JARO

REVIEW

Spontaneous Calcium Signalling in the Developing Mammalian Cochlea

Federico Ceriani[1] · Walter Marcotti[1,2]

Abstract

In mammals, the accurate and high-fidelity representation of sound largely depends on the cochlea, the sensory organ specialized for transducing acoustic signals into neural activity with remarkable temporal precision. Prior to hearing onset, which occurs around postnatal day 12 in most altricial rodents, the immature cochlea plays an active role in the refinement of neural circuits along the auditory pathway. To accomplish this function, sensory hair cells and glia-like supporting cells in the immature cochlea generate distinct patterns of spontaneous Ca^{2+} signals. Synchronized Ca^{2+}-dependent activity across multiple hair cells is conveyed to the ascending auditory neurons, where it contributes to the emergence of tonotopic maps that enable frequency discrimination. Within the cochlea itself, this spontaneous Ca^{2+} activity serves to promote cellular and synaptic refinement. In this review, we summarize the current insights into the cellular and molecular mechanisms responsible for generating and modulating these spontaneous Ca^{2+} signals in the developing cochlea, and how they regulate the activation of auditory afferent fibres.

Introduction

The mammalian auditory system is finely tuned to detect acoustic stimuli with remarkable precision and sensitivity. This capability emerges from the integrated function of specialized morphological structures, such as the basilar and tectorial membranes [1, 2], and the different cell types within the cochlea [3]. The sensory epithelium of the mammalian cochlea, the organ of Corti, contains a highly organized array of specialized sensory hair cells (inner and outer hair cells: IHCs and OHCs: Figs. 1,2) and glia-like supporting cells.

The IHCs serve as the primary sensory receptors, transmitting acoustic information to the central auditory pathway via type I afferent fibres [19], which represent the majority of the spiral ganglion neurons (SGNs) innervating the cochlea (~95 %) [20]. In the adult cochlea, type I SGNs form one-to-one axosomatic contact with a given IHC [21], and are directly modulated by the lateral olivocochlear (LOC) efferent system (Fig. 1) [22]. Unlike IHCs, the role of OHCs is to enhance cochlear sensitivity and frequency selectivity through somatic electromotility [23]. Adult OHCs are directly innervated by the medial olivocochlear (MOC) cholinergic efferent neurons (Fig. 2), which reduce cochlear amplification [22, 24]. OHCs are also sparsely contacted by type II afferent fibres (Fig. 2), constituting the remaining ~5 % of SGNs. These type II SGNs form extensive spiralling arborizations with numerous OHCs and are thought to be activated by noxious sound stimulations [25–27], possibly through ATP-gated P2x7 receptors [28]. The cochlear epithelium also contains a diverse population of non-sensory supporting cells that maintain the structural and functional integrity of the organ of Corti [29, 30]. Beyond their roles in the adult cochlea, both sensory and supporting cells have been shown to regulate the functional maturation of the developing cochlea and the central auditory pathway prior to hearing onset.

✉ Walter Marcotti
w.marcotti@sheffield.ac.uk

Federico Ceriani
f.ceriani@sheffield.ac.uk

1 School of Biosciences, University of Sheffield, S10 2TN, Sheffield, UK

2 Neuroscience Institute, University of Sheffield, S10 2TN, Sheffield, UK
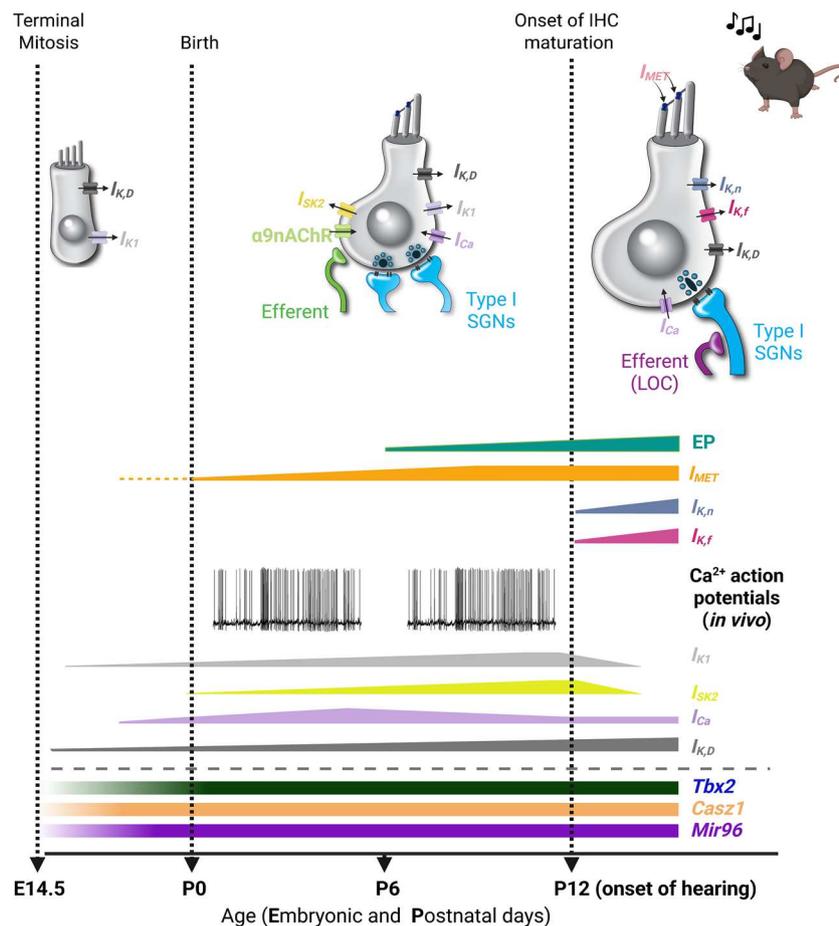
Fig. 1 Functional maturation of IHCs from the apical coil of the mouse cochlea. Diagram depicting some of the biophysical and morphological changes during IHC development. Note that similar changes occur in the basal cochlea, but with the onset shifted a few days earlier. The day of birth (P0) corresponds to E19.5 [4]. The maturation of IHCs into functional auditory receptors begins around P12. The shaded bars (bottom) represent the temporal expression of some of the known genes involved in IHC functional differentiation (*Tbx2*, *Casz1* and *Mir96*) [5–7]. The height of the horizontal bars indicates relative changes in the size of the different basolateral membrane currents, the mechanoelectrical transducer current (I_{MET}), and endocochlear potential (EP). During embryonic development, IHC total basolateral current is dominated by the inward rectifier (I_{K1}) and delayed-rectifier ($I_{K,D}$) K$^+$ currents. At this stage, afferent and efferent fibres extend within the sensory epithelium, but do not contact IHCs yet. Just before birth, IHCs begin to show a small Ca^{2+} current (I_{Ca}), which drives spontaneous Ca^{2+}-dependent action potentials (evidence from ex vivo work: [4, 8]). In vivo recordings showed that IHCs generate spontaneous Ca^{2+}-dependent transients (i.e. action potentials) throughout the pre-hearing stages of development [9, 10]. Postnatally, IHCs are transiently innervated by the efferent system, which includes the post-synaptic channels carrying the small conductance Ca^{2+}-activated K$^+$ current (I_{SK2}) and a current mediated by α9α10 nicotinic acetylcholine receptors (nAChRs) [11–14]. The mechanoelectrical transducer current (I_{MET}) appears at or just after birth. Around P0, both afferent and the transient efferent fibres establish synaptic contact with IHCs. During this time, type I SGN afferent fibres undergo pruning. Adult-type basolateral membrane currents (large conductance Ca^{2+}-activated $I_{K,f}$, and the negatively activating $I_{K,n}$) appear from around P12. Type I SGNs form one-to-one connections with IHCs, and the efferent system (LOC) forms synapses on the afferent terminals

As in other sensory systems [31], while the initial wiring of the auditory pathway depends on axon guidance molecules [32], circuit refinement is driven by spontaneous, patterned electrical activity that arises before the onset of external sensory input [33–35]. Indeed, neurons along the auditory pathway exhibit periodic bursts of spontaneous action potentials prior to hearing onset [36–38]. Multiple lines of evidence indicate that this spontaneous activity originates in the immature cochlea, as ablation of the sensory organ silences auditory neurons and disrupts their normal maturation [36, 39, 40]. This sensory-independent firing activity in the pre-hearing cochlea has been shown to be Ca^{2+}-dependent and is required not only for glutamate release from hair cell ribbon synapses onto afferent terminals but also for regulating cochlear development. In this review, we summarize the current understanding of the origin and modulation of spontaneous Ca^{2+}-dependent activity
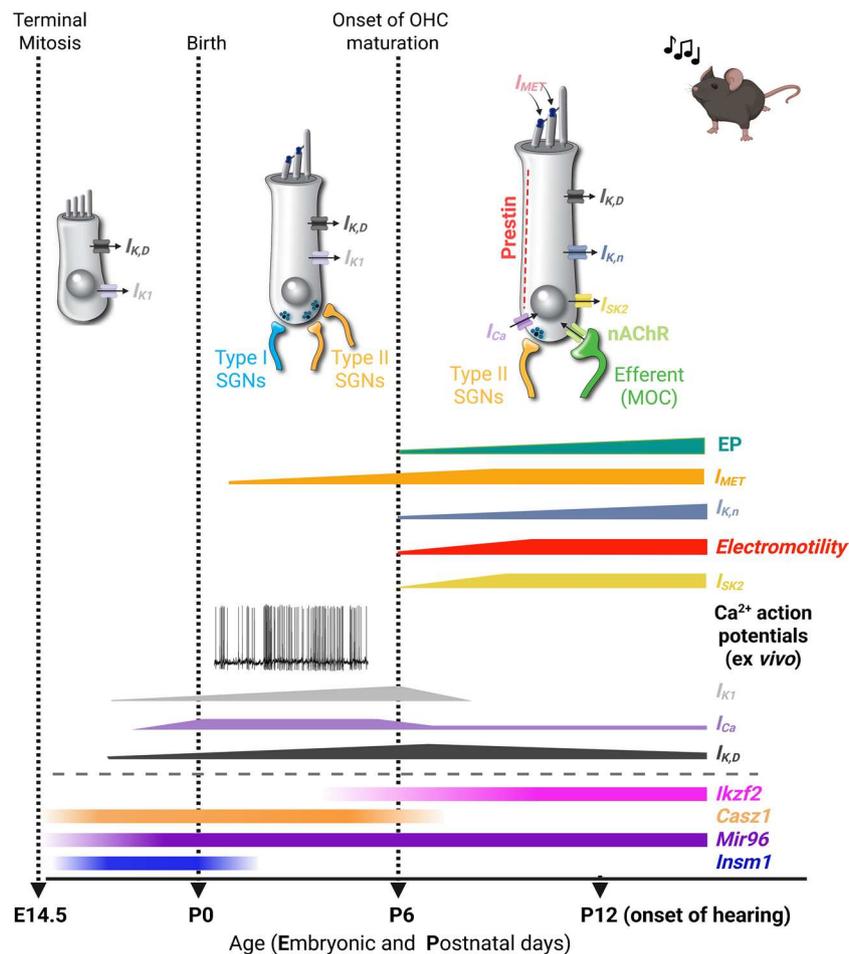
Fig. 2 Functional maturation of apical-coil OHCs of the mouse cochlea. This diagram follows the format of Fig. 1 but depicts the development of OHCs. The maturation of OHCs into functional auditory receptors begins around P6-P8. Shaded bars (bottom): expression of some of the known genes involved in OHC functional differentiation (*Insm1*, *Casz1*, *Mir96* and *Ikzf2*) [5, 7, 15]. During embryonic development, OHCs primarily exhibit the inward rectifier (I_{K1}) and delayed rectifier ($I_{K,D}$) K$^+$ currents. At this stage, afferent and efferent fibres extend within the sensory epithelium, but do not contact OHCs yet. Just before birth, OHCs begin to show a small Ca^{2+} current (I_{Ca}). Unlike IHCs (Fig. 1), early postnatal OHCs do not exhibit additional basolateral membrane currents; however, the increased size of I_{Ca} likely drives spontaneous Ca^{2+}-dependent transients (i.e. action potentials) for a brief period (from ex vivo work: [16]). I_{MET} appears

