Coordinated calcium signalling in cochlear sensory and non-sensory cells refines afferent innervation of outer hair cells

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Abstract

Outer hair cells (OHCs) are highly specialized sensory cells conferring the fine-tuning and high sensitivity of the mammalian cochlea to acoustic stimuli. Here, by genetically manipulating spontaneous Ca\textsuperscript{2+} signalling in mice in vivo, through a period of early postnatal development, we find that the refinement of OHC afferent innervation is regulated by complementary spontaneous Ca\textsuperscript{2+} signals originating in OHCs and non-sensory cells. OHCs fire spontaneous Ca\textsuperscript{2+} action potentials during a narrow period of neonatal development. Simultaneously, waves of Ca\textsuperscript{2+} activity in the non-sensory cells of the greater epithelial ridge cause, via ATP-induced activation of P2X\textsubscript{5} receptors, the increase and synchronization of the Ca\textsuperscript{2+} activity in nearby OHCs. This synchronization is required for the refinement of their immature afferent innervation. In the absence of connexin channels, Ca\textsuperscript{2+} waves are impaired, leading to a reduction in the number of ribbon synapses and afferent fibres on OHCs. We propose that the correct maturation of the afferent connectivity of OHCs requires experience-independent Ca\textsuperscript{2+} signals from sensory and non-sensory cells.

Keywords
calcium waves; hair cells; pre-hearing development; purinergic receptors; spontaneous activity

Subject Categories
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Introduction

Mammalian hearing depends upon two specialized sensory receptor cell types in the organ of Corti, the inner and outer hair cells, and their afferent and efferent neuronal connections. The differentiation, maturation and maintenance of these neuronal connections require precise timing and coordination between genetic programmes and physiological activity (Corns et al, 2014, 2018; Delacroix & Malgrange, 2015). Inner hair cells (IHCs) are the primary sensory receptor cells, and they relay sound information to spiral ganglion afferent neurons via the release of glutamate from vesicles tethered to pre-synaptic ribbons. By contrast, the role of outer hair cells (OHCs) is to extend the functional dynamic range of the mammalian cochlea and to enhance the sensitivity and the frequency tuning within the cochlear partition (Dallos, 1992). Adult OHCs are primarily innervated by cholinergic medial olivocochlear neurons (Liberman, 1980; Maison et al, 2003), the role of which is to modulate mechanical amplification in the adult cochlea (Guinan, 1996). However, OHCs are also innervated by type II afferent fibres that appear to be activated by acoustic trauma (Flores et al, 2015; Liu et al, 2015), unlike the type I fibres contacting IHCs that encode sound timing, intensity and frequency. In most altricial rodents, OHCs only begin to acquire the innervation pattern present in the mature cochlea towards the end of the first and the start of the second postnatal week (Simmons, 1994; Simmons et al, 1996). However, the molecular mechanisms responsible for the correct afferent innervation of OHCs remain poorly understood.

The refinement of sensory circuits during development is normally influenced by periods of experience-independent action potential (AP) activity before the onset of function (Katz & Shatz, 1996; Blankenship & Feller, 2010). Calcium-dependent APs have been shown to occur spontaneously in immature IHCs (Johnson et al, 1996; Blankenship & Feller, 2010). However, it is unclear whether calcium waves are present in OHCs in vivo. If spontaneous Ca\textsuperscript{2+} waves are a feature of OHCs, it is unclear what physiological role they may play and how they are elicited. Here, we use the genetically tractable mouse model to explore the role of spontaneous Ca\textsuperscript{2+} signalling in OHCs in vivo.
et al., 2011, 2017) but not in OHCs (Oliver et al., 1997; Marcotti & Kros, 1999; Weiss et al., 2012). One study reported spontaneous APs in OHCs of wild-type and ototoxin mutant mice, but mostly using elevated extracellular Ca²⁺ and high intracellular EGTA (Beurg et al., 2008).

We found that during a narrow, critical period of postnatal development (around birth), OHCs show spontaneous Ca²⁺ signals immediately preceding their functional maturation at ~P7–P8. This Ca²⁺ activity in immature OHCs can be modulated by Ca²⁺ waves travelling among non-sensory cells via the ATP-dependent activation of P2X₃ receptors. The Ca²⁺ waves, by increasing the Ca²⁺ signals in OHCs, were able to synchronize the activity of nearby OHCs. The reduction of spontaneous Ca²⁺ waves in non-sensory cells in vivo prevented the maturation of the OHC afferent innervation. We propose that precisely modulated Ca²⁺ signals between OHCs and non-sensory cells are necessary for the correct maturation of the neuronal connectivity to OHCs.

Results

The functional development of OHCs was studied primarily in the apical third of the mouse cochlea, corresponding to a frequency range in the adult mouse of ~6–12 kHz (Müller et al., 2005; Fig 1A). For comparison, some recordings were also made from the basal coil of the cochlea through the frequency range of ~25–45 kHz (Fig 1A). Spontaneous Ca²⁺ activity in immature OHCs and its modulation by non-sensory cells in the greater (GER) and lesser (LER) epithelial ridges (Fig 1B) was recorded from cochleae bathed in a perilymph-like extracellular solution (1.3 mM Ca²⁺ and 5.8 mM K⁺; Wangemann & Schacht, 1996) either near body temperature or at room temperature. Although the stereociliary bundles of hair cells (Fig 1B) are normally bathed in endolymph, which contains ~150 mM K⁺ and ~20 μM Ca²⁺ in the mature cochlea (Bosher & Warren, 1978; Wangemann & Schacht, 1996), during the first few days after birth endolymph has a similar ionic composition to that of the perilymph (Wangemann & Schacht, 1996).

Calcium-dependent activity in OHCs occurs spontaneously during a narrow period of development

Spontaneous, rapid Ca²⁺ transients were recorded from OHCs maintained at near-body (~35°C; Fig 1C, Movie EV1) and room temperature (~20°C; Fig 1D, Movie EV3, top panel) in acutely dissected cochleae from newborn mice loaded with the Ca²⁺ indicator Fluo-4. Similar Ca²⁺ transients were observed in OHCs in 37 separate recordings from 13 different mice. By combining cell-attached patch clamp recordings and Ca²⁺ imaging, we confirmed that the Ca²⁺ signals represent the optical readout of OHC firing activity, with bursts of APs causing large increases in the OHC Ca²⁺ level (Fig 1E). Although Ca²⁺ signals were present in OHCs along the entire cochlea at birth, the number of cells showing this activity decreased over time, with basal OHCs being the first to stop at around P4 and apical cells stopping a couple of days later (Fig 1F). This correlated with a decrease in the maximum Ca²⁺-related change in fluorescence intensity (ΔF/F₀) for Ca²⁺ measured from active OHCs (Fig 1G), which is likely due to a progressive disappearance of Ca²⁺ activity caused by the reduction of the Ca²⁺ current (Knirsch et al., 2007) and upregulation of the K⁺ currents (Marcotti & Kros, 1999) in OHCs with age.

