morphogenesis, actin filament-based processes, and Rho-GTPase signaling are upregulated in Kir2.1-OE mice. By manipulating in \textit{vivo} expression of Kir2.1 channels, we identify a “critical time period” during which intrinsic Ca\(^{2+}\) APs in IHCs regulate hair-bundle function.

**Keywords** calcium waves; development; hair cell; mechanoelectrical transduction; spontaneous action potentials

**Subject Category** Neuroscience

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**Introduction**

Inner hair cells (IHCs) are the primary sensory receptors of the adult mammalian cochlea and relay acoustic information onto type I spiral ganglion afferent neurons via the graded release of glutamate from their specialized ribbon synapses (Fuchs, 2005; Moser et al., 2020). Before hearing onset, however, which in most altricial rodents occurs at around postnatal day 12 (P12) (Mikaelian & Ruben, 1964; Ehret, 1983; Romand, 1983), IHCs exhibit patterned action potential activity that is elicited spontaneously in the absence of sound-induced stimulation by the activation of Ca\(_{v}1.3\) Ca\(^{2+}\) channels (Marcotti et al., 2003a; Tritsch et al., 2010; Johnson et al., 2011). This activity has been shown to drive the bursting-like firing pattern along the neural pathway of the immature auditory system (Lippe, 1994; Jones et al., 2007; Sonntag et al., 2009; Tritsch et al., 2010). As with other sensory systems (Katz & Shatz, 1996; Stellwagen & Shatz, 2002; Moody & Bosma, 2005; Blankenship & Feller, 2010), patterned peripheral firing activity was identified as being critical for the refinement of neural circuits in the brain (Clause et al., 2014, 2017; Müller et al., 2019; Maul et al., 2022). Additionally, Ca\(^{2+}\)-dependent APs in IHCs have been shown to instruct the normal functional differentiation of the IHCs themselves (Johnson et al., 2007, 2013), most likely via regulating gene expression (Dolmetsch et al., 1997). However, due to the complex extracellular modulation of the intrinsic Ca\(^{2+}\) action potentials in developing IHCs, the exact role of this activity is largely unknown.

Spontaneous intrinsic Ca\(^{2+}\) action potentials first appear in the IHCs of the mouse cochlea at late embryonic stages (Marcotti et al., 2003a), and their frequency and pattern are controlled by the transiently expressed small-conductance Ca\(^{2+}\)-activated K\(^{+}\) current \(I_{\text{SCa}}\) (Marcotti et al., 2004) and the inward rectifier K\(^{+}\) current \(I_{K1}\) (Marcotti et al., 1999). The frequency and pattern of the electrical activity in IHCs are also extrinsically evoked and modulated by the spontaneous release of ATP from the neighboring nonsensory cells (Tritsch et al., 2010; Wang et al., 2015; Johnson et al., 2017). This complex regulation makes it difficult to identify and separate the specific functional roles of the intrinsic and externally driven Ca\(^{2+}\)-dependent AP activity in IHCs.

In this study, we used a mouse model in which the inward rectifier K\(^{+}\) channel Kir2.1 (Yu et al., 2004) was selectively overexpressed...
in vivo in the IHCs under the control of doxycycline (DOX), lowering their membrane potential and preventing them from firing the intrinsic spontaneous Ca\(^{2+}\) action potentials. These “silent” IHCs, however, retained their ability to respond with AP activity to extrinsic modulation by the ATP-induced signaling from the nonsensory cochlear cells. Our results show that prehearing IHCs require spontaneous intrinsic Ca\(^{2+}\) firing to maintain the normal morphological and biophysical characteristics of the mechanoelectrical transducer apparatus for a period of time in the second postnatal week, before the onset of hearing. We also found several key genes that are upregulated in the absence of the intrinsic Ca\(^{2+}\) action potential activity in IHCs, several of which are involved in pathways related to maintaining cytoskeletal homeostasis.

**Results**

**Overexpression of Kir2.1 (Kir2.1-OE) in cochlear IHCs in vivo prevents spontaneous firing activity**

The role of spontaneous Ca\(^{2+}\) action potential activity in IHCs, which are spikes generated intrinsically as opposed to those induced by Ca\(^{2+}\) waves originating in the nonsensory cells, was investigated by conditionally overexpressing the inwardly rectifying K\(^+\) channel Kir2.1 (Kcnj2) in the IHCs, thereby hyperpolarizing their resting membrane potential.

DOX-induced overexpression of Kir2.1 channels in the IHCs was evident from the presence of Kir2.1 immunofluorescence in the basolateral membrane of P6 (Fig EV1B) and P11 Kir2.1-OE mice (OtofrT\(^{TVA/-}\); Kir2.1\(^{}\/-\); P6, Fig EV1A; P9-P11, Fig 1A). OHCs and nonsensory cells surrounding the hair cells showed no or very little overexpression of Kir2.1 (Appendix Fig S1), indicating specificity of the Otof promoter for targeting the IHCs. Prehearing IHCs overexpressing Kir2.1 showed a significantly larger inward K\(^+\) current compared with control cells but normal outward K\(^+\) currents (Fig 1C-G, P9-P11). The larger inward K\(^+\) current in the IHCs from Kir2.1-OE led to a hyperpolarized shift of the resting membrane potential \((V_m)\) of the IHCs of about 10 mV compared with control cells (Fig 1H). The slope conductance around the respective resting \(V_m\) values was also significantly increased in IHCs from Kir2.1-OE mice compared with control littermates (Fig 1I).

The overexpression of Kir2.1 in neonatal P4 mice had a similar effect on the biophysical properties of the IHC basolateral membrane (Fig EV1D) as that described in P9-P11 IHCs (Fig 1D). We also found that the number of presynaptic ribbons, postsynaptic glutamate receptors and their co-localization in prehearing IHCs was not affected by the overexpression of Kir2.1 channels (Fig EV2A-D). In agreement with the normal morphological profile of the synapses, exocytosis in IHCs was not significantly different between the two genotypes \((P = 0.4709, 2\)-way ANOVA, Fig EV2E and F). These data indicate that the overexpression of Kir2.1 channels is not affecting the expression of the ion channels that are normally present in developing IHCs or their ribbon synapses.

We then investigated the ability of IHCs to fire intrinsic and induced Ca\(^{2+}\) action potentials at near body temperature (34–37°C) with an in vivo endolymph-like solution surrounding the IHC hair bundles. IHC Ca\(^{2+}\) action potentials are elicited by the opening of Ca\(^{2+}\) channels that activate at around –60 mV (Marcotti et al., 2003a). During the first postnatal week, the ionic composition of the endolymph is comparable to that of the perilymph, which contains 1.3 mM Ca\(^{2+}\) (Wangemann & Schacht, 1996). Under these recording conditions, spontaneous Ca\(^{2+}\) spiking activity was recorded from P4 control IHCs (Fig 2A). The mean spike frequency of IHCs was 2.19 ± 1.09 Hz \((n = 6)\), and the coefficient of variation (CV) was 1.30 ± 0.63 \((n = 7)\), duration of the recordings 45–101 s), which being greater than one, is indicative of a bursting pattern of activity as previously demonstrated (Johnson et al., 2011). In P4 Kir2.1-OE mice, due to the more hyperpolarized resting \(V_m\), IHCs do not fire action potentials spontaneously, although they retain the ability to do so during large depolarizing current injections (Fig 2B).