starting around P1-P2. During this period, OHCs are contacted by both type I and type II SGNs. By the end of the first postnatal week, type I SGNs undergo pruning, and type I fibres are eliminated. The onset of functional maturation in OHCs is marked by the appearance of electromotile activity, which is driven by the motor-protein prestin located along the OHC lateral wall [17]. *Ikzf2* (see above), which encodes Helios, has been shown to be required for prestin expression. At the same time, the negatively activating delayed rectified K$^+$ current $I_{K,n}$ [18], is upregulated in maturing OHCs. Concurrent with the above basolateral changes, OHCs upregulate the efferent post-synaptic machinery, which includes the small conductance Ca^{2+}-activated K$^+$ current (I_{SK2}) and α9α10 nicotinic acetylcholine receptors (nAChRs). In the mature configuration, OHCs are innervated by both the efferent MOC system and type II SGNs

in the cochlea and discuss its role in establishing the mature sensory epithelium.

Functional Maturation of IHCs and OHCs

During embryonic stages, the development of the mammalian cochlea is guided by intrinsic genetic programmes [41, 42]. In mice, terminal mitosis begins at the cochlear base around embryonic day 12 (E12) and progresses towards

the apex over the following few days [43]. Since the onset of hearing occurs around postnatal day 12 (P12) in most altricial rodents, pro-sensory cells have approximately three weeks to functionally differentiate into sensory receptors capable of processing acoustic information (Figs. 1, 2). Within this developmental window, a combination of genetically programmed and physiologically driven mechanisms is required to generate functionally mature auditory receptors [41, 42, 44].

The initial differentiation between IHCs and OHCs is determined during embryonic stages by several transcription factors (INSM1, CASZ1, and TBX2) and the non-coding microRNA 96, which play a key role in consolidating the identity of both cell types (Figs. 1, 2) (recently reviewed by [45]). The final stage of hair cell maturation occurs postnatally with the acquisition of adult-like biophysical and morphological characteristics. The onset of IHC functional maturation, which occurs at about P10-P12, is marked by the appearance of the K^+ current $I_{K,n}$ and the fast-activating BK current $I_{K,f}$ (Fig. 1) [4, 46, 47]. IHC maturation also coincides with the progressive removal of their efferent innervation and the downregulation of the IHC post-synaptic components (Fig. 1) (recently reviewed by [45]). Immature IHCs are also innervated by highly branched type I SGNs via ribbon synapses. Synaptogenesis in IHCs mainly occurs during postnatal stages of development, through a range of structural and functional refinements [19, 48], including the assembly of the pre-synaptic active zones [49, 50], which eventually establish the characteristic and highly specialized monosynaptic axosomatic contact (Fig. 1). In OHCs, the onset of functional maturation at about P6-P8 is characterized by the appearance of electromotile activity and, similar to IHCs, the expression of $I_{K,n}$ (Fig. 2) (recently reviewed by [45]). While OHC somatic electromotility drives sound amplification within the cochlear partitions [51, 52], $I_{K,n}$ is crucial for setting the resting membrane potential [18], a role that in immature OHCs is fulfilled by the delayed rectifier ($I_{K,D}$) and the inward rectifier (I_{K1}) K^+ currents (Fig. 2) [18, 53]. Concurrently, the MOC efferent system establishes synaptic contacts with OHCs, a process that is accompanied by the expression of SK2 channels and α9α10-nAChRs at the postsynaptic basolateral membrane [22] (Fig. 2). The refinement of immature afferent innervation leads to a characteristic pattern consisting of type II fibres contacting several OHCs [25, 26].

Calcium Signals in Developing Cochlear Inner Hair Cells

Spontaneous activity in the developing cochlea has been extensively studied in mice and rats using *ex vivo* explants of the sensory epithelium. These pioneering studies showed that IHCs generate spontaneous and evoked Ca^{2+}-dependent action potentials prior to hearing onset (Fig. 1) [8, 46, 54–56]. These action potentials are elicited by an inward Ca^{2+} current flowing through $Ca_V1.3$ channels (I_{Ca}: Fig. 1 and Fig. 3a-d), which account for >90 % of the total Ca^{2+} current in IHCs [58]. Although pre-hearing IHCs also express a TTX-sensitive Na^+ current, this is not required for initiating action potentials but, instead, it modulates their frequency by shortening the time to reach threshold [8].

Action potential repolarization is mainly driven by a delayed rectifier K^+ current ($I_{K,D}$) [4, 46], an inward rectifier K^+ current (I_{K1}) [53] and a transiently expressed small conductance Ca^{2+}-activated K^+ current (I_{SK2}) [11–13] (Fig. 1).

Although *ex vivo* experiments have been invaluable for elucidating the biophysics underpinning Ca^{2+} action potentials in developing IHCs, they have been less effective in identifying the cellular mechanisms responsible for initiating and modulating this activity under *in vivo* conditions. For example, early studies showed that IHCs generate spontaneous action potentials only up to the end of the first postnatal week (Fig. 1). From the second postnatal week until hearing onset, IHCs maintained in cochlear explants undergo a progressive hyperpolarization of their resting membrane potential, reaching values more negative than the activation threshold of I_{Ca} (more negative than −65 mV) [59]. This shift, which is driven by the developmental increase in the size of I_{K1} (Fig. 1) [53], prevents spontaneous Ca^{2+} spiking. Consequently, in cochlear explants from mice aged P7-P12, action potentials could only be evoked by artificial depolarization of IHCs [4, 57, 60]. However, additional *ex vivo* work has shown that IHCs are intrinsically silent throughout pre-hearing development. Instead, Ca^{2+} action potentials in IHCs are driven by spontaneous ATP release from supporting cells [61, 62].

The origin of these Ca^{2+} action potentials has recently been addressed using an *in vivo* experimental approach that allowed the measurements of spontaneous Ca^{2+} signals in the developing cochlea in anaesthetised live mice expressing GCaMP in IHCs [9, 10]. This in vivo study demonstrated that IHCs can generate sporadic spontaneous Ca^{2+} transients (Fig. 3e,f) throughout pre-hearing stages of development, independently of supporting cell activation (Fig. 1) [62]. The discrepancy between *ex vivo* and *in vivo* conditions is likely due to the absence in cochlear explants of the distinct ionic compositions of the endolymph and perilymph [63], and consequently the lack of the endocochlear potential [64], which starts to appear from around the end of the first postnatal week (Fig. 1). The relatively low-Ca^{2+} concentration in the endolymph (~300 μM in prehearing mice) [60], combined with the developing endocochlear potential (~15–40 mV, P6-P10) [9, 64], likely promotes the opening of MET channels that are already functional during pre-hearing stages. This drives an inward depolarizing MET current into the immature IHCs (I_{MET}: Fig. 1), which contributes to setting the resting membrane potential of pre-hearing IHCs near the activation threshold of Ca^{2+} channels [59], thereby promoting spontaneous action potential activity. Furthermore, unlike cochlear explants, the *in vivo* approach preserves another critical aspect of cochlear IHC function, which is the inhibitory efferent cholinergic input originating in the brainstem, which transiently innervates IHCs during immature stages of development [11–14].

Spontaneous Activity in Developing OHCs

In the mouse cochlea, the onset of OHC functional maturation occurs at around P6-P7, nearly a week earlier than IHCs (Fig. 2). However, during the first postnatal week, whole-cell patch-clamp recordings from OHCs in cochlear explants have shown that action potentials can be elicited either by membrane depolarization [18] or by using highly elevated extracellular Ca^{2+} concentrations (10 mM) [65]. From P7 onwards, the presence of the outward K^+ current $I_{k.n}$ (Fig. 2), which is largely active at the resting membrane potential [18], likely prevents spiking activity in OHCs. Some evidence for the possible presence of spontaneous Ca^{2+} action potentials in OHCs during the first 3–4 postnatal days comes from two-photon imaging or cell-attached patch clamp experiments (Fig. 4) [16, 66]. These methods better preserve the intracellular milieu, including endogenous Ca^{2+} buffers, compared to the whole-cell approach. However, it remains unknown whether immature OHCs, like IHCs, can fire spontaneous Ca^{2+} action potentials *in vivo* and, if so, whether this activity is restricted to just a few days, as observed in cochlear explants.

Purinergic Signalling in the Supporting Cells of the Developing Cochlea

The sensory epithelium of the developing cochlea includes the greater epithelial ridge (GER) and the lesser epithelial ridge (LER) (Fig. 5), which become recognizable from around E16 [67]. The LER contains the OHCs and adjacent supporting cells in the lateral portion of the epithelium, whereas the GER includes IHCs and supporting cells located medially. During this developmental period, the GER contains Kölliker's organ, a transient structure that plays a critical role in early development and maturation of the auditory system [68]. Similar to astrocytes in the central nervous system [69], the supporting cells of Kölliker's organ actively generate complex Ca^{2+} signals that can propagate as intercellular Ca^{2+} waves throughout the sensory epithelium [16, 61, 70–72]. In the cochlea, this spontaneous Ca^{2+} activity ceases around the onset of hearing in mice [9, 71]. This follows the regression of Kölliker's organ, and the downregulation of several key molecules involved in the signalling cascade [62, 73, 74].