Calcium transients were abolished in Ca²⁺-free solution (Appendix Fig S1A–C; Movie EV2) and were absent in OHCs lacking the Ca²⁺.1.3 Ca²⁺ channel subunit (Appendix Fig S1D; Movie EV3, bottom panel), the main voltage-gated Ca²⁺ channel expressed in hair cells (Platzer et al., 2000; Michna et al., 2003). These results, together with the finding that Ca²⁺ activity was not prevented when blocking Ca²⁺ release from intracellular stores (Appendix Fig S1E), indicate their dependence on extracellular Ca²⁺.

Calcium waves from non-sensory cells coordinate OHC Ca²⁺ signals

Hair cells are embedded in a matrix of non-sensory, epithelial supporting cells (Fig 1B). The inner phalangeal cells surrounding the IHCs and the tightly packed columnar cells that form Kölliker’s organ are part of the GER, and they show spontaneous inward currents (Tritsch et al., 2007). This spontaneous activity is initiated by extracellular ATP, which is released via an extensive network of connexin hemichannels in non-sensory cells, activating purinergic autoreceptors on the same cells, causing an increase in intracellular Ca²⁺. It leads to spatially and temporally coordinated Ca²⁺ waves that are propagated across the epithelium (Tritsch et al., 2007). Calcium waves can also be triggered by the application of ATP to non-sensory cells surrounding the OHCs in the LER (e.g. Deiters’ cells: see Fig 1B), but these waves are thought not to occur spontaneously (Tritsch et al., 2007; Anselmi et al., 2008). Therefore, we investigated whether spontaneous Ca²⁺ waves originating in the GER of the developing mouse cochlea (Fig 2A, red arrow) can influence the Ca²⁺ activity in OHCs. In the proximity of large Ca²⁺ waves, OHCs showed an increased Ca²⁺ activity (Fig 2B, see also Fig 2G and H), which was most likely driven by their depolarization and subsequently increased AP firing rate (Fig 1E).

The increased OHC Ca²⁺ signals followed very closely the time course of the Ca²⁺ wave originating in the GER. As a consequence of the increased OHC Ca²⁺ signals, the otherwise uncorrelated spontaneous Ca²⁺ activity in nearby OHCs became highly correlated temporally during large Ca²⁺ waves (Fig 2B and D, Movie EV4). However, OHC Ca²⁺ signals remained uncorrelated in the absence of waves or during small Ca²⁺ waves in the GER (Fig 2C and E, Movie EV5). This suggests that Ca²⁺ waves may serve as an extrinsic pathway to coordinate the firing activity of otherwise independent nearby OHCs. To quantify the change in the synchronization of the OHC Ca²⁺ signals, we computed the average pairwise correlation coefficient (r²; see Materials and Methods). This correlation coefficient was measured between every pair of OHCs in the field of view (64 ± 5 OHCs, seven cochleae, six mice) during a time window of 13.2 s (400 frames, grey area in Fig 2B and C, right panels) centred on the maximum intensity of the spontaneous Ca²⁺ signal occurring in the GER. The average correlation coefficient in nearby OHCs showed a positive relationship with the longitudinal (i.e. along the tonotopic axis) extension of Ca²⁺ waves in the GER (Pearson’s correlation coefficient: 0.85, Fig 2F). While half of the smaller Ca²⁺ waves that spread over less than 75 μm (28 out of 56) had no significant effect on the correlation, all 19 of the larger waves analysed were able to synchronize the activity of several OHCs (Fig 2F).
In order to provide an estimate of the increased Ca\(^{2+}\) signal in OHCs during the Ca\(^{2+}\) waves from non-sensory cells, we quantified the time integral of the fluorescence traces recorded from OHCs (see Materials and Methods). We found that OHCs closer to the Ca\(^{2+}\) wave showed a larger increase in Ca\(^{2+}\) activity compared to those located far away (Fig 2G).
Figure 2.

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signals in OHCs positioned at around 100 μm from the Ca\(^{2+}\) waves that spread over < 75 μm with those that spread over > 150 μm (Fig. 2H), and found that larger Ca\(^{2+}\) waves caused a significantly increased Ca\(^{2+}\) activity in OHCs (P = 0.0054, t-test). We also found that both the increased OHC Ca\(^{2+}\) activity and the degree of correlation (r\(^m\)) were independent from the amplitude (ΔF/ΔF\(_0\)) of the Ca\(^{2+}\) waves (Appendix Fig S2A and B). Therefore, the coordination of the Ca\(^{2+}\) signals between nearby OHCs was dependent on the lateral spread, but not the amplitude, of the Ca\(^{2+}\) waves.

We then sought to identify how spontaneous Ca\(^{2+}\)\ activity from the non-sensory cells of the GER coupled to Ca\(^{2+}\)\ signalling in OHCs. Patch clamp recordings from Deiters’ cells, which surround the OHCs in the LER (Fig 2A), revealed spontaneous inward currents similar to those measured in non-sensory cells of the GER (Tritsch et al., 2007). During these spontaneous currents, Deiters’ cells depolarized by 16.7 ± 0.5 mV (range 5.4–43.5 mV, 339 events, n = 9) from an average resting membrane potential of −70.8 ± 1.6 mV (n = 9; Fig 3A and B). Simultaneous recordings showed that the inward currents in the Deiters’ cells appear synchronized with Ca\(^{2+}\) waves in the GER in both wild-type (Appendix Fig S3A and Ca\(_{\text{v1.3}}\) mice (Fig 3C). The absence of Ca\(^{2+}\) signals in OHCs from Ca\(_{\text{v1.3}}\) mice made it easier to see that the Ca\(^{2+}\) waves originating in the GER were able to travel to the LER and propagate through Deiters’ cells (Fig 3D; Appendix Fig S3B). Calcium waves originating in the GER reached the more distant Deiters’ cell in the radial direction with a delay of 1.67 ± 0.55 s (n = 11 recordings, 5 cochleae, 3 mice). In order to test whether Deiters’ cells mediate signal transfer from the GER to the OHCs, we analysed Ca\(^{2+}\) signals after removing a few Deiters’ cells beneath the area of interest (Fig 4C) using gentle suction via a small pipette (~ 3–4 μm in diameter). This procedure is widely used to gain access to the different cochlear cell types, including the OHCs (Marcotti & Kros, 1999). Importantly, this procedure does not affect OHC integrity, since they retained normal biophysical characteristics (e.g. resting membrane potential and ability to fire action potentials: Appendix Fig S4A–G), the ability to generate Ca\(^{2+}\) transients (Appendix Fig S4H) and sensitivity to extracellular ATP (Appendix Fig S4I). After the removal of the Deiters’ cells, we elicited Ca\(^{2+}\) waves by photo-damaging a small area of the GER at room temperature. This treatment was used as a proxy for spontaneous Ca\(^{2+}\) waves since they share the same connexin- and ATP-dependent molecular mechanism (Gale et al., 2004; Tritsch et al., 2007; Lahne & Gale, 2010), while allowing precise temporal and spatial control of Ca\(^{2+}\) wave occurrence. Moreover, spontaneous (Appendix Fig S5) and photo-damage-induced Ca\(^{2+}\) waves (Fig 4A and B, Movie EV6) have a qualitatively similar influence on OHC Ca\(^{2+}\) activity. We found that Ca\(^{2+}\) elevation in OHCs associated with induced Ca\(^{2+}\) waves in the GER was almost completely abolished when the nearby Deiters’ cells were removed (Fig 4C–E: P < 0.001 compared to when Deiters’ cells were present; post-test from one-way ANOVA) or in Ca\(_{\text{v1.3}}\) mice (Fig 4C–E). We conclude that Deiters’ cells are essential intermediaries for coupling activity from the GER to OHCs.