During the second postnatal week, spontaneous action potentials in IHCs disappear when using ex vivo cochlear preparations, which is due to a progressive hyperpolarization of the IHC resting \(V_m\) (Marcotti et al., 2003b) but could still be elicited by depolarizing current injections (Fig 2C, top panel). This membrane hyperpolarization is likely to be compensated in vivo by the resting open probability of the mechanoelectrical transducer (MET) channel (Johnson et al., 2012). This is because in vivo the endolympathic Ca\(^{2+}\) concentration during the second postnatal week has been estimated to be near 0.3 mM (Johnson et al., 2012), which will increase the open probability of the MET channels and thus cause the IHCs to depolarize to around the action potential threshold (Fig 2C, bottom panel; for spike frequency and CV see Fig 7E). We found that the IHCs from Kir2.1-OE mice failed to elicit spontaneous action potentials even in the estimated 0.3 mM endolympathic Ca\(^{2+}\) concentration, causing the IHCs to remain silent at rest (Fig 2D). IHCs are surrounded by nonsensory cells in the greater epithelial ridge (GER, also known as Kölliker’s organ: Fig 2E). The release of ATP from nonsensory cells of the GER leads to spatially and temporally coordinated Ca\(^{2+}\) waves that propagate across the epithelium and cause IHCs to depolarize as much as 28 mV (Tritsch et al., 2010). This depolarization has been shown to produce periodic bursts of Ca\(^{2+}\) action potentials in IHCs (Tritsch et al., 2007, 2010; Wang et al., 2015; Johnson et al., 2017). The frequency and duration of the Ca\(^{2+}\) waves in the nonsensory cells were not affected by the overexpression of the Kir2.1 channels (Fig EV3A and B). Therefore, we investigated whether the more hyperpolarized IHCs (by about 10 mV) from Kir2.1-OE mice retained the ability to respond to spontaneous Ca\(^{2+}\) waves originating in the GER. We found that in the presence of the estimated in vivo endolympath-like Ca\(^{2+}\) (0.3 mM), the Ca\(^{2+}\) signals caused by the opening of the Ca\(^{2+}\) channels in IHCs followed very closely the time course of the Ca\(^{2+}\) wave originating in the GER in both control (Fig 2F) and Kir2.1-OE (Fig 2G) P7-P9 mice. Moreover, the correlation between IHC Ca\(^{2+}\) activity and Ca\(^{2+}\) waves in the nonsensory cells was unaffected in Kir2.1 mice (Fig EV3C). This indicates that the large depolarization caused by the extracellular input of the nonsensory cells was necessary and sufficient to depolarize the IHCs in Kir2.1-OE mice and cause the opening of voltage-gated calcium channels.

**Progressive loss of mechanoelectrical transduction in IHCs lacking intrinsic Ca\(^{2+}\) action potentials**

MET currents were recorded from apical-coil IHCs by displacing their hair bundles using a 50 Hz sinusoidal force stimulus from a...
piezo-driven fluid jet (Corns et al., 2018; Carlton et al., 2021). A large MET current was elicited in all IHCs tested from control (Fig 3A) and Kir2.1-OE (Fig 3B) mice at P6-P7 when their stereociliary bundles were moved towards the taller stereocilia (i.e., in the excitatory direction) at negative membrane potentials. By stepping the bundles were moved towards the taller stereocilia (i.e., in the excitatory direction) at negative membrane potentials. By stepping the bundles were moved towards the taller stereocilia (i.e., in the excitatory direction) at negative membrane potentials. By stepping

Figure 1. Basolateral membrane properties of IHCs overexpressing Kir2.1 channels.

A, B Maximum intensity projections of confocal 2-stacks taken from the apical cochlear region of control (A) and littermate Kir2.1 overexpressing (B, Kir2.1-OE) mice at postnatal day 11. Inner hair cells (IHCs) were stained with antibodies against Kir2.1 (green) and the hair cell marker Myo7a (blue). At least 3 mice for each genotype were used. Scale bars: 10 μm.

C, D Currents from IHCs of control (C, P9) and Kir2.1-OE (D, P10) prehearing mice. Currents were elicited by using depolarizing and hyperpolarizing voltage steps, with a nominal increment of 10 mV, from a holding potential of −84 mV. Test potentials are shown next to some of the traces. Note that the large inward rectifier Kir2.1 current is only present in the IHC of the Kir2.1-OE mouse (D). The outward current is primarily carried by a delayed rectifier current. I, I, identifies the small inwardly rectifying K+ current normally expressed in IHCs.

E Steady-state current–voltage curves obtained from IHCs of control (P9-P11) and Kir2.1-OE (P9-P11) mice. F, G Size of the total steady-state inward (F, I, Control 2.88 ± 1.07 nA, n = 8; Kir2.1-OE 2.25 ± 0.97 nA, n = 6) and inward (G, Control, I, 0.30 ± 0.05 nA, n = 8; Kir2.1-OE, I, 3.13 ± 1.37 nA, n = 6) K+ currents measured at 0 mV and −124 mV, respectively. n.s. P = 0.2836.

H Resting membrane potential (V_m) measured in IHCs from Control (−62.6 ± 3.8 mV, n = 7) and Kir2.1-OE (−73.5 ± 3.5 mV, n = 5).

I Slope conductance of the current measured at around the respective resting V_m (Control 1.8 ± 0.4 nS, n = 8; Kir2.1-OE 25.5 ± 9.6 nA, n = 6).

Data information: In panels F–I, data are shown as means ± SD, and the single cell value recordings (open symbols) are plotted with the average data. All statistical tests were performed using the Student’s t-test. The number of IHCs investigated is shown above the average data points (6 control and 3 Kir2.1-OE mice).

Source data are available online for this figure.
Figure 2. Kir2.1 overexpression prevents spontaneous, but not induced, Ca\(^{2+}\) action potentials in IHCs.

A, B Whole-cell recordings of Ca\(^{2+}\) action potential activity in apical-coil IHCs from P4 control (A) and Kir2.1-OE (B) mice in the presence of 1.3 mM Ca\(^{2+}\) in the extracellular solution and at body temperature. Note that IHCs from control mice (A) fire spontaneous action potentials (40 s out of 142 s recording time), while those from overexpressing Kir2.1 IHCs (B) require a substantial current injection to elicit any spikes. For voltage-clamp data see also Fig EV1 C–I. Data in Panel A, B, and Fig EV1 C–I were obtained from 8 control IHCs (7 mice) and 11 Kir2.1-OE IHCs (6 mice).