Pharmacological studies have shown that these Ca^{2+} waves are mediated by the binding of extracellular ATP, which is released by the supporting cells in the GER, to G protein-coupled P2Y autoreceptors located on the endolymphatic surface of supporting cells. P2Y receptor activation triggers the phospholipase-C dependent generation of IP_3, which binds to its receptors on the ER, raising the concentration of cytosolic free Ca^{2+} and triggering additional ATP release (Fig. 6). The intercellular diffusion of IP_3 through gap-junction channels (connexin 26 and 30) enables the propagation of Ca^{2+} signals as an intercellular wave (recently reviewed by [45]). Interestingly, there is no evidence of spontaneous Ca^{2+} waves originating in the LER, even though supporting cells in both GER and LER share similar Ca^{2+} signalling cascade [82].

Spontaneous Ca^{2+} Waves Regulate IHC Firing Activity in the Prehearing Cochlea

Spontaneous ATP release from supporting cells into the extracellular space of the GER (Kölliker's organ) initiates the signalling cascade that drives the generation and propagation of discrete Ca^{2+} waves within the sensory epithelium. Although this mechanism has been observed using Ca^{2+} imaging in both cochlear explants and *in vivo*, the dynamics of these Ca^{2+} waves differ between the two conditions. In cochlear explants, supporting cells generate Ca^{2+} waves that are significantly larger and slower [9, 56, 61, 83] than those recorded *in vivo* [9, 10]. Given that supporting cells release ATP in response to cochlear damage [77, 78], it is possible that the large Ca^{2+} waves in explants are, at least partially, a direct consequence of tissue dissection.

Both *ex vivo* and *in vivo* studies have shown that spontaneous Ca^{2+} waves occurring near IHCs can depolarize them, leading to coordinated bursts of Ca^{2+} action potentials across several neighbouring cells [9, 61, 70, 81]. *Ex vivo* studies showed that ATP-induced activation of P_2Y_1 receptors in supporting cells leads to elevated intracellular Ca^{2+} and the opening of TMEM16A, which ultimately cause K^+ efflux in the extracellular space (Fig. 6). Elevated extracellular K^+ depolarizes adjacent IHCs, triggering their synchronous activity. Subsequently, the osmotic shrinkage of supporting cells expands the extracellular volume, diluting K^+ concentration and terminating IHC depolarization [81]. However, while the role of TMEM16A in synchronizing IHC activity is consistent across *ex vivo* studies, its direct involvement in generating Ca^{2+} waves is less clear, as conflicting evidence exists [62, 72].

In vivo recordings have confirmed this complex interaction between IHCs and supporting cells, showing that spontaneous Ca^{2+} waves are essential for synchronizing the firing activity of nearby IHCs. Yet, the long-range longitudinal propagation of Ca^{2+} signals within IHCs appears to occur independently of Ca^{2+} wave propagation through supporting cells (Fig. 7) [9]. The mechanism driving the long-range longitudinal propagation of Ca^{2+} signals in IHCs [9], which occurs more rapidly than Ca^{2+} wave propagation [71], remains unknow. It is possible that during sustained firing, K^+ efflux from active IHCs accumulates
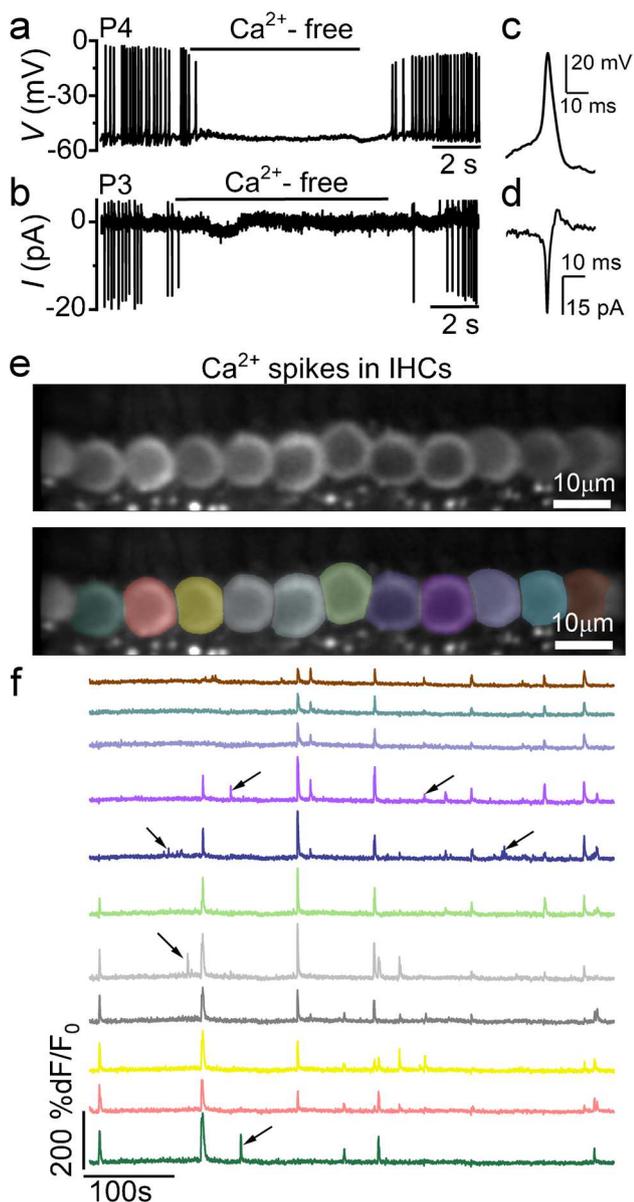
Fig. 3 Spontaneous action potentials in pre-hearing IHCs. (**a,b**) Spontaneous action potentials recorded from an IHC in a cochlear explant using whole-cell current clamp (**a**) and cell-attached voltage clamp (**b**). Recordings were performed at body temperature in the presence of 1.3 mM extracellular Ca^{2+}. Note that action potentials are reversibly abolished in Ca^{2+}-free extracellular solution. (**c,d**) Single action potentials from panels (**a**) and (**b**), respectively, shown on an expanded time scale. Image modified from [57]. (**e**) Top: average intensity projection of a timelapse recording showing GCaMP6f expression in apical-coil IHCs from a live *GCaMP6f^fl/fl^Myo15-Cre^±^* mouse (P6). Bottom: Regions of interest (ROIs) generated using a semi-automated identification approach. These ROIs were used to measure spontaneous Ca^{2+} signals from individual IHCs shown in the top panel. (**f**) Fluorescence time series computed as pixel-averages from the ROIs in panel (**e**), demonstrating spontaneous Ca^{2+} activity in IHCs in vivo (colours match those in panel (**e**)). Image modified from [9]

in the intercellular space, depolarizing neighbouring IHCs and thereby directly triggering their activation.

Much less is known about the regulation of Ca^{2+} signals in the LER. Although the LER itself does not appear to generate spontaneous Ca^{2+} waves (see above), recent studies in cochlear explants and organotypic cultures have shown that waves originating in Kölliker's organ can propagate into supporting cells of the LER (Fig. 8) [16, 84]. These GER-derived Ca^{2+} waves induce Ca^{2+} elevation in Deiters' cells, which lead to synchronized Ca^{2+} activity in OHCs of neonatal mice [16]. Whether this mechanism also operates under *in vivo* conditions remains unknown. Consistent with the absence of spontaneous Ca^{2+} waves in the LER, ATP release from supporting cells in this region has been reported primarily following exposure to noxious stimuli [77, 78], or in response to the extracellular application of ATP to hair cells [75, 85].

## Spontaneous Activity in Type I SGNs

During pre-hearing stages of development, IHCs are innervated by both the afferent and efferent systems (Fig. 1) [19, 22]. Type I SGN afferent fibres form axo-somatic contacts with IHCs shortly before birth around E16 in the basal turn, extending apically by E17-E18 [48]. At this age, IHCs generate broad, immature Ca^{2+} action potentials, that nevertheless trigger the fusion of glutamate-containing vesicles at their ribbon synapses [8], driving action potential activity in SGNs as early as P0 [86]. Before the onset of hearing, immature IHCs are innervated by highly branched type I SGNs, which eventually establish the characteristic one-to-one axosomatic contact following extensive pruning (Fig. 9) [19, 48, 87]. This loss of SGN terminals is accompanied by a corresponding decrease in the number of presynaptic ribbons [88, 89].

Ex vivo patch-clamp recordings from individual SGN terminals, pioneered by Glowatzki and colleagues (e.g., [90, 91]), have been instrumental in uncovering how IHC glutamate release shapes SGN responses [19, 92]. However, how the diverse Ca^{2+} dynamics in IHCs are faithfully transmitted to SGN terminals in the intact developing cochlea remains largely unknown. Ex vivo imaging studies in P7-P9 mice have reported sparse, long-lasting Ca^{2+} transients in SGN terminals, with little correlated activity across terminals contacting the same or neighbouring middle-apical IHCs [9]. This is surprising, given that IHCs appear capable of driving Ca^{2+}-induced glutamate release at ribbon synapses under similar experimental conditions (see above). In contrast, in vivo experiments have shown that SGN terminals display rapid and highly correlated Ca^{2+} transients that closely match IHC activity (Fig. 10) [9]. Furthermore, the synchronization of IHCs by Ca^{2+} waves in the supporting
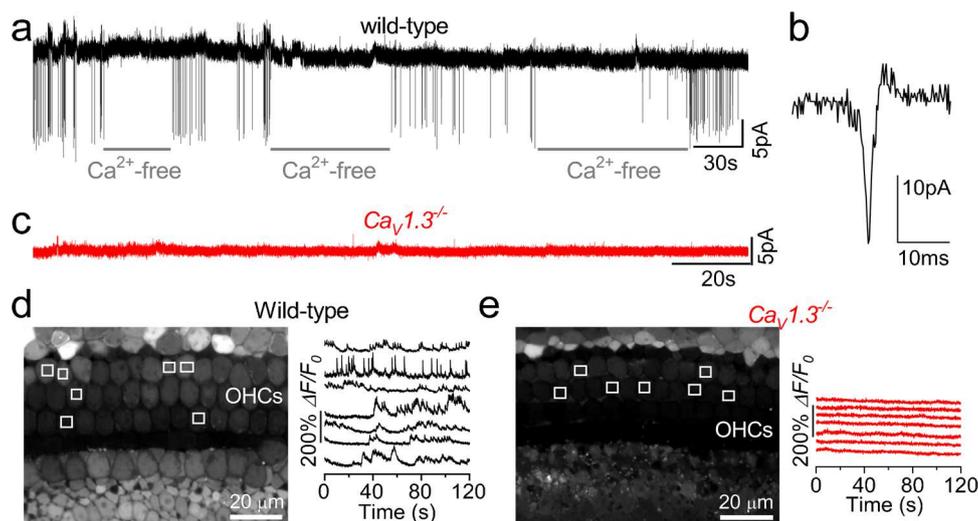
Fig. 4 Spontaneous action potentials in immature OHCs. (**a**) Spontaneous currents recorded from a P2 mouse OHC using cell-attached voltage clamp at body temperature with 1.3 mM extracellular Ca^{2+}. Note that the local perfusion of a Ca^{2+}-free extracellular solution reversibly abolishes the currents. (**b**) Expanded view of a single current transient from panel (**a**). (**c**) Cell-attached recordings from an OHC of a P1 $Ca_V1.3$ knockout mouse ($Ca_V1.3^{-/-}$), highlighting the dependence of spontaneous activity on Ca^{2+} channels. (**d**, **e**) Representative $\Delta F/F_0$ traces from OHCs of a P2 wild-type (**d**) and a P1 $Ca_V1.3^{-/-}$ (**e**) mouse. Traces are computed as pixel averages of regions of interest (white squares) centred on OHCs. Image modified from [66]
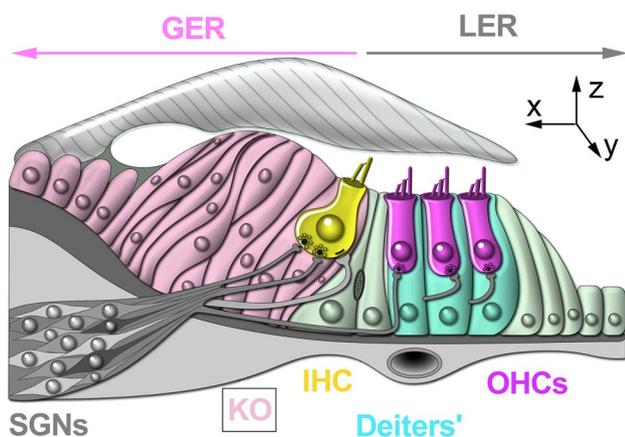