**ATP triggers OHC Ca\(^{2+}\) signals in the developing cochlea**

To determine the molecular mechanism linking activity in the Deiters’ cells with OHC synchronization, we pharmacologically probed the basolateral membrane of OHCs deprived of their surrounding Deiters’ cells (Fig 5A). Deiters’ cells release ATP via connexin hemichannels (Zhao et al., 2005), and immature OHCs exhibit depolarizing, ATP-gated currents (Glowatzki et al., 1997). In the absence of Deiters’ cells, we found that local perfusion of 10 μM ATP onto the basolateral membrane of OHCs triggered large Ca\(^{2+}\) responses (Fig 5B). The OHC response to ATP was abolished in Ca\(_{\text{v1.3}}\) mice even at 100 μM (Appendix Fig S6, Movie EV7). Under whole-cell patch clamp, 10 and 100 μM ATP caused OHCs to depolarize by 15.8 ± 2.9 mV (steady-state, n = 10, P1–P2; Fig 5C; Appendix Fig S6). Extracellular ATP can act on ionotropic (P2X) and metabotropic (P2Y) purinergic receptors, both of which are present in cochlear hair cells (Housley et al., 2006). We found that Ca\(^{2+}\) signals from OHCs were either abolished or greatly reduced when ATP was applied together with the purinergic receptor antagonists, suramin (200 μM: Fig 5D and I) and PPADS (Fig 5I). Under the whole-cell patch clamp configuration, suramin reduced the ATP responses by 89.8 ± 6.5% (n = 4; P1; Fig 5C). The absence of ATP-induced Ca\(^{2+}\) signals in wild-type OHCs bathed in a Ca\(^{2+}\)-free solution (Fig 5E and I) and in Ca\(_{\text{v1.3}}\) mice (Fig 5I, Appendix Fig S6, Movie EV7) indicates that P2Y receptors, which mobilize Ca\(^{2+}\) from intracellular stores, are unlikely to be involved in mediating OHC responses to...
ATP at this developmental stage (King & Townsend-Nicholson, 2003; Egan & Khakh, 2004). Consistent with this hypothesis, the application of the phospholipase C inhibitor U73122, which prevents the IP3-mediated Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores linked to the activation of P2Y receptors (Bleasdale & Fisher, 1993; Lahne & Gale, 2010), did not inhibit ATP-induced Ca\(^{2+}\) signals in OHCs (Fig 5F and I). The local application of 1 \(\mu\)M UTP, a selective agonist of P2Y receptors that mobilizes Ca\(^{2+}\) from intracellular stores in cochlear non-sensory cells (Piazza et al., 2007), onto Deiters’ cells triggered an increase in their intracellular Ca\(^{2+}\) levels, followed by increased Ca\(^{2+}\) activity in nearby OHCs (Fig 5G, Movie EV8). When the Deiters’ cells were removed, OHCs were not affected by UTP (Fig 5H: normalized maximal response: DCs intact: 1.00 \(\pm\) 0.23, \(n = 4\) recordings, 3 cochleae, 3 mice; DCs removed: 0.20 \(\pm\) 0.04, \(n = 5\) recordings, 3 cochleae, 3 mice; \(P < 0.01\), Mann–Whitney U-test), providing further evidence that ATP-induced Ca\(^{2+}\) signals from these non-sensory cells directly modulate OHC activity. Altogether these data indicate that ATP, acting through P2Y receptors in Deiters’ cells and P2X receptors in OHCs, can coordinate the activity of nearby OHCs.

We then sought to investigate whether the purinergic signalling from Deiters’ cells to OHCs during Ca\(^{2+}\) waves was also present in the intact cochlear preparation (i.e. without removing the Deiters’ cells as done for Fig 5A). We initially investigated the Deiters’ cell to OHC coupling while applying the non-selective purinergic receptor antagonist PPADS. Even though the occurrence of Ca\(^{2+}\) waves was reduced in the presence of PPADS (control: 2.09 \(\pm\) 0.21 events min\(^{-1}\)/ cochlea, 6 mice, P1–P2; PPADS: 0.81 \(\pm\) 0.14 events min\(^{-1}\)/ cochlea, 7 mice, P1–P2, \(P < 0.0001\), Mann–Whitney U-test), large Ca\(^{2+}\) waves originating in the GER were still able to reach the LER, but failed to increase, and as such synchronize, the Ca\(^{2+}\) signals in OHCs (Fig 6A, C and E). Of the other known P2X receptors present in the cochlea, P2X3 has been shown to be transiently expressed during early stages of development (Huang et al., 2006). We found that the specific P2X3 antagonist A317491 (Jarvis et al., 2004) was able to prevent the Ca\(^{2+}\) waves from affecting the Ca\(^{2+}\) signals in OHCs (Fig 6B, D and F). We further tested the presence of P2X3 receptors in OHCs by performing current-clamp recordings and found that A317491 fully and reversibly blocked ATP-induced OHC activity.

Figure 3. Calcium signalling from the GER travels to Deiters’ cells in the LER.

A, B Spontaneous activity recorded using the patch clamp technique from Deiters’ cells using whole-cell voltage clamp (A) and current clamp (B) in a P1 mouse cochlea. Recordings were made at RT.

C Simultaneous recording of whole-cell voltage responses in a Deiters’ cell in the LER (black trace) and a spontaneous Ca\(^{2+}\) wave from the GER (\(\Delta F/F_0\)) of a CaV\(^{1.3-/-}\) P1 mouse (for wild-type mouse cochlea, see Appendix Fig S1).