C, D Calcium action potentials in IHCs from control (C) and Kir2.1-OE (D) mice during the second postnatal week. IHC voltage responses were recorded during the application of a solution containing 1.3 mM Ca\(^{2+}\) (top panels) or 0.3 mM Ca\(^{2+}\) (bottom panels). The latter Ca\(^{2+}\) concentration (0.3 mM), which was used to mimic the estimated in vivo Ca\(^{2+}\) concentration in the endolymphatic compartment (Johnson et al., 2012), caused control IHCs, but not those from Kir2.1-OE mice, to elicit spontaneous action potentials (40 s out of 56 s recording time).

E Diagram showing a cross-section of an immature organ of Corti. IHCs: inner hair cells; GER: greater epithelial ridge, which includes nonsensory cells surrounding the IHCs. Red arrows indicate the propagation of ATP-induced Ca\(^{2+}\) waves from the GER towards the IHCs, which leads to their depolarization (Tritsch et al., 2007; Wang et al., 2015; Johnson et al., 2017).

F, G Representative \(\Delta F/F_0\) traces from the IHCs and GER of P7-P9 control (F) and Kir2.1-OE (G) mice in the presence of 0.3 mM Ca\(^{2+}\). Spontaneous ATP-dependent Ca\(^{2+}\) waves from the GER (green traces) were eliciting coordinated Ca\(^{2+}\) signals in the IHCs from both controls and Kir2.1-OE mice. For each genotype, two separate sets of recordings from 2 mice are shown (top and bottom right), with the top traces being linked to the images on the left: before [1], during [2] and after [3]) the generation of a large Ca\(^{2+}\) wave from the GER. For details about the frequency and duration of the Ca\(^{2+}\) waves, and the number of mice and recordings see Fig EV3. All recordings were obtained at body temperature. Traces are computed as pixel averages of regions of interest centred on IHCs.

Source data are available online for this figure.
The MET current was significantly reduced (−124 mV: P < 0.0001; +96 mV: P = 0.0003, Fig 4D) and the resting open probability increased (−124 mV: P = 0.0080; +96 mV: P = 0.0130, Fig 4E) in IHCs from Kir2.1-OE mice compared with controls. Since an increased resting open probability of the MET channel could be associated with changes, specifically a reduction, in the free Ca²⁺ inside the stereocilia, we tested this possibility by changing the intracellular Ca²⁺ buffering capacity by using different concentrations of the fast Ca²⁺ chelator BAPTA. Increasing the intracellular BAPTA from 0.1 to 5 mM significantly augmented the resting open probability of the MET channel in IHCs from both genotypes, although at both BAPTA concentrations it was significantly higher in the IHCs of Kir2.1-OE mice (Fig EV4). This indicates that in the absence of spontaneous intrinsic firing activity in the IHCs of Kir2.1-OE mice, the MET channels are likely to have a reduced Ca²⁺ sensitivity during the second postnatal week. By P10-P11, we found that the MET current in the IHCs of Kir2.1-OE mice was very small or absent (Fig 4F–I). At this stage, IHCs from P10-P11 Kir2.1-OE mice also failed to load with the styryl dye FM1-43 (Fig 4J), which is a permeant blocker of the hair cell MET channel and functions as an optical readout for the presence of the resting MET current (Gale et al., 2001).

**IHCs from Kir2.1-OE mice undergo progressive loss and fusion of the stereocilia**

We investigated whether the rapid reduction in the MET current was caused by defects in the growth and/or maintenance of the stereociliary bundles in IHCs. Using scanning electron microscopy we found that the hair bundles of the IHCs from Kir2.1-OE mice were able to develop a staircase structure composed of rows of stereocilia that were indistinguishable from those present in control cells (arrows: Fig 5A and B). This is consistent with the presence of a normal MET current at least up to the end of the first postnatal
week (Fig 3). However, from about P9 onwards, IHCs from Kir2.1-OE mice started to lose the shorter third row of stereocilia (Fig 5B). A few IHCs also started to exhibit stereocilia fusion, which became more pronounced at older ages. By P26, none of the IHCs in the Kir2.1-OE mice showed normal-looking bundles, which instead exhibited profound stereocilia fusion (Fig 5C and D). Kir2.1−/− mice that were not crossed with OtofrtTA+/− mice showed normal hair-bundle development when treated with DOX, highlighting the specificity of the Kir2.1-OE strategy (Fig EV5). These data indicate that spontaneous Ca2+ actions potential activity during the second
postnatal week is required for the maintenance of the stereociliary bundles in the mature IHCs.

Localisation of bundle proteins is not affected in Kir2.1-OE mice

To establish whether the progressive loss and fusion of the stereocilia were linked to the mislocalization of some of the key proteins expressed in the hair bundles, we performed immunostaining experiments on both genotypes. Stereocilia fusion has previously been documented in hair cells from mice lacking Myo6, the gene encoding for the (F-actin) minus-end-directed unconventional myosin 6 (Self et al., 1999). We found that MYOSIN VI was expressed in the stereocilia of the IHCs from both control and littermate Kir2.1-OE mice (Fig 6A and C). The disorganized hair bundle of the IHCs from Kir2.1-OE mice also showed a normal distribution at the tip of the taller rows of stereocilia of EPS8, MYOSIN XV-isoform 1 and WHIRLIN (Fig 6B and D); key proteins required for growth and maintenance of stereocilia (Belyantseva et al., 2005; Delprat et al., 2005; Manor et al., 2011; Zampini et al., 2011).

IHC action potentials exert their developmental role in stereocilia maintenance during a critical period

Next, we tested whether Ca²⁺ action potential activity in IHCs was regulating hair-bundle maintenance during a specific time window or “critical period” of prehearing maintenance. This was achieved by downregulating Kir2.1-OE in vivo by removing DOX from the drinking water at a specific developmental time point. Considering that the hair bundles of the IHCs in Kir2.1-OE mice were able to acquire a staircase structure and have normal MET current at the end of the first postnatal week, we sought to test whether the role of the Ca²⁺-action potentials was restricted to the second week, just before hearing onset at ~P12.

As for the above investigation, DOX was continuously supplied to the females from the time of conception, but for this set of experiments, it was then removed from the drinking water when the pups were P5. We found that 2–3 days without DOX was sufficient to strongly downregulate Kir2.1 from the membrane of the IHCs of Kir2.1-OE mice (Fig 7A and B). This indicates that the overexpression of Kir2.1 in IHCs was primarily occurring during the first postnatal week under these conditions. We then investigated whether the downregulation of Kir2.1 channels following DOX removal (Appendix Fig S2) re-established the ability of IHCs to fire intrinsic spontaneous action potentials. In 1.3 mM extracellular Ca²⁺, action potentials only occurred during depolarizing current injections in the IHCs from both control and Kir2.1-OE mice in the second postnatal week (Fig 7C and D; see also Fig 2C and D). In the presence of the in vitro endolymph-like Ca²⁺ concentration (0.3 mM), spontaneous intrinsic firing was present not only in the IHCs of control mice (Fig 7E; see also Fig 2C) but also in Kir2.1-OE mice (Fig 7F). For long-lasting current clamp recordings, spikes occurred in a bursting pattern in both controls and Kir2.1-OE mice. The mean spike frequency (1.17 ± 0.47 Hz, n = 4) and CV (1.12 ± 0.17, n = 4) IHCs, duration of the recordings 62–125 s) in control mice were not significantly different from those measured in Kir2.1-OE mice (frequency: 1.29 ± 0.83 Hz, n = 5 IHCs, P = 0.7766; CV: 1.15 ± 0.11, n = 5, duration of the recordings 32–101 s, P = 0.7954). The IHC resting membrane potential was not significantly different between control and Kir2.1-OE mice in the presence of both 1.3 mM and 0.3 mM Ca²⁺ (Fig 7G). These data indicate that the removal of DOX was effective in downregulating Kir2.1 channels and restoring the normal physiology of the IHCs. We also found that when DOX was removed at P5, IHCs were able to maintain their hair-bundle structure after the onset of hearing (Fig 7H–K). These findings indicate that Ca²⁺ regulation via action potentials in IHCs is required for the final maturation and maintenance of the hair bundles after a critical point just before hearing onset.