Fig. 5 Diagram of the pre-hearing sensory epithelium of the cochlea. Diagram depicting a cross-section of an immature organ of Corti, illustrating the location of the sensory hair cells and supporting cells. Spontaneous Ca^{2+} waves are generated in the supporting cells present in the greater epithelial ridge (GER) but not in the lesser epithelial ridge (LER). Image modified from [16]

cells has been shown to increase the number of SGN terminals activated by a single IHC in live mice [9]. This is likely to reinforce the refinement of neuronal projections to discrete areas of the developing auditory pathway [33, 35].

In the adult mammalian cochlea, type I SGNs exhibit a wide range of spontaneous rate (SR) activity, with low-SR neurons typically having higher activation thresholds compared to those with high SR [93–96]. SGN subtypes segregate around the IHC synaptic region, with low-SR fibres contacting the modiolar side (facing the central axis of the cochlea), while high-SR fibres contact their pillar side (facing the OHCs) [94, 97]. This functional diversity is likely required to convey the wide dynamic range of sound intensity encoded by each IHC [98]. In mice, single-cell RNA sequencing studies have identified three SGN subtypes based on unique molecular marker combinations, the identity of which is primarily defined during pre-hearing stages of development (e.g., [96, 99–101]). However, electrophysiological measurements in adult mice did not reveal three distinct functional subclasses with strict spatial segregation around IHCs [96]. Instead, the authors identified a group of *Lypd1*-expressing SGNs with low SRs that preferentially innervate the IHC modiolar side, and a second *Calb2*-positive population exhibiting a wider range of SRs that preferentially innervates the IHC pillar side [96]. Although the bimodal distribution in type I afferent SRs identified in cats is less evident in mice, some degree of spatial segregation appears to exist in both in adult [96] and pre-hearing mice [102]. In vivo recordings have also revealed that the frequency and amplitude of Ca^{2+} transients are higher in SGN terminals on the pillar side of the IHCs compared to those on the modiolar side (Fig. 10) [9]. This further supports the notion that, alongside molecular identity, the functional segregation of SGNs is likely established during pre-hearing stages of development.

In addition to the afferent system, IHCs form transient axo-dendritic synapses with the cholinergic efferent fibres, which
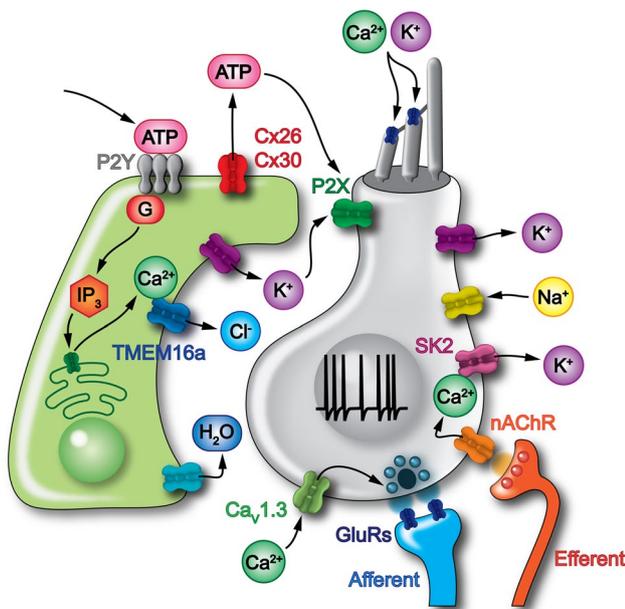
Fig. 6 Supporting cell-IHC interaction in pre-hearing mice. Schematic illustrating the key molecular components and ionic currents in the supporting cells (left) and IHCs (right) required for their function. Supporting cells can release ATP into the extracellular space either spontaneously or in response to hair-cell stimulation [75], mechanical stress [76], or damage [77, 78]. Connexin hemichannels are the most likely candidates as the conduit, however different mechanisms have been proposed [79, 80]. This localized release of ATP likely initiates the intercellular cascade that drives the propagation of Ca^{2+} waves in supporting cells through paracrine activation of G protein-coupled P2Y1 receptors [71, 81]. ATP-induced Ca^{2+} increase opens TMEM16A Ca^{2+}-activated Cl^- channels, driving Cl^- efflux followed by water and K^+ efflux to maintain osmotic balance and electroneutrality [62]. Accumulation of K^+ in the intercellular space between supporting cells and IHCs has been proposed to increase the frequency of spontaneous action potentials in IHCs and to coordinate the firing of several adjacent IHCs (for more details see main text). In IHCs, currents are as described in Fig. 1. IHCs are innervated by type I afferent fibres, which release glutamate onto post-synaptic glutamate receptors (GluRs). Pre-hearing IHCs are also transiently innervated by the efferent fibres, which release acetylcholine (ACh) onto pre-synaptic $\alpha9\alpha10$ nicotinic acetylcholine receptors (nAChRs). Supporting cells express IP_3 receptor (IP_3R), transmembrane protein 16A (TMEM16A, also known as Anoctamin-1, ANO1), Ca^{2+}-activated Cl^- channels, connexin 26 (Cx26), connexin 30 (Cx30) and G-coupled metabotropic purinergic receptors (P2YR)

originate in the brainstem [22]. Acetylcholine (ACh) released by efferent terminals activates $\alpha9\alpha10$ nicotinic ACh receptors (nAChRs) on IHCs, causing Ca^{2+} influx that, in turn, opens small-conductance Ca^{2+} activated K^+ channels (SK2). The resulting K^+ efflux hyperpolarizes IHCs, thereby reducing their excitability (Fig. 6) (e.g., [11–13]). Ex vivo studies using artificial activation of the efferents have demonstrated that this system provides an inhibitory feedback mechanism capable of directly modulating spontaneous Ca^{2+} activity in developing

IHCs. However, the exact physiological role of the efferent system in regulating IHC firing in vivo remains unknown.

Refinement of Type II SGN Connections

Mature OHCs receive cholinergic input via medial olivocochlear (MOC) efferent neurons originating in the brainstem [22]. OHCs are also innervated by type II SGNs, which turn towards the base of the cochlea to form en-passant ribbon synaptic contacts with 5–30 OHCs (Fig. 9) [26, 27]. Each OHC receives two or three SGN terminals with juxtaposed ribbons (Fig. 9) [27, 103]. This mature morphological organization is established towards the end of the first postnatal week following extensive axon outgrowth, target selection, refinement, and pruning of SGN peripheral processes [25, 104]. During this refinement, approximately 25 % of the type II SGNs innervating OHCs are lost, while the type I SGN terminals that transiently innervate immature OHCs are eliminated (Fig. 9) [25, 105]. Although the role of type II SGNs remains largely unclear, they appear to respond to sound levels capable of causing cochlear damage, suggesting they function as cochlear nociceptors [106, 107]. This conclusion is derived from *ex vivo* electrophysiological recordings demonstrating that type II SGNs are only weakly depolarized by OHCs, suggesting that the synchronized activation of several OHCs connected to the same afferent fibre is likely required to reach the firing threshold [27, 106, 108]. Recent evidence indicates that type II SGNs can be directly modulated by MOC efferent terminals via the release of GABA, instead of the classical neurotransmitter ACh [109], the role of which could be to fine tuning the output of the OHCs.

Role of Spontaneous Calcium Signalling in the Developing Cochlea

Spontaneous Ca^{2+} activity in the developing mammalian cochlea has been implicated in multiple processes, including the maturation of hair cells [70, 110–112], the survival of nascent neurons and the refinement of the neural circuitry (recently reviewed by [45, 113].