D Representative \(\Delta F/F_0\) traces (right traces) from the GER and one Deiters’ cell (arrowhead in the middle panel) of a P1 CaV\(^{1.3-/-}\) mouse. The red triangle (left panel) shows the fluorescence level before the Ca\(^{2+}\) wave, and the red circle (middle panel) shows the response during the wave. Note that the response in the GER occurs earlier than that in the Deiters’ cell (left), even though the rapid onset of the voltage change in the Deiters’ cell (C) gives the impression of an overlapping event. Traces are computed as pixel averages from a single non-sensory cell in the GER and a Deiters’ cell positioned in the radial direction to the Ca\(^{2+}\) wave. The onset of the Ca\(^{2+}\) waves was defined by a fluorescence increase of five times the standard deviation of the background noise compared to baseline.
The above data show that Ca\(^{2+}\) waves originating in the GER are able to travel to the LER, where the OHCs reside, and induce the release of ATP from the non-sensory Deiters’ cells. ATP activates P2X\(_{3}\) receptors in the basolateral membrane of OHCs, leading to OHC depolarization and an increased open probability of voltage-gated Cav1.3 Ca\(^{2+}\) channels. This depolarization will increase the action potential frequency of the OHCs within the area of the Ca\(^{2+}\) wave, thereby increasing the probability of synchronized firing among adjacent OHCs.

The frequency of large Ca\(^{2+}\) waves is reduced in Cx30\(^{-/-}\) mice

In the sensory epithelium of the mammalian cochlea, gap junctions are formed by connexin 26 (Cx26) and Cx30 (Lautermann et al., 1998). To test the role of gap junctions in the spread of spontaneous Ca\(^{2+}\) activity, we used Cx30\(^{-/-}\) mice (Teubner et al., 2003) in which the mRNA and protein expression of Cx30 are abolished and those of Cx26 are reduced to only ~10% of normal levels during pre-hearing stages (Boulay et al., 2013). Despite the loss of connexins, spontaneous and rapid Ca\(^{2+}\)-dependent signals in developing OHCs were still recorded (Fig 7A). These Ca\(^{2+}\) signals occurred in both apical and basal OHCs as shown in wild-type mice (Fig 1), and the number of active OHCs decreased with age (Fig 7B). Electrophysiological recordings from OHCs of Cx30\(^{-/-}\) mice showed that their resting membrane potential, ability to fire action potentials and size of the K\(^{+}\) currents were not significantly different to those recorded from wild-type cells (Appendix Fig S7). Thus, the intrinsic Ca\(^{2+}\) firing activity in developing OHCs was unaffected by the loss of connexins in the non-sensory cells. In agreement with previous findings (Rodriguez et al., 2012), the average frequency of Ca\(^{2+}\) waves in the GER of Cx30\(^{-/-}\) mice (1.3 ± 0.1 events min\(^{-1}\), n = 36...
Figure 5.

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recordings, 11 cochleae, 11 mice, P1–P2) was significantly reduced compared to that of wild-type mice (2.09 ± 0.21 events min⁻¹, n = 32 recordings, 7 cochleae, 6 mice, P1–P2, P = 0.003, Mann–Whitney U-test). Furthermore, the frequency of the larger Ca²⁺ events (> 75 µm: see Fig 2) required for the synchronization of several OHCs was reduced ~ 5-fold in Cx30⁻/⁻ (0.10 ± 0.03 events min⁻¹, Fig 7C) compared to wild-type mice (0.53 ± 0.11 events min⁻¹, Fig 2, P < 0.0001). The few remaining large Ca²⁺ waves in Cx30⁻/⁻ mice were still able to synchronize the bursting activity of adjacent OHCs (Fig 7C). Similar to wild-type mice (Fig 2G and H), Ca²⁺ signals in OHCs from Cx30⁻/⁻ mice were significantly stronger for larger Ca²⁺ waves (P = 0.0106, t-test; Fig 7D). However, the increased Ca²⁺ signals in OHCs were not significantly different between wild-type (Fig 2H) and Cx30⁻/⁻ (Fig 7D) mice (overall 2-way ANOVA: P = 0.4526; Tukey’s post-test: < 75 µm: P = 0.9972; > 150 µm: P = 0.8866). As seen in wild-type mice (Appendix Fig S2A and B), the f⁰peat was independent of the amplitude (ΔF/F₀) of the Ca²⁺ signal measured as a pixel average over the entire spread of the Ca²⁺ wave (Appendix Fig S2C and D). Overall, these findings show that the Ca²⁺ signalling from non-sensory cells, although not required for generating spontaneous Ca²⁺ activity in OHCs, is crucial for synchronizing this activity.

The biophysical characteristics of OHCs from Cx30⁻/⁻ mice develop normally

In OHCs, the onset of maturation occurs at around P7–P8 when they begin to express a negatively activated K⁺ current Iₖ,n and acquire electromotile activity (Marcotti & Kros, 1999; Abe et al., 2007). The expression of the motor protein prestin (Zheng et al., 2000; Liberman et al., 2002), which drives the somatic motility of OHCs, was normal between wild-type and Cx30⁻/⁻ mice (see Fig 10). The total K⁺ current in mature OHCs was similar between the two genotypes (Fig 8A–C). The maturation of OHCs is also associated with an increase in cell membrane capacitance (Marcotti & Kros, 1999), which was observed in both genotypes (Fig 8C: P = 0.4350). The total outward K⁺ current (Iₖ,o) and the isolated Iₖ,n recorded from OHCs of Cx30⁻/⁻ mice (P10–P12) were similar in size to that of wild-type cells (Iₖ,o: P = 0.8445; Iₖ,n: P = 0.2238, Fig 8C). Mature OHCs are the primary target of the inhibitory olivocochlear efferent fibres that release the neurotransmitter acetylcholine (ACh; Simmons et al., 1996). Efferent inhibition of OHCs by ACh is achieved by Ca²⁺ influx through 99x10-nAChRs activating a hyperpolarizing SK2 current (Oliver et al., 2000; Katz et al., 2004; Lioudyno et al., 2004; Marcotti et al., 2004). Mouse OHCs first become highly sensitive to ACh from around the end of the first postnatal week (Katz et al., 2004; Marcotti et al., 2004), which coincides with their onset of functional maturation (Marcotti & Kros, 1999). In the presence of ACh, depolarizing and hyperpolarizing voltage steps from a holding potential of −84 mV elicited an instantaneous current in wild-type OHCs. This ACh-activated instantaneous current is mainly carried by SK2 channels but also by nAChRs since it is blocked by apamin and strychnine, respectively (Marcotti et al., 2004). The ACh-activated current was present in OHCs from wild-type (Fig 8D) and Cx30⁻/⁻ mice (Fig 8E). The sensitivity of OHCs to ACh was quantified by measuring the steady-state slope conductance at −84 mV of the ACh-sensitive current (Iₖ,ACCh), which was obtained by subtracting the control currents from the currents in the presence of 100 µM ACh (Fig 8D and E; see also Marcotti et al., 2004). Iₖ,ACCh was similar between wild-type (8.4 ± 1.7 nS, n = 4, P12) and Cx30⁻/⁻ (8.4 ± 1.4 nS, n = 6, P10–P12; P = 0.9843). We further confirmed that the ACh-induced currents in Cx30⁻/⁻ OHCs were carried by the nAChRs and SK2 channels since they were blocked by strychnine (at −90 mV: Fig 8F) and a Ca²⁺-free solution (at −40 mV: Fig 8G), respectively, as previously shown in hair cells (Glowatzki & Fuchs, 2000; Oliver et al., 2000; Marcotti et al., 2004).