Identification of genes regulated by the intrinsic Ca²⁺ action potentials using RNA-sequencing

To understand the molecular pathways underpinning the changes in the hair-bundle structure observed in the absence of the intrinsic Ca²⁺ action potential activity in IHCs, we performed RNA-seq on P9 controls and littermate Kir2.1-OE mice. At this age, most of the hair bundles still showed a normal-looking structure, but with some IHCs having lost the 3rd row of stereocilia and some showing stereocilia fusion (Fig 5B). This was associated with the onset of MET

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**Figure 4. Rapid disappearance of the MET current in Kir2.1 overexpressing IHCs during the second postnatal week.**

A, B Saturating MET currents recorded from apical IHCs of P8 control (A) and Kir2.1-OE (B) mice. IHC hair bundles were stimulated as described in Fig 3.

C Peak-to-peak MET current–voltage curves from P8–P9 apical-coil IHCs of 12 control (17 IHCs) and 13 littermates Kir2.1-OE mice (24 IHCs). The two sets of data are significantly different: P < 0.0001, 2-way ANOVA.

D The maximum size of the MET current measured in IHCs at ~124 mV (left panel) and ~96 mV (right panel) from Kir2.1-OE mice was significantly reduced compared to that of control cells.

E The resting open probability (P_{open}) of the MET current in IHCs was significantly increased in Kir2.1-OE compared with control cells at both ~124 mV (left) and ~96 mV (right).

F, G Saturating MET currents recorded from apical IHCs of control (F, P10) and Kir2.1-OE (G, P11) mice. IHC hair bundles were stimulated as described in Fig 3.

H Peak-to-peak MET current–voltage curves from P10–P11 apical-coil IHCs of 13 control (16 IHCs) and 4 littermate Kir2.1-OE mice (9 IHCs). The two sets of data are significantly different: P < 0.0001, 2-way ANOVA.

I The maximum size of the MET current measured in IHCs at ~124 mV (left panel) and ~96 mV (right panel) from Kir2.1-OE mice is significantly reduced compared to that of control cells.

J Example of FM1-43 uptake by IHCs from P11 control (top) and Kir2.1-OE (bottom) mice, showing the lack of fluorescence labeling in the latter, which is an indication of the lack of MET channels open at rest at this stage in the IHCs overexpressing the Kir2.1 channels. At least 3 mice for each genotype were used.

Data information: In panels D, E, I, data are shown as means ± SD, and the single cell value recordings (open symbols) are plotted with the average data. The number of IHCs investigated is shown above the average data points from 12 control and 13 littermates Kir2.1-OE mice (panels D and E) and 13 control and 4 littermate Kir2.1-OE mice (panel I). Statistical tests in panels D, E, I was done using the t-test. The * defines the presence of statistical significance, with the P-value shown above the data. Source data are available online for this figure.
Figure 5. IHC bundle morphology progressively deteriorates in Kir2.1 overexpressing mice.

A, B Scanning electron microscope (SEM) images showing the IHC hair-bundle structure in the apical coil of the cochlea of P11 control (A) and P8-P11 Kir2.1-OE (B) mice. Control IHCs and the large majority of P8 IHCs from Kir2.1-OE mice show a normal hair-bundle structure composed of three rows of stereocilia: tall, intermediate and short (arrow). From about P9 in Kir2.1-OE mice, some IHCs start to lose the third row of stereocilia (arrowheads) and some already exhibit some fusion of the stereocilia (asterisk). These changes in hair-bundle structure became more prominent at P11. At least 3 mice for each genotype were used. In these panels and those below, asterisks are used to define some of the abnormal hair bundles.

C, D SEM images of both IHCs and OHCs from the cochlea of P26 control (C, upper panel) and P26 Kir2.1-OE (D, upper panel) mice. Lower panels show a higher-magnification view of the hair bundle of IHCs from both genotypes, highlighting the profound disruption of the stereocilia in IHCs overexpressing Kir2.1 channels. At least 3 mice for each genotype were used.

Source data are available online for this figure.
Figure 6. Hair-bundle proteins involved in stereociliary elongation are not affected in IHCs from Kir2.1-OE mice.

A, B Maximum intensity projections of confocal z-stacks showing images of the hair bundles from apical-coil IHCs of P6 and P11 control (A) and Kir2.1-OE (B) mice immunostained with antibodies against MYOSIN VI (blue) and EPS8 (magenta). At least 3 mice for each genotype were used.

C, D Confocal images of the hair bundles of P11 IHCs from control (C) and Kir2.1-OE (D) mice immunostained with antibodies against MYOSIN XV-isoform 1 (blue) and WHIRLIN (magenta). In all panels (A–D), stereocilia are labeled with phalloidin (green). Note that despite the disrupted hair-bundle structure in the IHCs overexpressing Kir2.1 channels, the stereocilia retained a normal distribution of these bundle proteins. At least 3 mice for each genotype were used.

Source data are available online for this figure.
current reduction in at least some of the IHCs (Fig 4A–E). We reasoned that by profiling animals at this age we could understand the early molecular response that leads to abnormal hair-bundle morphology.

RNA-sequencing was performed on three replicates, each with eight pooled organs of Corti from four mice. Total RNA was extracted and sent for library preparation and sequencing. Sequence data were mapped to the mouse genome (mm10) using the

Figure 7.
Ca²⁺ spikes in IHCs regulate bundle morphology over a critical period during the second postnatal week of development.

A, B Maximum intensity projections of confocal z-stacks showing the IHCs of the apical cochlear region from control (A) and Kir2.1-OE (B) pups with the females being in the continuous presence of DOX in the drinking water from conception up to when the pups were P5 (upper panels). Middle and bottom panels show IHCs at P7 and P14 following the removal of DOX at P5 for both control (A) and Kir2.1-OE mice (B). IHCs were stained with antibodies against the K⁺ channel Kir2.1 (green) and Myosin 7a (Myo7a, blue cell marker). Note that after 2 days following the removal of DOX, Kir2.1 overexpression was already largely downregulated. At least 3 mice for each genotype were used.