Disrupting spontaneous Ca^{2+}-dependent signalling in the developing cochlea is known to affect the functional maturation of the sensory epithelium. For example, altering the temporal pattern of IHC Ca^{2+} action potentials during the second postnatal week of cochlear development impairs maturation of their synaptic machinery [110, 114]. Moreover, suppression of IHC spiking during the same period disrupts the normal morphological and functional maturation of their stereociliary bundles
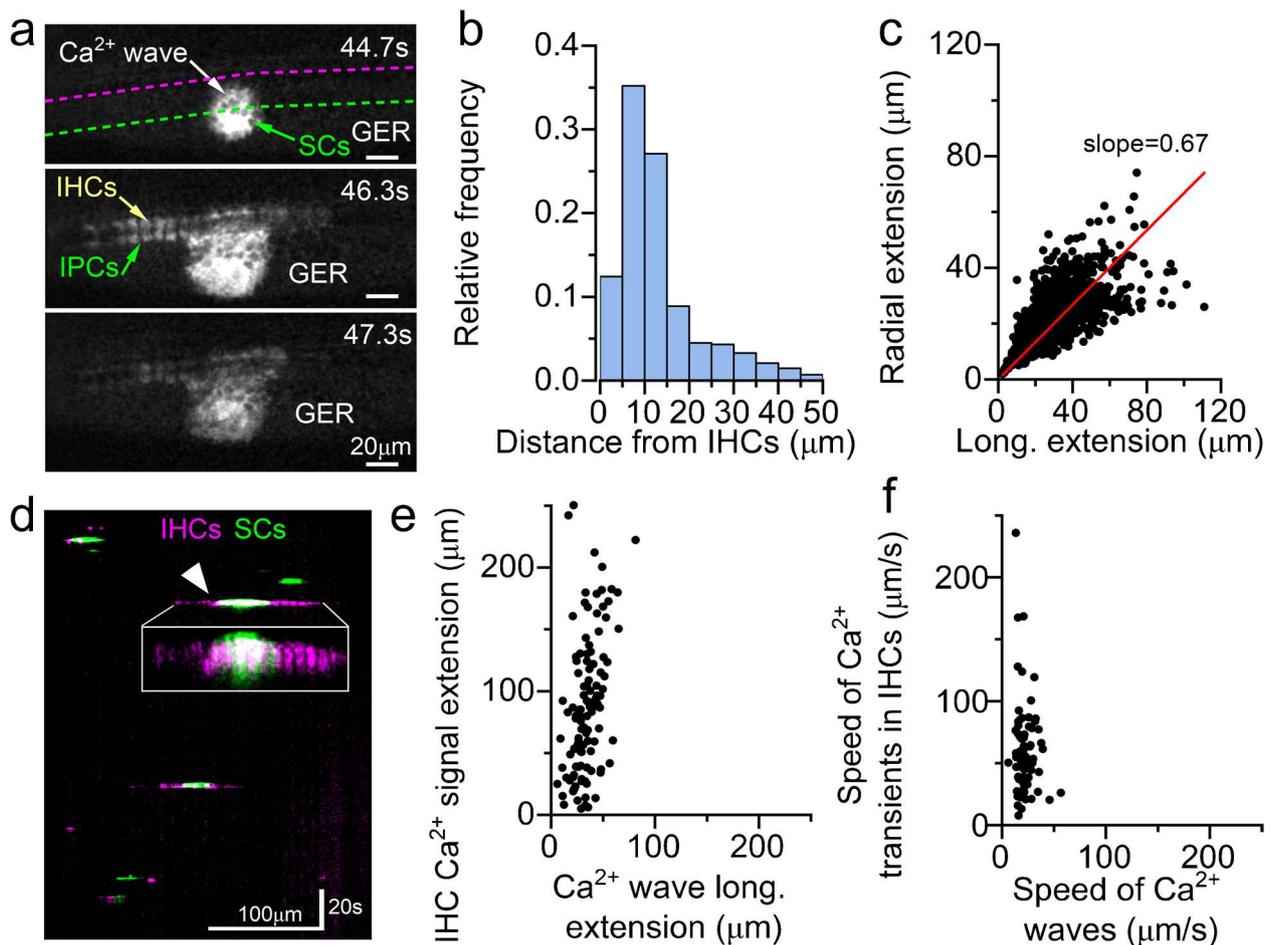
Fig. 7 Spontaneous Ca^{2+} signals in supporting cells and IHCs in pre-hearing live mice. (**a**) Representative images showing spontaneous Ca^{2+} waves propagating from supporting cells in the GER towards the IHCs of a live P4 *GCaMP6f$^{f/fl}$Pax2-Cre$^{\pm}$* mouse. SCs: supporting cells within the GER; IPCs: inner phalangeal cells located in between the IHCs. (**b**) Histogram displaying the number of Ca^{2+} waves as a function of their distance relative to the IHCs. (**c**) Maximal longitudinal extension of Ca^{2+} waves from panel (**b**) plotted as a function of their radial extension. (**d**) Kymograph constructed by drawing lines across the IHCs (magenta) and GER (green) regions from panel (**a**). Inset: magnification of the Ca^{2+} wave indicated by the arrowhead, highlighting the larger longitudinal spread of the Ca^{2+} signal in the IHCs compared to that of the GER. (**e**, **f**) Relationship between the spread (**e**) and speed (**f**) of Ca^{2+} waves in the supporting cells of the GER and Ca^{2+} signals in IHCs, demonstrating the faster and wider extension of Ca^{2+} signals in the latter. Image modified from [9]

[112]. Furthermore, mice lacking the MET current, which normally drives depolarization in IHCs during the second postnatal week due to the establishment of the endocochlear potential (Fig. 1) [60, 64], fail to upregulate mature basolateral membrane currents ($I_{K,n}$ and $I_{K,f}$) [115, 116]. Together, these findings support the existence of a "critical period" in the second postnatal week, just prior to hearing onset, during which Ca^{2+} activity is essential for IHC maturation. Much less is known about OHCs, but recent work showed that abolishing their Ca^{2+}-induced firing activity, which unlike in IHCs, is restricted to the first postnatal week, leads to dysregulation of type II SGN afferent connectivity [16].

A recent study has also demonstrated that the developing stereocilia of both IHCs and OHCs exhibit spontaneous Ca^{2+} transients [117]. Interestingly, these Ca^{2+} signals occur not only at the tip of transducing stereocilia, where the MET channel is located [118], but also along the stereocilia shaft and within the microvilli-like structures on the hair cell surface [117]. These Ca^{2+} transients are not triggered by the propagating Ca^{2+} waves from the GER, they are short-lived and unable to induce hair-cell depolarization. Although it is currently unknown whether these Ca^{2+} signals are present in the mouse cochlea *in vivo*, they could potentially be involved in regulating hair bundle development [119, 120].

Spontaneous cochlear activity is also critical for the refinement of the central auditory pathway. Peripheral activity propagates centrally along the auditory pathway [86, 121], since functional synapses are already established in the brainstem at birth [122], and neurons in the superior olivary
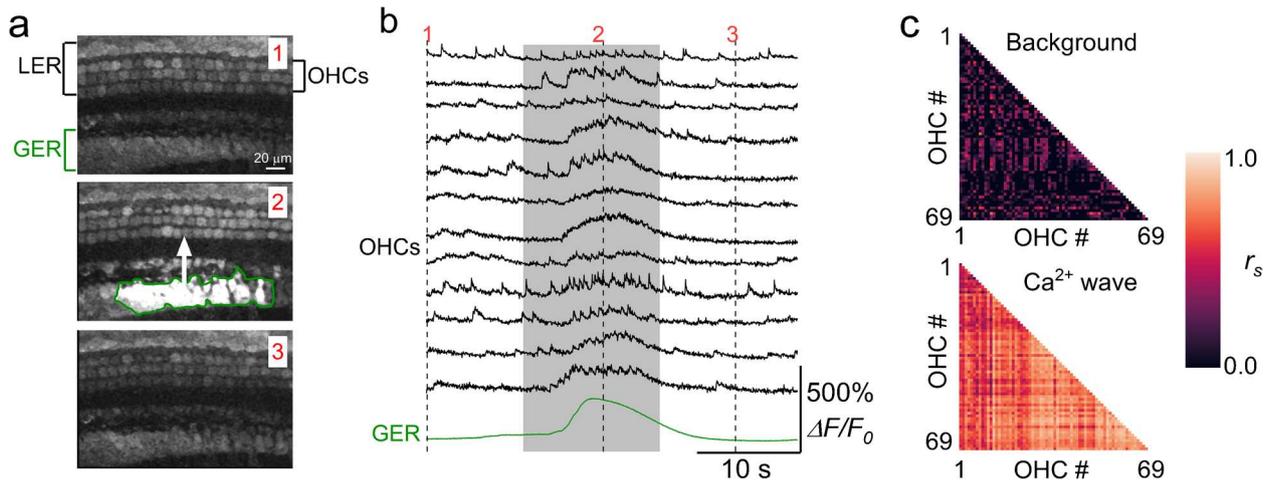
Fig. 8 Calcium waves and OHC Ca^{2+} signalling from cochlear explants. (**a**) Three representative images (left panels) obtained before (1), during (2) and after (3) the spontaneous appearance of a large Ca^{2+} wave in the GER in the apical coil of a P2 wild-type mouse. (**b**) Representative $\Delta F/F_0$ traces from 12 OHCs (black traces) and that originating from the Ca^{2+} wave in the GER (green traces). The grey-shaded area highlights the time window used for correlation analysis. Recordings were made at 31 °C. (**c**) Correlation matrices computed from the Ca^{2+} fluorescence traces of 69 OHCs. Correlation coefficients were computed before (top panels: background) and during (bottom panels: Ca^{2+} wave) the occurrence of the Ca^{2+} wave in nearby supporting cells. Each matrix element represents the Spearman's rank correlation coefficient of one pair of OHCs. Image modified from [16]
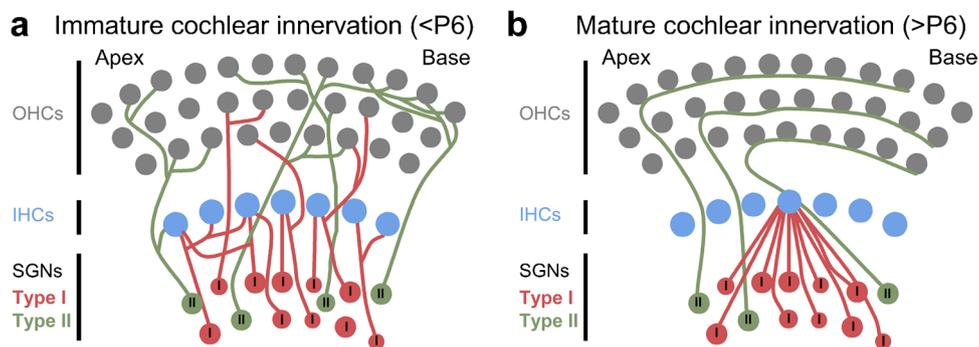
Fig. 9 Diagram illustrating the developmental refinement of SGN afferent terminals in the pre-hearing sensory epithelium. Diagram showing the innervation pattern targeting IHCs and OHCs during the first (**a**) and second (**b**) postnatal week of mouse cochlear development. Following extensive pruning of SGN afferent fibres, the mature configuration is established. This consists of: Type I SGN fibres (~95 %), which are unbranched, myelinated neurons that connect with a single IHC (approximately 10–20 neurons per IHC); and Type II SGN fibres (the remaining ~5 %), which are unmyelinated neurons that spiral towards the base of the cochlea to contact multiple OHCs

complex can fire action potentials in pre-hearing rodents [123]. Cochlear ablation or block of spontaneous activity in the sensory epithelium abolishes firing in the auditory centres and leads to substantial loss of cochlear nucleus neurons. Furthermore, removing efferent feedback to IHCs disrupts tonotopic map refinement in the medial nucleus of the trapezoid body (MNTB) and impairs bilateral coupling in the inferior colliculi (recently reviewed by [45, 113]).