OHC ribbon synapses and afferent fibres are reduced in Cx30⁻/⁻ and Cav1.3⁻/⁻ mice

The above results demonstrate that pre-hearing Cx30⁻/⁻ mice, in which OHCs retain their intrinsic Ca²⁺ activity but which have reduced and more spatially confined Ca²⁺ waves in the GER, were able to develop functionally mature OHCs. During the same time window (~ P0–P12), immature IHCs from Cx30⁻/⁻ mice have been shown to be normal (Johnson et al., 2017), indicating that Ca²⁺ waves in the non-sensory cells do not interfere with the normal pre-hearing development of hair cells. Indeed, it has been suggested that the modulation of AP activity in IHCs by the Ca²⁺ waves could be used to refine the afferent auditory pathway (Tritsch et al., 2007). Therefore, we made use of the fact that hair cells from Cx30⁻/⁻ mice...
**Figure 6.** P2X₃ receptors are implicated in the ATP-induced modulation of the OHC firing activity.

A, B Representative ΔF/F₀ traces from apical OHCs of wild-type mice (selected from the images in the left panel) in the continuous presence of the non-selective purinergic antagonist PPADS (A, P₂) or the selective P₂X₃ antagonist A317491 (B, P₁). Note the lack of synchronized Ca²⁺ activity in OHCs despite the presence of a large Ca²⁺ wave in the GER.

C, D Correlation matrices computed from the Ca²⁺ fluorescence traces of 75 (C: PPADS) and 70 (D: A317491) OHCs from panel (A and B), respectively. See Fig 2D and E legend for more details.

E, F Average Spearman’s rank correlation coefficient (ravg; see Materials and Methods) between the OHC activity as a function of the longitudinal extension of spontaneous Ca²⁺ waves in the GER from the apical coil of P₁–P₂ mouse cochleae in the presence of 50 µM PPADS (E, 16 recordings, 11 cochleae, 7 mice) or 50 µM A317491 (F, 17 recordings, 7 cochleae, 6 mice, 1,267 OHCs). Grey and black dots are as described in Fig 2. Solid lines represent a linear fit to the data. Slopes were not significantly different from zero [E: (−0.05 ± 0.13)10⁻³ μm⁻¹ P = 0.699; F: (0.02 ± 0.03)10⁻³ μm⁻¹ P = 0.444].

G Voltage responses in whole-cell current clamp from a P₂OHC of a wild-type mouse during the extracellular application of 10 µM ATP alone or together with 10 µM of the P₂X₃ receptor antagonist A317491. A317491 reversibly blocked the ATP-induced OHC depolarization.
are normal during pre-hearing stages to investigate whether the Ca\textsuperscript{2+} signalling in the GER contributes to the refinement of OHC afferent innervation.

The OHC afferent ribbon synapses from wild-type and Cx30\textsuperscript{−/−} mice were investigated before (P4) and after (P10) their onset of functional maturation at ~P8 (Simmons, 1994). At P4, both wild-type and Cx30\textsuperscript{−/−} OHCs showed a similar number of ribbons (\(P = 0.24\), t-test: Fig 9A,B and E; CtbP2 puncta in red; Myo7a, blue, was used as the hair cell marker). In mature OHCs (P10), the number of ribbons in Cx30\textsuperscript{−/−} OHCs was about half of that in wild-type OHCs (\(P < 0.0001\), t-test: Fig 9C,D and E). As a comparison, we also looked at IHCs and found a similar number of ribbons between the two genotypes at both P4 (\(P = 0.30\), t-test: Appendix Fig S8A,B and E) and P10 (\(P = 0.43\), t-test: Appendix Fig S8C,D and E), further supporting the evidence that at this age immature IHCs are unaffected by the absence of Ca\textsuperscript{2+} waves (Johnson et al, 2017). The requirement for OHC Ca\textsuperscript{2+} signals for the maturation of the afferent synapses was further tested by using knockout mice for Ca\textsubscript{v}1.3 Ca\textsuperscript{2+} channels (Ca\textsubscript{v}1.3\textsuperscript{−/−}), which are required for hair cell exocytosis. OHCs in P11 Ca\textsubscript{v}1.3\textsuperscript{−/−} mice are present and healthy (Appendix Fig S9). We found a significant reduction in the number of ribbons at P10 (\(P < 0.0001\), one-way ANOVA; \(P < 0.001\) post-test for wild-type vs. both Ca\textsubscript{v}1.3\textsuperscript{−/−} and Cx30\textsuperscript{−/−} \(P > 0.05\) post-test for Ca\textsubscript{v}1.3\textsuperscript{−/−} vs. Cx30\textsuperscript{−/−}) but not P4 (one-way ANOVA \(P = 0.4497\)) compared to wild-type cells (Fig 9E–G).

We then looked at whether the reduction in ribbon synapses was also associated with abnormalities in the afferent fibres innervating mature OHCs. Prestin was used as the OHC marker (Fig 10A–C). At P11 in the apical cochlear region, afferent fibres from spiral ganglion neurons form outer spiral fibres that terminate on the OHCs after long spiral courses (Simmons & Liberman, 1988). Peripherin has been shown to specifically target type II neurons innervating mature OHCs (Hafidi, 1998; Mou et al, 1998; Maison et al, 2016). We found that type II fibres show peripherin immunoreactivity and course radially from the spiral ganglion to the organ of Corti, cross along the floor of the tunnel of Corti and spiral in a basal direction before giving rise to punctate endings on OHCs (Fig 10D). In the apical region, there were 14.0 ± 2.0 (mean ± SD, \(n = 3\) mice)
tunnel-crossing outer spiral fibres per 100 μm along the length of the organ of Corti. Compared to wild-type, Cx30\(^{−/−}\) mice had fewer peripherin-labelled outer spiral fibres (Fig 10E), which was matched by a reduction in labelled fibres crossing the tunnel of Corti (7.0 ± 1.0 fibres per 100 μm, n = 3 animals) compared to wild-type mice (P < 0.01). This result is in agreement with the significantly lower number of ribbon synapses in OHCs from Cx30\(^{−/−}\) mice (Fig 9E). We also found that the cochlea from Cav1.3\(^{−/−}\) mice had a lower number of ribbon synapses in OHCs from Cav1.3\(^{−/−}\) mice (P < 0.05) compared to wild-type mice and E), outer spiral fibres in Cav1.3\(^{−/−}\) mice spiralled in both apical and basal directions (Fig 10F). The above results provide evidence that the maturation of OHCs ribbon synapses and associated afferents is, at least in part, influenced by the synchronized Ca\(^{2+}\) signals in OHCs caused by the Ca\(^{2+}\) waves originating in the GER.