C, D Calcium action potentials in IHCs from control (C) and Kir2.1-OE (D) mice during the second postnatal week (P8–P9). IHC voltage responses were recorded during the application of 1.3 mM Ca²⁺ extracellular solution. The voltage-clamp data recorded from the same two IHCs displayed in panels C and D are shown in Appendix Fig S2; the IHCs from Kir2.1-OE mice show a strongly reduced Kir2.1 current. DOX was removed from the drinking water at P5.

E, F Spontaneous Ca²⁺ action potentials in IHCs from control (E, 60 s out of 92 s recording time) and Kir2.1-OE (F, 60 s out of 103 s recording time) mice during the second postnatal week in the presence of the in vivo endolymph-like 0.3 mM Ca²⁺. Note that in contrast to when DOX was present throughout development (Fig 2A–D), the removal of DOX at P5 restored the ability of IHCs from Kir2.1-OE mice to generate spontaneous intrinsic Ca²⁺ action potentials.

G, H IHC resting membrane potentials from 2 control (4 IHCs) and 3 Kir2.1-OE (7 IHCs) mice in the presence of 1.3 mM Ca²⁺ (left) or 0.3 mM Ca²⁺ (right) in the extracellular solution. One-way ANOVA followed by the Bonferroni’s post-test: ns, P > 0.9990 (1.3 mM Ca²⁺); ns, P = 0.8864 (0.3 mM Ca²⁺); all other comparisons were *P < 0.0001.

I, J Maximum intensity projections of confocal z-stacks showing images of the hair bundles from apical-coil IHCs of P14 control (I) and Kir2.1-OE (J) mice stained with phalloidin. DOX was removed from the mother’s drinking water when the pups were P5. At least 3 mice for each genotype were used.

K, L SEM images showing the normal structure of the hair bundles of the IHCs in the apical coil of the cochlea of P14 control (L) and P14 Kir2.1-OE (K) mice. DOX was removed from the mother’s drinking water when the pups were P5. Note that the morphological profile of the hair bundles in IHCs is interchangeable between control and Kir2.1-OE mice, indicating that the removal of the intrinsic Ca²⁺ action potentials prior to the second postnatal week has no effect on the mechanoelectrical transduction apparatus. At least 3 mice for each genotype were used.

Source data are available online for this figure.

NextFlow RNA pipeline and gene counts were performed using Salmon (see Materials and Methods). These raw counts were then used as the input for differential gene expression analysis using DESEQ2 (Love et al., 2014). After performing principal component analysis (PCA) on the top 1,000 expressed genes in the samples, we observed a clear separation between the different genotypes with PC1, which explained 85% of the observed variance. Conversely, PC2, which mostly separated the different biological replicates, explained 8% of variance between samples (Fig 8A). As expected, we observed a 13-fold increase in Kcnj2 (Fig 8B), validating the overexpression of the Kir2.1.

We next performed differential gene expression analysis (Padj < 0.05 and fold-changes >1.5), yielding 589 upregulated genes and 30 downregulated genes (Dataset EV1; Appendix Fig S3). Pathway analysis showed an enrichment of GO terms related to cell morphogenesis, actin filament-based processes and Rho-GTPase signaling (Fig 8C). Among the differentially expressed genes were 118 upregulated genes with annotations related to actin filament or microtubule regulation (Fig 8D). We also noted several genes related to the Golgi body and the trans-Golgi network (TGN), for example, Gola3, Golgap4, and Trip11, which are all hypothesized to play a role in maintaining Golgi structure. In line with the stereocilia phenotype, we observed the upregulation of some components of the stereocilia, Myo7a and Pdxb15 (2.25 and 2.15-fold, respectively) (Fig 8E).

We also performed network analysis on known protein–protein interactions on the differentially expressed genes (Fig 8G, Dataset EV2). Chromatin remodeling genes were also overrepresented among the upregulated genes, including DNA methylation (Dnmt1, Dnmt3a) and demethylation (Tet1, Tet2, Tet3) and histone modifying enzymes (Hdac4, Setd2, Setd5). Several components of the LINC complex that connects the nuclear lamina to the cytoskeletal network, including the subunits of the laminin complex (Lama 1, 2, 4, 5, Lamb1,2, Lamc1 and 2) and the Nesprin family (Synec1, Synec2, Synec3) that connect the cytoskeletal network to laminin, were upregulated (Fig 8G, Dataset EV2). Mechanical signals are directly transduced from extracellular stimulus to the nuclear interior through the interaction of the nesprin proteins (Khilan et al., 2021). Moreover, the maintenance of nuclear structure and...
Figure 8.
organization is regulated by laminins, which also help to transduce mechanical strain forces into a transcriptional response.

We next sought to determine which transcription factors (TFs) might be mediating the upregulated genes in Kir2.1 overexpression. Using the list of 589 upregulated genes, we used HOMER (Heinz et al., 2010) to scan the region ± 2,000 bp from the transcriptional start site (TSS) of each gene for TF binding motifs. Within the top fifteen enriched motifs were several hair cell-enriched TFs, such as Isl1, Sox9, and Gfi1 (Fig 8F). Several classic targets of SOX9, including Acan, Col2a1, Col4a1, Col5a1, Col11a1/2, Col23a1, and several ankyrin family proteins (genes: Ank1, Ank2, Ankr11, Ankr12) and Myo9b, were found to be SOX9 target genes in chromatin immunoprecipitation with sequencing (ChIP-seq) studies conducted in rib chondrocytes (Ohba et al., 2015). Of the 602 upregulated genes, 65% overlapped with SOX9 target genes in chondrocytes. Similarly, SOX9 ChIP-seq in the developing testis found SOX9 bound on Myo7a (Li et al., 2014). We also observed enrichment for the RXF family, which plays a conserved role in ciliogenesis in many different organisms (Lemeille et al., 2020).

Discussion

Here we show that spontaneous intrinsic Ca2+ action potential activity present in the developing IHCs, and thus Ca2+ regulation, is crucial for the final stages of maturation and maintenance of the stereociliary hair bundles. The absence of the intrinsic action potentials during the second postnatal week led to a progressive re-absorption of stereocilia in the short 3rd row and a fusion of the tallest rows, generating “giant” stereocilia. The functional consequence of this hair-bundle disruption was a complete loss of mechanoelectrical transduction prior to the onset of hearing at P12. Furthermore, we show that this intrinsic regulation of IHC development occurs during a critical time window that spans the second postnatal week of development, just before hearing onset. The RNA-seq analysis highlighted that absence of intrinsic APs caused the upregulation of genes involved in cytoskeleton and Rho-GTPase-related pathways, several of which have not been previously associated with cochlear development.

Calcium-dependent activity in the developing cochlea

The initial morphological and functional differentiation of cochlear sensory hair cells depends on intrinsic genetic programs that are coordinated by a combination of transcription factors, including Atoh1 (Bermingham et al., 1999), Helios (Chessum et al., 2018) and Tbx2 (Garcia-Anoveros et al., 2022), and microRNAs such as miR-96 (Kuhn et al., 2011). However, evidence from other sensory systems, especially from the visual system (e.g., Grubb & Thompson, 2004; Blankenship & Feller, 2010), shows that the final maturation of sensory pathways is driven by experience-independent Ca2+-dependent activity, which occurs during a critical period of development. This early electrical activity has been shown to regulate several cellular responses (Berridge et al., 2000), including the remodeling of synaptic connections (Zhang & Poo, 2001) and ion-channel expression (Moody & Bosma, 2005).