Conclusions and Future Work

A combination of single-cell and systems neuroscience approaches, applied to both *in vivo* and *ex vivo* models, has considerably furthered our understanding of the mechanisms driving early spontaneous activity in the cochlea and its role in the maturation of the auditory system. The emerging picture suggests that the interactions between Ca^{2+} and electrical activity in the developing cochlea influence gene expression

Fig. 10 Spontaneous Ca^{2+} signals in postsynaptic afferent terminals from pre-hearing live mice. (**a**) Average intensity projections displaying GCaMP6f expression in vivo from a P4 *GCaMP6f^{fl/fl}NeuroD-Cre^±* mouse. Image shows superimposed segmentation mask highlighting ROIs for identified synaptic terminals colour-matched to their associated IHC body. (**b**) Fluorescence traces of pillar (blue) and modiolar (black) afferent terminals identified by the ROIs shown in panel (**a**). Lines on the right of the traces indicate the terminals belonging to the colour-matched IHC in panel (**a**). While single IHC Ca^{2+} events show scattered activity in individual afferent terminals, coordinated Ca^{2+} events across multiple IHCs consistently recruit a large number of terminals. (**c**) Average intensity projection displaying GCaMP6 signal in vivo from a P4 *GCaMP6f^{fl/fl}NeuroD-Cre^+* mouse. Bottom panel: ROIs highlighting individual afferent terminals assigned to the IHCs in the bottom panel. (**d**) Relationship between the frequency of Ca^{2+} transients in the afferent terminals positioned in the modiolar and pillar side of individual IHCs. (**e**) Fluorescence traces of the pillar (blue) and modiolar (black) ROIs numbered 1–5 in panel (**c**). Image modified from [9]
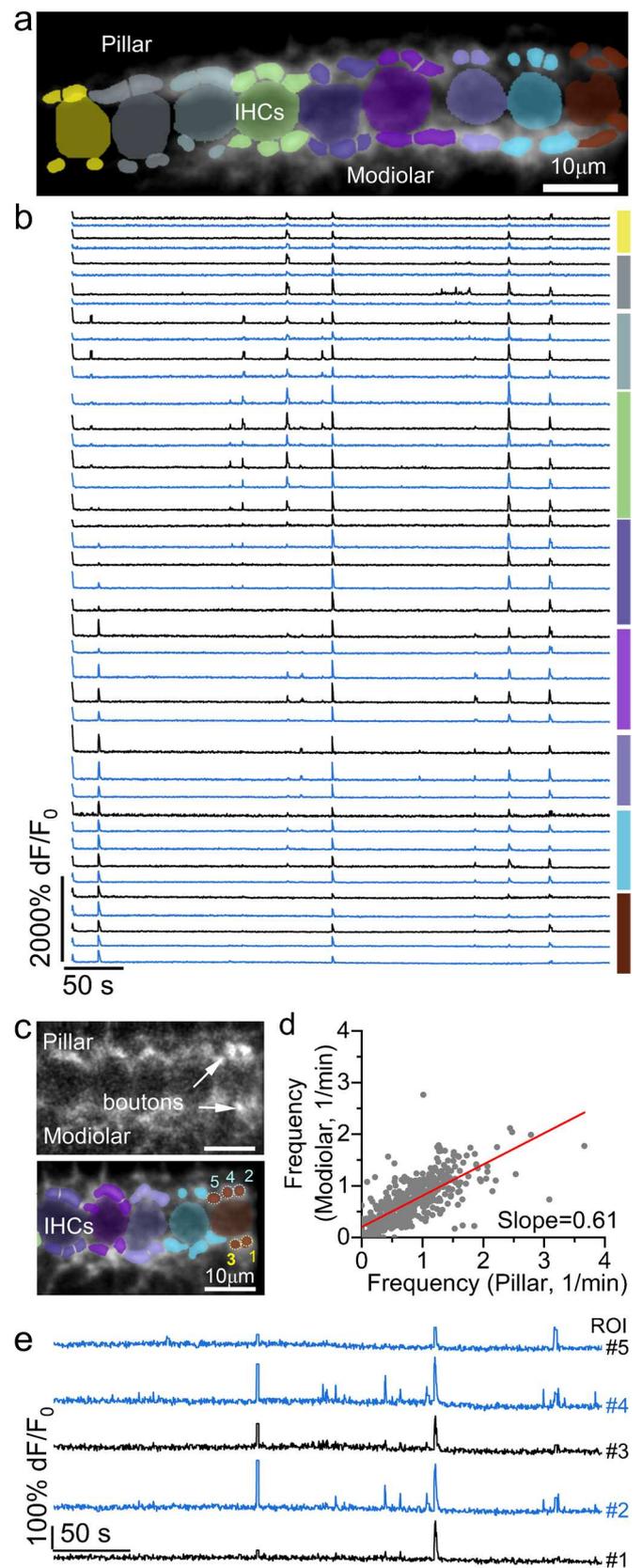
programs and the refinement of immature neural connections and sensory domains along the auditory pathway.

Nevertheless, several fundamental questions remain currently unaddressed. For example, what initiates ATP release from developing supporting cells, and what are the mechanisms underpinning it? How does the inhibitory efferent system modulate the spiking activity of developing IHCs, and what is the activity pattern of the efferent fibres *in vivo*? Does glutamate release dynamics under in vivo conditions mirror findings obtained using *ex-vivo* preparations? Furthermore, there is evidence showing that action potentials occurring in IHCs during the second postnatal week are required for their correct morphological and physiological maturation by the onset of hearing [110, 112]. However, the intracellular signals regulating this pre-hearing functional maturation are still largely unknown. Regarding OHCs, it remains unclear whether they also exhibit spontaneous Ca^{2+} signals *in vivo*, and, if so, whether this activity is required for their maturation and the refinement of the type II fibres, analogous to the role proposed for IHCs. Recent experimental developments using live mice [9, 10, 124, 125] have the potential to address many of these remaining questions with unprecedented resolution. This approach will be instrumental in bridging the current understanding of single-cell physiology with systems-level data from the cochlea.

Declarations

Competing interests The Authors declare no conflict of interest.

References

1. Fettiplace R, Hackney CM (2006) The sensory and motor roles of auditory hair cells. Nat Rev Neurosci 7:19–29. https://doi.org/10.1038/nrn1828
2. Hudspeth AJ (2014) Integrating the active process of hair cells with cochlear function. Nat Rev Neurosci 15:600–614. https://doi.org/10.1038/nrn3786
3. Jang MW, Lim J, Park MG, Lee JH, Lee CJ (2022) Active role of glia-like supportingcells in the organ of Corti: membrane proteins and their roles in hearing. Glia 70:1799–1825. https://doi.org/10.1002/glia.24229
4. Marcotti W, Johnson SL, Holley MC, Kros CJ (2003) Developmental changes in the expression of potassium currents of embryonic, neonatal and mature mouse inner hair cells. J Physiol 548:383–400. https://doi.org/10.1113/jphysiol.2002.034801
5. Sun Y, Ren M, Zhang Y, Li S, Luo Z, Sun S, He S, Wang G, Zhang D, Mansour SL, Song L, Liu Z (2025) Casz1 is required for both inner hair cell fate stabilization and outer hair cell survival. Science 388:eado4930. https://doi.org/10.1126/science.ado4930
6. García-Añoveros J, Clancy JC, Foo CZ, García-Gómez I, Zhou Y, Homma K, Cheatham MA, Duggan A (2022) Tbx2 is a master regulator of inner versus outer hair cell differentiation. Nature 605:298–303. https://doi.org/10.1038/s41586-022-04668-3
7. Kuhn S, Johnson SL, Furness DN, Chen J, Ingham N, Hilton JM, Steffes G, Lewis MA, Zampini V, Hackney CM, Masetto S, Holley MC, Steel KP, Marcotti W (2011) MiR-96 regulates the progression of differentiation in mammalian cochlear inner and outer hair cells. Proc Natl Acad Sci USA 108:2355–2360. https://doi.org/10.1073/pnas.1016646108
8. Marcotti W, Johnson SL, Rusch A, Kros CJ (2003) Sodium and calcium currents shape action potentials in immature mouse inner hair cells. J Physiol 552:743–761. https://doi.org/10.1113/jphysiol.2003.043612
9. De Faveri F, Ceriani F, Marcotti W (2025) In vivo spontaneous Ca2+ activity in the pre-hearing mammalian cochlea. Nat Commun 16:29. https://doi.org/10.1038/s41467-024-55519-w
10. De Faveri F, Marcotti W, Ceriani F (2025) Spontaneous Ca2+ signals in the developing mammalian cochlea of live mice under different anaesthetic regimes. Exp Physiol. https://doi.org/10.1113/EP093267
11. Glowatzki E, Fuchs PA (2000) Cholinergic synaptic inhibition of inner hair cells in the neonatal mammalian cochlea. Science 288:2366–2368. https://doi.org/10.1126/science.288.5475.2366
12. Katz E, Elgoyhen AB, Gomez-Casati ME, Knipper M, Vetter DE, Fuchs PA, Glowatzki E (2004) Developmental regulation of nicotinic synapses on cochlear inner hair cells. J Neurosci 24:7814–7820. https://doi.org/10.1523/JNEUROSCI.2102-04.2004
13. Marcotti W, Johnson SL, Kros CJ (2004) A transiently expressed SK current sustains and modulates action potential activity in immature mouse inner hair cells. J Physiol 560:691–708. https://doi.org/10.1113/jphysiol.2004.072868
14. Simmons DD, Mansdorf NB, Kim JH (1996) Olivocochlear innervation of inner and outer hair cells during postnatal maturation: evidence for a waiting period. J Comp Neurol 370:551–562. https://doi.org/10.1002/(SICI)1096-9861(19960708)370:4<551::AID-CNE10>3.0.CO;2-M
15. Chessum L, Matern MS, Kelly MC, Johnson SL, Ogawa Y, Milon B, McMurray M, Driver EC, Parker A, Song Y, Codner G, Esapa CT, Prescott J, Trent G, Wells S, Dragich AK, Frolenkov GI, Kelley MW, Marcotti W et al (2018) Helios is a key transcriptional regulator of outer hair cell maturation. Nature 563:696–700. https://doi.org/10.1038/s41586-018-0728-4