**Discussion**

We have identified distinct, coordinated Ca\(^{2+}\)-dependent mechanisms that influence the refinement of OHC innervation. Our evidence shows that the morphological maturation of the afferent synapses and innervation of OHCs requires spontaneous ATP-induced Ca\(^{2+}\) waves in the non-sensory cells of the GER, which increase and synchronize the Ca\(^{2+}\) activity between several OHCs. Similar Ca\(^{2+}\) signalling mechanisms are used to drive the maturation of IHCs (Johnson et al, 2013), but they do not appear to be required for the refinement of the pre-synaptic ribbons and post-synaptic afferents. Moreover, in contrast to the ATP-dependent activity modulating IHC action potentials (Wang et al, 2015), that influencing OHC Ca\(^{2+}\) signals is mediated by ATP-induced activation of p2X receptors, which has distinct functional consequences for OHC maturation and is separated by developmental timing, with OHCs preceding IHCs (Johnson et al, 2011, 2017). The data suggest that several distinct patterns of spontaneous, experience-independent Ca\(^{2+}\) activity across the auditory sensory epithelium orchestrate the differential maturation of OHCs (afferent innervation) and IHCs (sensory cells Johnson et al, 2013) to shape the final stages of auditory organ development.

**OHC activity is synchronized by ATP-induced Ca\(^{2+}\) signalling in non-sensory cells**

We show that spontaneous intercellular Ca\(^{2+}\) signalling activity originating in the non-sensory cells of the greater epithelial ridge (GER) synchronizes Ca\(^{2+}\) activity between nearby OHCs via release of ATP from Deiters’ cells. This ATP acts directly via P2X receptors on the OHCs from Deiters’ cells. This ATP acts directly via P2X receptors on the OHCs from Deiters’ cells. This ATP acts directly via P2X receptors on the OHCs from Deiters’ cells. This ATP acts directly via P2X receptors on the OHCs from Deiters’ cells.
autoreceptors expressed in the non-sensory cells surrounding the IHCs, which leads to the opening of TMEM16A Ca2+-activated Cl−/C0 channels and the efflux of K+ in the intercellular space (Wang et al., 2015). The expression of these TMEM16A channels seems to follow closely the development of IHCs, and they are absent in the LER (Wang et al., 2015). Although P2X2 are the most abundant purinergic receptors in the cochlea, they are mainly expressed in hair cells from the second postnatal week onward throughout adult stages (Ja¨rlebark et al., 2000), so they are unlikely to mediate the ATP-induced signalling in developing OHCs. P2X4 receptors have been suggested to be present in the developing cochlea based on pharmacological assays, but these findings have not been confirmed with expression studies (Lahne & Gale, 2010). Of the other known P2X receptors, only P2X3 (Huang et al., 2006) and P2X7 (Nikolic et al., 2003) have been shown to be transiently expressed during early stages of development. The expression time course of P2X3 receptors seems to match our Ca2+-imaging experiments (Fig 1), since a previous study has shown that by P3 they are still present in apical, but no longer in basal OHCs (Huang et al., 2006). Indeed, our pharmacological and imaging experiments (Fig 6) demonstrated that P2X3 receptors play a crucial role in mediating the modulation of OHC activity by Ca2+ waves originating in the GER. Interestingly, P2X3

Figure 9. Ribbon synapses are reduced in Cx30−/− and CaV1.3−/− OHCs.
A–D Maximum intensity projections of confocal z-stack images that were taken from apical OHCs before (P4) and after (P10) their onset of functional maturation at P8 in wild-type (A and C) and Cx30−/− (B and D) mice. Immunostaining for ribbon synapses (CtBP2) is shown in red; Myo7a (blue) was used as the hair cell marker.
E, F Maximum intensity projections as in (A–D) from apical-coil OHCs at P4 (E) and P10 (F) of CaV1.3−/− mice.
G Number of ribbons (CtBP2 puncta) in wild-type, Cx30−/− and CaV1.3−/− OHCs at P4 and P10. Values are mean ± SEM. Number of OHCs analysed is shown above each average data point; four mice were used for each experimental condition. *** indicates P < 0.001, one-way ANOVA, Bonferroni post-test. Scale bars 10 μm.
receptors have previously been implicated in early development in the peripheral and central nervous system (Kidd et al., 1998).

Although ATP-induced Ca\(^{2+}\) signalling from non-sensory cells is crucial for promoting the maturation of IHCs after the onset of hearing (Johnson et al., 2017), our data suggest that it does not contribute directly to OHC maturation. However, the time course of maturation of OHCs and IHCs is very different (Knirsch et al., 2007; Corns et al., 2014). During the time over which OHCs become sensory competent, IHCs are still immature and fire Ca\(^{2+}\) action potentials that are thought to drive the functional refinement of the auditory pathway, which mainly occurs during the first week of postnatal development (tonotopic organization in the brainstem: Snyder & Leake, 1997; Kim & Kandler, 2003; spiral ganglion neuron survival: Zhang-Hooks et al., 2016; spiral ganglion neuron subtype refinement: Shrestha et al., 2018; Sun et al., 2018). However, recent results have also indicated that the neuronal diversification process of type I SGNs is already established at birth in mice and as such it is independent of electrical activity (Petitpré et al., 2018). IHCs begin to mature towards the end of the second postnatal week (at the onset of hearing), indicating that Ca\(^{2+}\) waves from the non-sensory cells are likely to drive different signals to the hair cells during early and later stages of pre-hearing development. There are thus temporally distinct critical windows for the influence of spontaneous Ca\(^{2+}\) activity in non-sensory cells on IHCs and OHCs.

OHC afferent innervation is shaped by ATP-induced intercellular Ca\(^{2+}\) signalling in non-sensory cells

In the cochlea, the onset of OHC function is associated with type II spiral ganglion afferent terminals forming extensive arborizations with several OHCs (Perkins & Morest, 1975; Echteler, 1992). Unlike type I afferent fibres contacting IHCs, type II afferent fibres seem to respond only to the loudest sounds (Robertson, 1984; Brown, 1994), which has led to the assumption that they represent the cochlear nociceptors (Weisz et al., 2009; Liu et al., 2015). Our imaging experiments show that large spontaneous Ca\(^{2+}\) waves originating in the GER (Fig 2F) are able to increase and synchronize bursting activity between OHCs. Considering that the average spiral processes of type II fibres span 215 µm (Weisz et al., 2012; Martinez-Monedero et al., 2016) and that they contact more than a dozen OHCs (Perkins & Morest, 1975), these Ca\(^{2+}\) waves should be sufficient to increase the Ca\(^{2+}\) activity of most of the pre-synaptic, immature OHCs that form synapses with each developing afferent fibre. Since OHCs provide an infrequent and weak synaptic input to type II afferent fibres, their suprathreshold excitation would require the summation of the input coming from all OHCs contacting each fibre (Weisz et al., 2009, 2012), which in the developing cochlea could be provided by the Ca\(^{2+}\) waves originating in the GER. The synchronized activity among nearby OHCs would lead to periodic stimulation of the type II afferent fibres and the activity-dependent refinement of synaptic connections as also seen in the visual system (Katz & Shatz, 1996; Spitzer, 2006). Indeed, we found that the absence of connexins in non-sensory cells, which reduces the frequency and spatial extent of the Ca\(^{2+}\) waves and as such OHC synchronization (Cx30\(^{-/-}\) mice: Fig 7), leads to a reduced number of ribbon synapses and type II afferent fibres. This finding was also supported by similar results in Cav1.3\(^{-/-}\) mice, in which OHCs are unable to drive vesicle fusion at their pre-synaptic site. A similar phenotype in the type II afferent innervation was also seen in mice lacking Deiters’ cells (Mellado Lagarde et al., 2013), corroborating our finding that these non-sensory

![Image 10: Afferent fibres are reduced in Cx30\(^{-/-}\) and Cav1.3\(^{-/-}\) mice.](image-url)
cells are crucial for the transfer of information from the GER to the OHCs.