In the mammalian cochlea, spontaneous Ca2+-dependent action potentials have been recorded throughout the postnatal development of the IHCs (Kros et al., 1998; Glowatzki & Fuchs, 2000; Beutner & Moser, 2001; Marcotti et al., 2003a; Brandt et al., 2007). The firing activity of neighboring IHCs is normally synchronized by spontaneous intercellular Ca2+ signaling originating in the nonsensory cells via the release of ATP (Tritsch et al., 2007; Johnson et al., 2011, 2017; Wang et al., 2015; Eckrich et al., 2018). ATP acts on purinergic autoreceptors expressed in the nonsensory cells surrounding the IHCs, which leads to the opening of TMEM16A Ca2+-activated Cl− channels and the efflux of K+ in the intercellular space, causing IHC depolarization (Wang et al., 2015). Although Ca2+ action potential activity in developing IHCs has been linked to the refinement of the tonotopic organization in the brainstem (Clause et al., 2014, 2017; Müller et al., 2019; Maul et al., 2022) and auditory neuron survival (Zhang-Hooks et al., 2016), its direct role in regulating and/or maintaining IHC development is still largely unknown. A previous study has shown that increasing the IHC firing activity prevented linearization of their exocytotic Ca2+ dependence in the adult cochlea (Johnson et al., 2013), although both the intrinsic and ATP-dependent mechanisms could have contributed.

The mouse model used in this study (Kir2.1-OE) has allowed us to specifically silence the intrinsic Ca2+ action potentials in developing IHCs in vivo while retaining the ability of nonsensory cells to depolarize the IHCs via ATP-dependent Ca2+ signaling. We found that the absence of spontaneous Ca2+ action potentials that are intrinsically generated by the IHCs prevented the full maturation and maintenance of the hair bundles in IHCs, thus abolishing the mechanoelectrical transducer current that is required for the conversion of acoustic stimuli into electrical signals. We found that such crucial control over the hair-bundle structure and function is only established after a critical time point in the second postnatal week, just before hearing onset.

Role of Ca2+-dependent action potentials in the maturation of hair cells

Calcium-dependent electrical activity regulates several cellular responses (Berridge et al., 2000). Changes in intracellular Ca2+ signals mediated by L-type Ca2+ channels have been implicated in regulating gene expression in many intracellular pathways including those associated with remodeling and development (Bading et al., 1993; Dolmetsch et al., 1997; Fields et al., 2005; Hagenston & Bading, 2011). Here we show using RNA-seq analysis that the dysregulation of Ca2+ in prehearing IHCs, which only retain the extrinsic modulation of the Ca2+ signals from the nonsensory cells, led to 589 upregulated and 30 downregulated genes.

One of the characteristic phenotypes of the Kir2.1-OE mouse cochlea was the formation of giant or fused stereocilia, which was previously reported in knockout mice for the protein TRIOBP (Kita-jiri et al., 2010), which is a component of the stereocilia rootlets (Pacentine et al., 2020), for the unconventional MYO6 (Self et al., 1999) that localizes at the base and all along the length of the stereocilia (Hertzano et al., 2008) and for a protein associated with the shaft connections located between stereocilia (PTPRQ, Goodyear et al., 2003). RNA-seq analysis did not show any significant changes in the genes encoding the above three proteins in the cochlea of Kir2.1-OE mice but did identify 118 upregulated genes with annotations related to actin filament or microtubule regulation. This included cytoskeletal genes Map2, Spib, PLEC, and Nefl, Kinesin
superfamily proteins, which are microtubule-dependent molecular motors (Kif1a, Kif5a, Kif5c, Kif21a, Kif21b), and several components of the Rho-GTPase pathway (Rock1, lqpap1, lqpap2, ltp1, ltp2, ltp3, Arhgap13, Argef11, Argef17, Trio, and Kalrn). Although most of the identified genes are possible novel candidates involved in hair cell development, we found some that have previously been associated with hair-bundle morphology. For example, the actin-binding protein spectrin isoform SPTBN1 (Spbn1), which is expressed in the rootlets actin filaments of the stereocilia (Furness et al., 2008), and together with TRIOBP contributes to strengthen their insertion point into the apical membrane of the hair cells (Pacentine et al., 2020), is required for the correct hair-bundle morphology. In the absence of SPTBN1 mice are deaf (Liu et al., 2019). Furthermore, the actin crosslinking family protein 7 (ACF7) and the microtubule-associated protein 1A (MAP-1A), which are encoded by the genes Macf1 and Macp1a, respectively, are also involved in the organization of the cuticular plate of the hair cells (Jaeger et al., 1994; Antonelli et al., 2014), which is the point of stereocilia insertion of the hair bundles. Finally, the non-muscle myosin Type IIA (MYH9) has been linked to both syndromic and nonsyndromic hearing loss due to the disruption of the hair cell stereociliary bundles (Mhatre et al., 2006). Among the identified transcription factors, we found enrichment for regulatory factor X (RFX), which plays a role in ciliogenesis in many different organisms (Lemeille et al., 2020). In mammals, Rfx3 is involved in ciliary assembly and motility, and Rfx4 is known to modulate Shh signaling and regional control of ciliogenesis (Ashique et al., 2009). Moreover, recent work has shown that the RFX family is essential for hearing in mice, with mice at 3 months of age showing a loss of stereocilia structure (Elkon et al., 2015).

Altogether, these results indicate that the intrinsic Ca^{2+}-dependent action potential activity in IHCs during the second postnatal week is necessary to drive their full morphological and functional maturation into auditory sensory receptors. The absence of such activity led to the upregulation of the genetic pathways involved in the maintenance of cytoskeletal homeostasis, possibly as an attempt to repair or compensate for the progressive deterioration of the actin-based hair bundles. Moreover, we found that the MET channel of the IHCs from Kir2.1-OE mice acquire a reduced Ca^{2+} sensitivity, which could be a potential compensatory mechanism for maintaining resting MET current size as the MET current is rapidly declining. Although genetic compensation responses following the mutation of genes have been described in many organisms including zebrafish and mice (e.g., El-Brolosy & Stainier, 2017; Buglo et al., 2020), their underlying mechanisms remain largely unknown.

### Materials and Methods

#### Reagents and Tools table

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**Methods and Protocols**

**Ethics statement**
Animal work was licensed by the Home Office under the Animals (Scientific Procedures) Act 1986 (PPL_PCC8E5E93) and was approved by the University of Sheffield Ethical Review Committee (180626_Mar). For *ex vivo* experiments mice were killed by cervical dislocation followed by decapitation. Mice had free access to food and water and a 12 h light/dark cycle.