16. Ceriani F, Hendry A, Jeng J-Y, Johnson SL, Stephani F, Olt J, Holley MC, Mammano F, Engel J, Kros CJ, Simmons DD, Marcotti W (2019) Coordinated calcium signalling in cochlear sensory and non-sensory cells refines afferent innervation of outer hair cells. EMBO J 38:e99839. https://doi.org/10.15252/embj.201899839

17. Zheng J, Shen W, He DZ, Long KB, Madison LD, Dallos P (2000) Prestin is the motor protein of cochlear outer hair cells. Nature 405:149–155. https://doi.org/10.1038/35012009

18. Marcotti W, Kros CJ (1999) Developmental expression of the potassium current IK,n contributes to maturation of mouse outer hair cells. J Physiol 520:653–660. https://doi.org/10.1111/j.1469-7793.1999.00653.x

19. Johnson SL, Safieddine S, Mustapha M, Marcotti W (2019) Hair cell afferent synapses: function and dysfunction. Cold Spring Harb Perspect Med 9:a033175. https://doi.org/10.1101/cshperspect.a033175

20. Ryugo DK (1992) The auditory nerve: peripheral innervation, cell body morphology, and central projections. In: Webster DB et al (eds) The mammalian auditory pathway: neuroanatomy. Springer, New York, pp 23–65. https://doi.org/10.1007/978-1-4612-4416-5_2

21. Meyer AC, Frank T, Khimich D, Hoch G, Riedel D, Chapochnikov NM, Yarin YM, Harke B, Hell SW, Egner A, Moser T (2009) Tuning of synapse number, structure and function in the cochlea. Nat Neurosci 12:444–453. https://doi.org/10.1038/nn.2293

22. Fuchs PA, Lauer AM (2019) Efferent inhibition of the cochlea. Cold Spring Harb Perspect Med 9:a033530. https://doi.org/10.1101/cshperspect.a033530

23. Ashmore J (2019) Outer hair cells and electromotility. Cold Spring Harb Perspect Med 9:a033522. https://doi.org/10.1101/cshperspect.a033522

24. Guinan JJ Jr (1996) Physiology of olivocochlear efferents. In: Dallos P, Popper A, Fay R (eds) The cochlea. Springer, New York, NY, pp 435–502. https://doi.org/10.1007/978-1-4612-0757-3_8

25. Barclay M, Ryan AF, Housley GD (2011) Type I vs type II spiral ganglion neurons exhibit differential survival and neuritogenesis during cochlear development. Neural Dev 6:33. https://doi.org/10.1186/1749-8104-6-33

26. Zhang KD, Coate TM (2017) Recent advances in the development and function of type II spiral ganglion neurons in the mammalian inner ear. Semin Cell Dev Biol 65:80–87. https://doi.org/10.1016/j.semcdb.2016.09.017

27. Weisz CJ, Lehar M, Hiel H, Glowatzki E, Fuchs PA (2012) Synaptic transfer from outer hair cells to type II afferent fibers in the rat cochlea. J Neurosci 32:9528–9536. https://doi.org/10.1523/JNEUROSCI.6194-11.2012

28. Liang C, Zhai TY, Chen J, Fang S, Zhu Y, Liu LM, Yu N, Zhao HB (2025) ATP-gated P2x7 receptors express at type II auditory nerves and required for efferent hearing control and noise protection. Proc Natl Acad Sci U S A 122:e2421995122. https://doi.org/10.1073/pnas.2421995122

29. Raphael Y, Altschuler RA (2003) Structure and innervation of the cochlea. Brain Res Bull 60:397–422. https://doi.org/10.1016/s0361-9230(03)00047-9

30. Jagger DJ, Forge A (2015) Connexins and gap junctions in the inner ear - it's not just about K+ recycling. Cell Tissue Res 360:633. https://doi.org/10.1007/s00441-014-2029-z

31. Huberman AD, Feller MB, Chapman B (2008) Mechanisms underlying development of visual maps and receptive fields. Annu Rev Neurosci 31:479–509. https://doi.org/10.1146/annurev.neuro.31.060407.125533

32. Fekete DM, Campero AM (2007) Axon guidance in the inner ear. Int J Dev Biol 51:549–556. https://doi.org/10.1387/ijdb.072341df

33. Clause A, Kim G, Sonntag M, Weisz CJC, Vetter DE, Rübsamen R, Kandler K (2014) The precise temporal pattern of prehearing spontaneous activity is necessary for tonotopic map refinement. Neuron 82:822–835. https://doi.org/10.1016/j.neuron.2014.04.001

34. Kersbergen CJ, Babola TA, Rock J, Bergles DE (2022) Developmental spontaneous activity promotes formation of sensory domains, frequency tuning and proper gain in central auditory circuits. Cell Rep 41:111649. https://doi.org/10.1016/j.celrep.2022.111649

35. Müller NIC, Sonntag M, Maraslioglu A, Hirtz JJ, Friauf E (2019) Topographic map refinement and synaptic strengthening of a sound localization circuit require spontaneous peripheral activity. J Physiol 597:5469–5493. https://doi.org/10.1113/JP277757

36. Lippe WR (1994) Rhythmic spontaneous activity in the developing avian auditory system. J Neurosci 14:1486–1495. https://doi.org/10.1523/JNEUROSCI.14-03-01486.1994

37. Sonntag M, Englitz B, Kopp-Scheinpflug C, Rübsamen R (2009) Early postnatal development of spontaneous and acoustically evoked discharge activity of principal cells of the medial nucleus of the trapezoid body: an in vivo study in mice. J Neurosci 29:9510–9520. https://doi.org/10.1523/JNEUROSCI.1377-09.2009

38. Tritsch NX, Rodríguez-Contreras A, Crins TT, Wang HC, Borst JG, Bergles DE (2010) Calcium action potentials in hair cells pattern auditory neuron activity before hearing onset. Nat Neurosci 13:1050–1052. https://doi.org/10.1038/nn.2604

39. Mostafapour SP, Cochran SL, Del Puerto NM, Rubel EW (2000) Patterns of cell death in mouse anteroventral cochlear nucleus neurons after unilateral cochlea removal. J Comp Neurol 426:561–571. https://doi.org/10.1002/1096-9861(20001030)426:4<561::aid-cne5>3.0.co;2-g

40. Tierney TS, Russell FA, Moore DR (1997) Susceptibility of developing cochlear nucleus neurons to deafferentation-induced death abruptly ends just before the onset of hearing. J Comp Neurol 378:295–306. https://doi.org/10.1002/(sici)1096-9861(19970210)378:2<295::aid-cne11>3.0.co;2-r

41. Pyott SJ, Pavlinkova G, Yamoah EN, Fritzsch B (2024) Harmony in the molecular orchestra of hearing: developmental mechanisms from the ear to the brain. Annu Rev Neurosci 47:1–20. https://doi.org/10.1146/annurev-neuro-081423-093942

42. Nelson JC, Hosamani IV, Groves AK (2025) Control of sensory cell differentiation in the inner ear by extracellular signals and transcriptional regulators. Curr Top Dev Biol 165:1–44. https://doi.org/10.1016/bs.ctdb.2025.03.002

43. Driver EC, Kelley MW (2020) Development of the cochlea. Development 147:dev162263. https://doi.org/10.1242/dev.162263

44. Corns LF, Bardhan T, Houston O, Olt J, Holley MC, Masetto S, Johnson SL, Marcotti W (2014) Functional development of hair cells in the mammalian inner ear. In: Romand R, Varela-Nieto I (eds) Development of auditory and vestibular systems. Academic, New York, pp 155–188. https://doi.org/10.1016/B978-0-12-408088-1.00006-3

45. Ceriani F, Wood KC, Johnson SL, Kros CJ, Marcotti W (2025) Mechanisms driving the functional maturation of the developing mammalian auditory pathway. Curr Top Dev Biol 165:185–233. https://doi.org/10.1016/bs.ctdb.2025.03.005

46. Kros CJ, Ruppersberg JP, Rüsch A (1998) Expression of a potassium current in inner hair cells during development of hearing in mice. Nature 394:281–284. https://doi.org/10.1038/28401

47. Oliver D, Knipper M, Derst C, Fakler B (2003) Resting potential and submembrane calcium concentration of inner hair cells in the isolated mouse cochlea are set by KCNQ-type potassium channels. J Neurosci 23:2141–2149. https://doi.org/10.1523/JNEUROSCI.23-06-02141.2003

48. Pujol R, Lavigne-Rebillard M, Lenoir M (1998) Development of sensory and neural structures in the mammalian cochlea. In: Rubel EW, Popper AN, Fay R (eds) Development of the auditory system. Springer, New York, pp 145–192. https://doi.org/10.1007/978-1-4612-2186-9_4

49. Michanski S, Smaluch K, Steyer AM, Chakrabarti R, Setz C, Oestreicher D, Fischer C, Möbius W, Moser T, Vogl C, Wichmann C (2019) Mapping developmental maturation of inner hair cell ribbon synapses in the apical mouse cochlea. Proc Natl Acad Sci USA 116(13):6415–6424. https://doi.org/10.1073/pnas.1812029116

50. Kapoor R, Kim H, Garlick E, Madrbf L, Esch K, Ruhwedel T, Möbius W, Wolf F, Moser T (2025) Charting the nanotopography of inner hair cell synapses using MINFLUX nanoscopy. Sci Adv 11(45):eady4344. https://doi.org/10.1126/sciadv.ady4344

51. Fettiplace R, Hackney CM (2006) The sensory and motor roles of auditory hair cells. Nat Rev Neurosci 7:19–29. https://doi.org/10.1038/nrn1828

52. Brownell WE, Bader CR, Bertrand D, de Ribaupierre Y (1985) Evoked mechanical responses of isolated cochlear outer hair cells. Science 227:194–196. https://doi.org/10.1126/science.3966153

53. Marcotti W, Géeléeoc GS, Lennan GW, Kros CJ (1999) Transient expression of an inwardly rectifying potassium conductance in developing inner and outer hair cells along the mouse cochlea. Pflugers Arch 439:113–122. https://doi.org/10.1007/s004249900157

54. Beutner D, Moser T (2001) The presynaptic function of mouse cochlear inner hair cells during development of hearing. J Neurosci 21:4593–4599. https://doi.org/10.1523/JNEUROSCI.21-13-04593.2001

55. Sendin G, Bourien J, Rassendren F, Puel JL, Nouvian R (2014) Spatiotemporal pattern of action potential firing in developing inner hair cells of the mouse cochlea. Proc Natl Acad Sci USA 111:1999–2004. https://doi.org/10.1073/pnas.1319615111

56. Eckrich T, Blum K, Milenkovic I, Engel J (2018) Fast Ca2+ transients of inner hair cells arise coupled and uncoupled to Ca2+ waves of inner supporting cells in the developing mouse cochlea. Front Mol Neurosci 11:264. https://doi.org/10.3389/fnmol.2018.00264