In summary, we propose that in the immature mammalian cochlea, the refinement of the OHC afferent innervation pattern is caused by the increased and synchronized Ca\(^{2+}\) activity between neighbouring OHCs, which is provided via Deiters’ cells from large Ca\(^{2+}\) waves originating in the GER. Overall, our results reveal extraordinary physiological regulation of spontaneous Ca\(^{2+}\) signalling in the developing cochlea over discrete and separate time periods, to ensure the correct functional differentiation of neuronal and sensory cells in the maturing auditory system.

Materials and Methods

Ethics statement

The majority of the animal studies were performed in the UK and licensed by the Home Office under the Animals (Scientific Procedures) Act 1986 and were approved by the University of Sheffield Ethical Review Committee. Some experiments were performed in the USA, and the animal work was licensed by the Baylor University IACUC (Institutional Animal Care and Use Committee) as established by U.S. Public Health Service.

Tissue preparation

Apical- and basal-coil OHCs from wild-type mice or transgenic mice of either sex were studied in acutely dissected organs of Corti from postnatal day 0 (P0) to P13, where the day of birth is P0. Transgenic mice include Cx30\(^{-/-}\) (MGI:2447863; Teubner et al, 2003) and Ca\(_{v}1.3\)\(^{-/-}\) mice (Platzer et al, 2000). The genotyping protocols for these transgenic mice were performed as previously described (Platzer et al, 2000; Teubner et al, 2003). Mice were killed by cervical dislocation, and the organ of Corti dissected in extracellular solution composed of (in mM): 135 NaCl, 5.8 KCl, 1.3 CaCl\(_2\), 0.9 MgCl\(_2\), 0.7 NaH\(_2\)PO\(_4\), 5.6 D-glucose and 10 Hepes-NaOH (pH 7.5; 308 mmol kg\(^{-1}\)). Sodium pyruvate (2 mM), amino acids and vitamins were added from concentrates (Thermo Fisher Scientific, UK). The pH was adjusted to 7.5 (osmolality was 294 mmol kg\(^{-1}\)). In the experiments designed to investigate the effect of extracellular ATP, Na\(_2\)ATP was omitted from the above solution. For cell-attached recordings, the pipette contained (in mM): 140 NaCl, 5.8 KCl, 1.3 CaCl\(_2\), 0.9 MgCl\(_2\), 0.7 NaH\(_2\)PO\(_4\), 5.6 D-glucose and 10 Hepes-NaOH (pH 7.5; 308 mmol kg\(^{-1}\)). Data acquisition was controlled by pCLAMP software (RRID:SCR_011323) using Digidata 1320A, 1440A or 1550 boards (Molecular Devices, USA). Recordings were low-pass filtered at 2.5 kHz (8-pole Bessel) and sampled at 5 kHz and stored on computer for offline analysis (Origin: OriginLab, USA, RRID:SCR_002815). Membrane potentials in whole-cell recordings were corrected for the residual series resistance \(R_s\) after compensation (usually 70–90%) and the liquid junction potential (LJP) of −4 mV measured between electrode and bath solution. The extracellular application of a Ca\(^{2+}\)-free solution or solutions containing 40 mM KCl, ATP (Tocris Bioscience, UK) or acetyicholine (Sigma-Aldrich, UK) was performed with a multibarrelled pipette positioned close to the patched cells.

Two-photon confocal Ca\(^{2+}\) imaging

For calcium dye loading, acutely dissected preparations were incubated for 40 min at 37°C in DMEM/F12, supplemented with Fluo-4 AM (final concentration 10–20 μM; Thermo Fisher Scientific). The incubation medium contained also pluronic F-127 (0.1%, w/v, Sigma-Aldrich, UK) and sulfinipyrazone (250 μM) to prevent dye sequestration and secretion (Corns et al, 2018). Preparations were then transferred to the microscope stage and perfused with extracellular solution for 20 min before imaging to allow for de-esterification.

Ca\(^{2+}\) 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 800 nm, 80-MHz pulse repetition rate and <100-fs pulse width (Mai Tai HP DeepSee, Spectra-Physics, USA). Images were formed by a 60× objective, 1.1 NA (LUMFLN60XW, Olympus, Japan) using a GaAsP PMT (Hamamatsu) coupled with a 525/40 bandpass filter (FF02-525/40-25, Semrock). Images were analysed offline using custom-built software routines written in Python (Python 2.7; Python Software Foundation, RRID:SCR_014795) and ImageJ (NIH) (Schindelin et al, 2012). Ca\(^{2+}\) signals were measured as relative changes of fluorescence emission intensity \((ΔF/F_0)\). \(ΔF = F - F_0\), where \(F\) is fluorescence at time \(t\) and \(F_0\) is the fluorescence at the onset of the recording.

The extracellular application of solutions containing ATP, ryanodine, the P2X antagonist suramin and PPADS (Tocris), the P2Y agonist UTP (Sigma, UK), the phospholipase C inhibitor U73122 (Tocris Bioscience, UK) and the P2X\(_3\) antagonist A-317491 (Sigma) was performed using a Picospritzer or bath application. The pipettes used for local perfusion (diameter 2–4 μm) were pulled from borosilicate glass using a two-step vertical puller (Narishige, USA; Axopatch 200B). The extracellular dislocation, and the organ of Corti dissected in extracellular solution were normalized to control experiments, carried out on the same day under the same imaging and dye-loading conditions.