**Transgenic mice**
The transgenic mouse line *OtofrtTA* expressing rtTA driven by the *Otof* promoter was constructed by Ozgene Pty Ltd (Bentley WA, Australia). In these mice, the expression of a target gene is controlled by a reverse tetracycline-controlled transactivator rtTA (Tet-On system: Baron & Bujard, 2000). *Otof* encodes for the ribbon synaptic Ca$^{2+}$ sensor otoferlin, in which the cochlea is expressed exclusively in hair cells but primarily in IHCs from around birth (Roux et al, 2006). Homozygous *OtofrtTA* mice were paired with heterozygous *tetO-Kir2.1-ires-tau-lacZ* mice (*Kir2.1*: Jackson laboratories, 009136, Yu et al, 2004). Both mouse lines (*OtofrtTA* and *Kir2.1*) were maintained on the C57BL/6N background. The resultant compound heterozygous mice, which we named *Kir2.1-OE* (*Kir2.1-OverExpression*) mice for simplicity, allowed cell-specific overexpression of the inward rectifier K$^+$ channel *Kir2.1* in the IHCs when mice were treated with doxycycline (DOX). Littermate heterozygous *OtofrtTA* mice treated with DOX were used as controls. Pregnant, breast-feeding females and weaned pups (controls and *Kir2.1-OE*) were given 0.5 mg/ml of DOX daily in their drinking water, a dose that was previously optimized for the mouse cochlea (Johnson et al, 2013).

**Tissue preparation**
Cochleae were dissected out from the inner ear of the mouse using an extracellular solution composed of (in mM): 135 NaCl, 5.8 KCl, 1.3 CaCl$_2$, 0.9 MgCl$_2$, 0.7 Na$_2$HPO$_4$, 5.6 D-glucose, 10 HEPES, Sodium pyruvate (2 mM), amino acids, and vitamins were added from concentrates (Thermo Fisher Scientific, UK). The pH was adjusted to 7.48 with 1 M NaOH (osmolality ~308 mOsm/kg). The dissected cochleae were transferred to a microscope chamber and immobilized via a nylon mesh attached to a stainless-steel ring as previously described (Marcotti et al, 2003b). The chamber (volume ~ 2 ml) was perfused from a peristaltic pump and mounted on the stage of an upright microscope (Olympus BX51, Japan; Leica DMLFLS, Germany) equipped with Nomarski Differential Interference Contrast (DIC) optics (60× or 64× water immersion objective) and 15× eyepieces. The microscope chamber was continuously perfused with the extracellular solution by a peristaltic pump (Cole-Palmer, UK).

**Whole-cell electrophysiology**
Patch clamp experiments were performed from hair cells positioned at the 9–12 kHz region of the cochlear apical coil (Müller et al, 2005). Recordings were performed at room temperature (20–24°C) using an Optopatch amplifier (Cairn Research Ltd, UK) as previously described (Jeng et al, 2020; Carlton et al, 2021). Patch pipettes were pulled from soda glass capillaries, which had a typical resistance in the extracellular solution of 2–3 MΩ. The intracellular solution used for the patch pipette contained (in mM): 131 KCl, 3 MgCl$_2$, 1 EGTA-KOH, 5 Na$_2$ATP, 5 HEPES, 10 Na-phosphocreatine (pH was adjusted with 1 M KOH to 7.28; 294 mOsm/kg). Data acquisition was controlled by pClamp software using Digidata 1440A (Molecular Devices, USA). In order to reduce the electrode capacitance, patch electrodes were coated with surf wax (Mr Zoggs SexWax, USA). Recordings were low-pass filtered at 2.5 kHz (8-pole Bessel), sampled at 5 kHz, and stored on a computer for offline analysis (Clampfit, Molecular Devices; Origin 2021: OriginLab, USA). Membrane potentials under voltage-clamp conditions were corrected offline for the residual series resistance $R_s$ after compensation (usually 80%)
and the liquid junction potential (LJP) of –4 mV, which was measured between electrode and bath solutions. Voltage-clamp protocols are referred to a holding potential of –84 mV unless otherwise stated.

Real-time changes in membrane capacitance (ΔCm) were tracked at body temperature as previously described (Johnson et al., 2005, 2017). Briefly, a 4 kHz sine wave of 13 mV RMS was applied to IHCs from the holding potential of –81 mV and was interrupted for the duration of the voltage step. The capacitance signal from the Optopatch was filtered at 250 Hz and sampled at 5 kHz. ΔCm was measured by averaging the Cm trace over a 200 ms period following the voltage step and subtracting the pre-pulse baseline. Data were acquired using pClamp software and a Digidata 1440A (Molecular Devices). ΔCm experiments were performed during the local perfusion of the IHCs with 30 mM TEA, 15 mM 4-AP (Fluka) to block the outward K+ currents (Johnson et al., 2005), and 5 mM CsCl to block the inward rectifier K+ current (Marcotti et al., 1999).

For mechanoelectrical transducer (MET) current recordings, the hair bundles of hair cells were displaced using a fluid-jet system from a pipette driven by a 25 mm diameter piezoelectric disc (Corns et al., 2014, 2018; Carlton et al., 2021). For these experiments, the intracellular solution contained (in mM): 131 CsCl, 3 MgCl2, 1 EGTA-KOH, 5 Na2ATP, 10 Na-phosphocreatine (pH was adjusted with 1 M CsOH to 7.28; 290 mOsm/kg). The extracellular solution was as described above, although for most of the recordings we included 5 mM CsCl, which was used to block the inward rectifier K+ current (Marcotti et al., 1999). In order to maintain the osmolality of the extracellular solution constant, NaCl was reduced to 130 mM in this case.

The fluid-jet pipette tip had a diameter of 8–10 μm and was positioned near the hair bundles to elicit a maximal MET current (typically 10 μm). Mechanical stimuli were applied at 50 Hz sinusoids (filtered at 1 kHz, 8-pole Bessel). Prior to the positioning of the fluid jet by the hair bundles, any steady-state pressure was removed. The use of the fluid jet allows for the efficient displacement of the hair bundles in both the excitatory and inhibitory directions, which is essential to perform reliable measurements of the resting open probability of the MET channels.

### Two-photon confocal Ca2+ imaging

Acutely dissected cochleae were incubated for 40 min at 37°C in DMEM/F12, supplemented with fluo-4 AM at a final concentration of 10 μM (Thermo Fisher Scientific) as recently described (Ceriani et al., 2019). The incubation medium contained also pluronic F-127 (0.1%, w/v) and sulfonpyrazone (250 μM) to prevent dye sequestration and secretion. Calcium signals were recorded using a two-photon laser-scanning microscope (Bergamo II System B232, Thorlabs Inc., USA) based on a mode-locked laser system operating at 925 nm, 80-MHz pulse repetition rate, < 100-fs pulse width (Mai Tai HP DeepSee, Spectra-Physics, USA). Images were captured with a 60× objective (LUMFLN60XW, Olympus, Japan) using a GaAsP PMT (Hamamatsu) coupled with a 525/40 bandpass filter (FF02-52S5/40-25, Semrock). Images were analyzed offline using custom-built software routines written in Python (Python 2.7, Python Software Foundation) and ImageJ (NIH). Calcium signals were measured as relative changes in fluorescence emission intensity (ΔF/F0).