Each fluorescence recording consisted of 4,000 frames taken at 30.3 frames per second from a 125 × 125 μm \((512 \times 512\) pixels\) region. OHC fluorescence traces were computed as pixel averages
from square ROIs (side = 3.7 µm) centred on each OHC. OHCs were classified as either active or inactive using the following algorithm: (i) imaging traces were smoothed using a moving average temporal filter of length 3. (ii) Slow Ca\textsuperscript{2+} variations and the exponential decay in fluorescence due to photo-bleaching were removed by subtracting a polynomial fit of order 5 to each trace. Detrended traces were normalized to the maximum value in the recording. (iii) The noise floor level was estimated by calculating the power spectral density of the signal using Welch’s method and averaging over the large frequencies (greater than 66% of the Nyquist frequency). (iv) A spike inference algorithm [spikes (Pnevmatikakis et al., 2016); module in the SIMA python package (Kaifosh et al., 2014)] was used to estimate the (normalized) spike count $s$. We then calculated the cumulative spike count $S = \sum s_i$ for each trace and considered the cell as active (inactive) if $S$ was above (below) a predetermined threshold. (v) Cells that were classified as active (or inactive) and had a maximum signal below (or above) 4 standard deviations were manually sorted. (vi) The entire dataset was independently reviewed by two experimenters. Cells that had discordant classification based on the above criteria (69 out of 2,229 at body temperature and 30 out of 5,217 at room temperature) were removed from the analysis. For the experiments in which we calculated the Ca\textsuperscript{2+} spike frequency from Ca\textsuperscript{2+} imaging data (Appendix Fig S1E), we first estimated the number of spikes from the posterior marginal distribution of 1,000 samples of spike trains produced by the Markov chain Monte Carlo (MCMC) spike inference algorithm described in Pnevmatikakis et al. (2016). The average frequency was then computed by dividing the number of spikes by the total duration of the recording (133 s).

For recording spontaneous activity in the GER, we increased the field of view to a 182 × 182 µm region, which was dictated by the ability to detect the full extension of a Ca\textsuperscript{2+} wave in the GER and to maintain a sufficient spatial resolution to resolve the activity of individual OHCs with good signal-to-noise ratio. Under these conditions, the average length of apical coil used for these experiments was 188 ± 4 µm, since some preparations were positioned diagonally in the field of view. Under this recording condition, some large Ca\textsuperscript{2+} waves were underestimated because they travelled beyond the field of view. Time-series images were corrected for motion using a rigid-body spatial transformation, which does not distort the image (spm12; www.fil.ion.ucl.ac.uk/spm). Recordings showing large drifts of the preparation were discarded from the analysis to avoid potential artefacts in the computation of correlation. Calcium waves were manually identified using thresholding, and a ROI was drawn around the maximum extension of each multicellular calcium event. Only events that initiated within the field of view of the microscope were considered for this analysis. GER fluorescence traces were computed as ROI pixel averages, and as such they give an indication of the average cytosolic calcium increase in non-sensory cells participating in the propagation of the Ca\textsuperscript{2+} wave. To measure the degree of correlation between OHCs during Ca\textsuperscript{2+} activity in the GER, we first computed the pairwise Spearman’s rank correlation coefficient $r_s$ between every pair of OHCs in the field of view (Fig 2D and E). We then averaged $r_s$ using Fisher’s z-transformation

$$z = \text{arctanh}(r_s)$$

$$r_s^{\text{avg}} = \text{tanh}(z)$$

We take $r_s^{\text{avg}}$ as a measure of the average degree of coordination of the activity of neighbouring OHCs.

To test for the increase in coordinated OHC activity, we used the Mann–Whitney U-test (one sided) to check whether OHC correlation coefficients during spontaneous Ca\textsuperscript{2+} activity in the GER were significantly ($P < 0.001$) greater than those computed over a time window of 13.2 s (400 frames) during which no Ca\textsuperscript{2+} waves were observed in the GER.

To quantify the change in OHC activity during the Ca\textsuperscript{2+} waves in non-sensory cells, we measured the integral of the Ca\textsuperscript{2+} trace in the same 400 frames (see above) in the absence of Ca\textsuperscript{2+} waves (baseline) and during Ca\textsuperscript{2+} waves. Traces were smoothed using the Savitzky–Golay filter (window length = 11, polynomial order = 1) and normalized to the baseline $F_0$ before computing the integral.

Photo-damage-induced Ca\textsuperscript{2+} waves were triggered by applying high-intensity laser pulses using a second mode-locked laser system operating at 716 nm (Mai Tai HP, Spectra-Physics, USA). The laser was merged into the excitation light path using a long-pass dichroic mirror (FF773-Di02, Semrock) and focused on the preparation through the imaging objective (LUMFLN60XW, Olympus, Japan). Two galvanometric mirrors were used to steer the laser beam across the photo-damage area (6.6 × 8.4 µm), which typically comprised one or two non-sensory cells of the GER. The number of repetitions, and thus the total amount of energy delivered, was set to the minimum able to trigger a Ca\textsuperscript{2+} wave (typically five repetitions, lasting 165 ms in total).

**Immunofluorescence microscopy**

Dissected inner ears from wild-type and Cx30\textsuperscript{−/−} and Cx41.3\textsuperscript{−/−} mice (n ≥ 3 for each set of experiment) were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) for 5–20 min at room temperature. Cochleae were microdissected, rinsed three times for 10 min in PBS and incubated for 1 h at room temperature in PBS supplemented with 5% normal goat or horse serum and 0.3% Triton X-100. The samples were then incubated overnight at 37°C with the primary antibody in PBS supplemented with 1% of the specific serum. Primary antibodies were as follows: mouse anti-myosin7a (1:1,000, DSHB, #138-1), rabbit anti-myosin7a (1:200, Proteus Biosciences, #25-6790), rabbit anti-peripherin (#AB1530, 1:200, Millipore), mouse anti-CtBP2 (1:200, Biosciences, #612044) and rabbit anti-prestin (1:2,000, kindly provided by Robert Fettkether). All primary antibodies were labelled with species appropriate Alexa Fluor secondary antibodies for 1 h at 37°C. Samples were then mounted in VECTASHIELD. The z-stack images were captured either with a LSM 800 with Airyscan (Carl Zeiss) system with GaAsP detectors or with a Nikon A1 confocal microscope. Image stacks were processed with Fiji Image Analysis software.

**Statistical analysis**

Statistical comparisons of means were made by Student’s two-tailed t-test or, for multiple comparisons, analysis of variance (one-way or 2-way ANOVA followed by Bonferroni’s test) and Mann–Whitney U-test (when normal distribution could not be assumed) were applied. $P < 0.05$ was selected as the criterion for statistical significance. Mean values are quoted in text and figures as means ± SEM (electrophysiology and imaging) and ±SD (fi cle counting). Only mean
values with a similar variance between groups were compared. Animals of either sex were randomly assigned to the different experimental groups. No statistical methods were used to define sample size, which was defined based on previous published similar work from our laboratory. Animals were taken from multiple cages and breeding pairs. The electrophysiological and morphological (but not imaging) experiments were performed blind to animal genotyping.

Expanded View for this article is available online.

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Author contributions
FC, AH, J-YJ, SLJ, FS, JE, JD, DDS and WM collected and analysed the data. All authors helped with the interpretation of the results. FC, AH, MCH, CJK, DDS and WM wrote the paper. FM helped with the design of the 2-photon imaging system. WM conceived and coordinated the study. SLJ is a Royal Society University Research Fellow.

Conflict of interest
The authors declare that they have no conflict of interest.

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