### Scanning electron microscopy (SEM)

The isolated inner ear was very gently perfused with fixative for 1–2 min through the round window. A small hole in the apical portion of the cochlear bone was made prior to perfusion to allow the fixative to flow out from the cochlea. The fixative contained 2.5% vol/vol glutaraldehyde in 0.1 M sodium cacodylate buffer plus 2 mM CaCl2 (pH 7.4). The inner ears were then immersed in the above fixative and placed on a rotating shaker for 2 h at room temperature. After fixation, the organ of Corti was exposed by removing the bone from the apical coil of the cochlea and then immersed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h. For osmium impregnation, which avoids gold coating, cochleae were incubated in solutions of saturated aqueous thio-carbohydrazide (20 min) alternating with 1% osmium tetroxide in buffer (2 h) twice (the OTOTO technique: Furness & Hackney, 1986). The cochleae were then dehydrated through an ethanol series and critical point dried using CO2 as the transitional fluid (Leica EM CPD300) and mounted on specimen stubs using conductive silver paint (Agar Scientific, Stansted, UK). The apical coil of the organ of Corti was examined at 10 kV using a Tescan Vega3 LMU scanning electron microscope in the electron microscopy unit at the University of Sheffield.

### Immunofluorescence microscopy

As for SEM, the isolated inner ear was initially gently perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) through the round window. Following this initial short fixation, the inner ear was fixed for 20 min at room temperature and then washed three times in PBS for 10 min. The apical coil of the organ of Corti was then washed in PBS, removed by fine dissection, and incubated in PBS supplemented with 5% normal goat or horse serum and 0.5% Triton X-100 for 1 h at room temperature. The samples were immunolabeled with primary antibodies overnight at 37°C, washed three times with PBS, and incubated with the secondary antibodies for 1 h at 37°C. Antibodies were prepared in 1% serum and 0.5% Triton X-100 in PBS. Primary antibodies were mouse-IgG anti-Eps8 (1:1,000, BD Biosciences, 610,143), rabbit-igG anti-WHIRLIN (1:200, gift from Dr. Thomas Friedman, NIH, USA); rabbit-igG anti-MYO6 (1:150, Proteus Biosciences, 25–6,791); rabbit-igG anti-MYO15 isoform 1 (1:1,000, gift from Dr. Thomas Friedman, NIH, USA) mouse-IgG1 anti-Kir2.1 channel (1:100, Alomone Lab, Israel, APC026); mouse IgG1 anti-CtBP2 (1:200, Biosciences, 612044A) and mouse IgG2a anti-GluR2 (1:200, Millipore, MAB397). F-actin was stained with Texas Red-X phalloidin (1:400, Thermofisher, T7471) in the secondary antibody solution. Secondary antibodies were species-appropriate Alexa Fluor or Northern Lights secondary antibodies. Samples were mounted in VECTASHIELD (H-1000). The images from the apical cochlear region (8–12 kHz) were captured with Nikon A1 confocal microscope equipped with a Nikon CFI Plan Apo 60× Oil objective or a Zeiss LSM 880 Airyscan equipped with Plan-Apochromat 63× Oil DIC M27 objective for super-resolution images of hair bundles. Both microscopes are part of the Wolfson Light Microscope Facility at the University of Sheffield. Image stacks were processed with Fiji ImageJ software.

### FM1-43 staining

A 3 mM stock solution of the dye FM1-43 (T3163, Molecular Probes) was prepared in water. The dissected organs of Corti (aged P11–P12) were transferred to the bottom of a chamber filled with

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extracellular solution, and held in position using a nylon mesh, as described above (see above: Tissue preparation). All experiments were performed at room temperature (20–24°C), as previously described (Gale et al., 2001). Briefly, the solution bathing the cochlea was very rapidly exchanged with that containing 3 μM FM1-43 for 10 s and immediately washed several times with normal extracellular solution. The cochlea were then viewed with an upright microscope equipped with epifluorescence optics and FITC filters (excitation 488 nm, emission 520 nm) using a 63× water immersion objective and a CCD camera.

**RNA isolation and library preparation**

The sensory epithelium from four control and four littermates Kir2.1-OE mice under DOX were microdissected in DNase-free ice-cold PBS 1× and immediately snap frozen in liquid nitrogen. RNA was extracted using RNeasy Plus Micro Kit (Qiagen) according to the manufacturer’s instructions. RNA quantity was established using a Nanodrop spectrophotometer and RNA integrity number (RIN) was calculated using a BioAnalyzer. All samples had RIN score greater than 9.1. Preparation of the mRNA library was performed using poly A enrichment and sequenced on the Illumina NovaSeq sequencer using paired-end 150 bp reads.

**RNA-sequencing analysis and differential gene expression**

The sequencing libraries were processed using the nf-core RNA pipeline (Ewels et al., 2020, https://nf-co.org/rnaseq/usage) using the standard parameters. Reads were mapped to the mouse genome (mm10). The resulting gene counts were determined using Salmon (Patro et al., 2017) and used for downstream analysis with DESeq2 (Love et al., 2014). Metascape (Zhou et al., 2019) and Reactome (Gillespie et al., 2022) were used to query for enriched GO terms and pathways in the list of differentially expressed genes. Homer (Heinz et al., 2010) was used to find known and de novo motifs among the upregulated genes in a 2000 bp window up and downstream of the transcriptional start site (TSS).

**Statistical analysis**

Statistical comparisons of means were made by the Student’s two-tailed t-test or, for multiple comparisons, the analysis of variance (one-way or two-way ANOVA followed by a suitable post-test) and Mann–Whitney U test (when normal distribution could not be assumed) were used. P < 0.05 was selected as the criterion for statistical significance. Only mean values with a similar variance between groups were compared. Average values are quoted in text and figures as means ± S.D. Animals of either sex were randomly assigned to the different experimental groups. No statistical methods were used to define sample size, which was determined based on previously published similar work from our laboratory. Animals were taken from several cages and breeding pairs over a period of several months. Most of the electrophysiological and morphological (but not imaging) experiments were performed blind to animal genotyping and in most cases, experiments were replicated at least 3 times.

**Data availability**

The data that support the findings of this study are available from the corresponding author. RNA-sequencing data have been deposited in GEO under accession number (GSE215951; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE215951).

**Expanded View** for this article is available online.

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**Author contributions**


**Disclosure and competing interests statement**

The authors declare that they have no conflict of interest.

**References**


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Maul A, Huebner AK, Strenzke N, Moser T, Rübsamen R, Jovanovic S, Hübner CA (2022) The Ci-channel TMEM16A is involved in the generation of cochlear Ca²⁺ waves and promotes the refinement of auditory brainstem networks in mice. Elife 11: e72251


Moser T, Grabner CP, Schmitz F (2020) Sensory processing at ribbon synapses in the retina and the cochlea. Physiol Rev 100: 103 – 144


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