57. Johnson SL, Eckrich T, Kuhn S, Zampini V, Franz C, Ranatunga KM, Roberts TP, Masetto S, Knipper M, Kros CJ, Marcotti W (2011) Position dependent patterning of spontaneous action potentials in immature cochlear inner hair cells. Nat Neurosci 14:711–717. https://doi.org/10.1038/nn.2803

58. Platzer J, Engel J, Schrott-Fischer A, Stephan K, Bova S, Chen H, Zheng H, Striessnig J (2000) Congenital deafness and sinoatrial node dysfunction in mice lacking class D L-type Ca2+ channels. Cell 102:89–97. https://doi.org/10.1016/s0092-8674(00)00013-1

59. Zampini V, Johnson SL, Franz C, Lawrence ND, Münkner S, Engel J, Knipper M, Magistretti J, Masetto S, Marcotti W (2010) Elementary properties of CaV1.3 Ca2+ channels expressed in mouse inner hair cells. J Physiol 588:187–199. https://doi.org/10.1113/jphysiol.2009.181917

60. Johnson SL, Kennedy HJ, Holley MC, Fettiplace R, Marcotti W (2012) The resting transducer current drives spontaneous activity in prehearing mammalian cochlear inner hair cells. J Neurosci 32:10479–10483. https://doi.org/10.1523/JNEUROSCI.0803-12.2012

61. Tritsch NX, Yi E, Gale JE, Glowatzki E, Bergles DE (2007) The origin of spontaneous activity in the developing auditory system. Nature 450:50–55. https://doi.org/10.1038/nature06233

62. Wang HC, Lin CC, Chong R, Zhang-Hooks Y, Agarwal A, Ellis-Davies G, Rock J, Bergles DE (2015) Spontaneous activity of cochlear hair cells triggered by fluid secretion mechanism in adjacent support cells. Cell 163:1348–1359. https://doi.org/10.1016/j.cell.2015.10.070

63. Wangemann P, Schacht J (1996) Homeostatic mechanisms in the cochlea. In: Dallos P, Popper A, Fay R (eds) The cochlea. Springer, New York, pp 130–185. https://doi.org/10.1007/978-1-4612-0757-3_3

64. Li Y, Liu H, Zhao X, He DZ (2020) Endolymphatic potential measured from developing and adult mouse inner ear. Front Cell Neurosci 14:584928. https://doi.org/10.3389/fncel.2020.584928

65. Beurg M, Safieddine S, Roux I, Bouleau Y, Petit C, Dulon D (2008) Calcium and otoferlin-dependent exocytosis by immature outer hair cells. J Neurosci 28:1798–1803. https://doi.org/10.1523/JNEUROSCI.4653-07.2008

66. Jeng J-Y, Ceriani F, Hendry A, Johnson SL, Yen P, Simmons DD, Kros CJ, Marcotti W (2020) Hair cell maturation is differentially regulated along the tonotopic axis of the mammalian cochlea. J Physiol 598:151–170. https://doi.org/10.1113/JP279012

67. Lim DJ, Anniko M (1985) Developmental morphology of the mouse inner ear. Ascanning electron microscopic observation. Acta Otolaryngol Suppl 422:1–69

68. Wang HC, Bergles DE (2015) Spontaneous activity in the developing auditory system. Cell Tissue Res 361:65–75. https://doi.org/10.1007/s00441-014-2007-5

69. Bazargani N, Attwell D (2016) Astrocyte calcium signaling: the third wave. Nat Neurosci 19:182–189. https://doi.org/10.1038/nn.4201

70. Johnson SL, Ceriani F, Houston O, Polishchuk R, Polishchuk E, Crispino G, Zorzi V, Mammano F, Marcotti W (2017) Connexin-mediated signaling in nonsensory cells is crucial for the development of sensory inner hair cells in the mouse cochlea. J Neurosci 37:258–46. https://doi.org/10.1523/JNEUROSCI.2251-16.2016

71. Babola TA, Li S, Wang Z, Kersbergen CJ, Elgoyhen AB, Coate TM, Bergles DE (2021) Purinergic signaling controls spontaneous activity in the auditory system throughout early development. J Neurosci 41:594–612. https://doi.org/10.1523/JNEUROSCI.2178-20.2020

72. Maul A, Huebner AK, Strenzke N, Moser T, Rübsamen R, Jovanovic S, Hübner CA (2022) The Cl-channel TMEM16A is involved in the generation of cochlear Ca2+ waves and promotes the refinement of auditory brainstem networks in mice. Elife 11:e72251. https://doi.org/10.7554/eLife.72251

73. Huang LC, Thorne PR, Vlajkovic SM, Housley GD (2010) Differential expression of P2Y receptors in the rat cochlea during development. Purinergic Signal 6:231–248. https://doi.org/10.1007/s11302-010-9191-x

74. Hool SA, Jeng JY, Jagger DJ, Marcotti W, Ceriani F (2023) Age-related changes in P2Y receptor signalling in mouse cochlear supporting cells. J Physiol 601:4375–4395. https://doi.org/10.1113/JP284980

75. Anselmi F, Hernandez VH, Crispino G, Seydel A, Ortolano S, Roper SD, Kessaris N, Richardson W, Rickheit G, Filippov MA, Monyer H, Mammano F (2008) ATP release through connexin hemichannels and gap junction transfer of second messengers propagate Ca2+ signals across the inner ear. Proc Natl Acad Sci U S A 105:18770–18775. https://doi.org/10.1073/pnas.0800793105

76. Zhao HB, Yu N, Fleming CR (2005) Gap junctional hemichannel-mediated ATP release and hearing controls in the inner ear. Proc Natl Acad Sci USA 102:18724–18729. https://doi.org/10.1073/pnas.0506481102

77. Gale JE, Piazza V, Ciubotaru CD, Mammano F (2004) A mechanism for sensing noise damage in the inner ear. Curr Biol 14:526–529. https://doi.org/10.1016/j.cub.2004.03.002

78. Lahne M, Gale JE (2010) Damage-induced cell–cell communication in different cochlear cell types via two distinct

ATP-dependent ca 2+ waves. Purinergic Signal 6:189–200. https://doi.org/10.1007/s11302-010-9193-8

79. Chen J, Zhu Y, Liang C, Chen J, Zhao HB (2015) Pannexin1 channels dominate ATP release in the cochlea ensuring endoc- ochlear potential and auditory receptor potential generation and hearing. Sci Rep 5:10762. https://doi.org/10.1038/srep10762

80. Zorzi V, Paciello F, Ziraldo G, Peres C, Mazzarda F, Nardin C, Pasquini M, Chiani F, Raspa M, Scavizzi F, Carrer A, Crispino G, Ciubotaru CD, Monyer H, Fetoni AR, Salvatore AM, Mammano F (2017) Mouse Panx1 is dispensable for hearing acquisi- tion and auditory function. Front Mol Neurosci 10:379. https://doi.org/10.3389/fnmol.2017.00379

81. Babola TA, Kersbergen CJ, Wang HC, Bergles DE (2020) Purinergic signaling in cochlear supporting cells reduces hair cell excitability by increasing the extracellular space. Elife 9:e52160. https://doi.org/10.7554/eLife.52160

82. Rodriguez L, Simeonato E, Scimemi P, Anselmi F, Calì B, Cris- pino G, Ciubotaru CD, Bortolozzi M, Ramirez FG, Majumder P, Arslan E, De Camilli P, Pozzan T, Mammano F (2012) Reduced phosphatidylinositol 4, 5-bisphosphate synthesis impairs inner ear Ca2+ signaling and high-frequency hearing acquisition. Proc Natl Acad Sci USA 109:14013–14018. https://doi.org/10.1073/pnas.1211869109

83. Harrus AG, Ceccato JC, Sendin G, Bourien J, Puel JL, Nouvian R (2018) Spiking pattern of the mouse developing inner hair cells is mostly invariant along the tonotopic axis. Front Cell Neurosci 12:407. https://doi.org/10.3389/fncel.2018.00407

84. Majumder P, Crispino G, Rodriguez L, Ciubotaru CD, Anselmi F, Piazza V, Bortolozzi M, Mammano F (2010) ATP-mediated cell–cell signaling in the organ of Corti: the role of connexin channels. Purinergic Signal 6:167–187. https://doi.org/10.1007/s11302-010-9192-9

85. Ceriani F, Pozzan T, Mammano F (2016) Critical role of ATP- induced ATP release for Ca2+ signaling in nonsensory cell networks of the developing cochlea. Proc Natl Acad Sci USA 113:E7194–E7201. https://doi.org/10.1073/pnas.1616061113

86. Tritsch NX, Bergles DE (2010) Developmental regulation of spontaneous activity in the mammalian cochlea. J Neurosci 30:1539–1550. https://doi.org/10.1523/JNEUROSCI.3875-09.2010

87. Meyer AC, Frank T, Khimich D, Hoch G, Riedel D, Chapoch- nikov NM, Yarin YM, Harke B, Hell SW, Egner A, Moser T (2009) Tuning of synapse number, structure and function in the cochlea. Nat Neurosci 12:444–453. https://doi.org/10.1038/nn.2293

88. Sobkowicz HM, Rose JE, Scott GL, Levenick CV (1986) Dis- tribution of synaptic ribbons in the developing organ of Corti. J Neurocytol 15:693–714. https://doi.org/10.1007/BF01625188

89. Huang LC, Barclay M, Lee K, Peter S, Housley GD, Thorne PR, Montgomery JM (2012) Synaptic profiles during neurite exten- sion, refinement and retraction in the developing cochlea. Neural Dev 7:38. https://doi.org/10.1186/1749-8104-7-38

90. Glowatzki E, Fuchs PA (2002) Transmitter release at the hair cell ribbon synapse. Nat Neurosci 5:147–154. https://doi.org/10.1038/nn796

91. Grant L, Yi E, Glowatzki E (2010) Two modes of release shape the postsynaptic response at the inner hair cell ribbon synapse. J Neurosci 30:4210–4220. https://doi.org/10.1523/JNEUROSCI.4439-09.2010

92. Reijntjes DOJ, Pyott SJ (2016) The afferent signaling complex: regulation of type I spiral ganglion neuron responses in the audi- tory periphery. Hear Res 336:1–16. https://doi.org/10.1016/j.heares.2016.03.011

93. Liberman MC (1978) Auditory-nerve response from cats raised in a low-noise chamber. J Acoust Soc Am 63:442–455. https://doi.org/10.1121/1.381736

94. Liberman LD, Wang H, Liberman MC (2011) Opposing gra- dients of ribbon size and AMPA receptor expression underlie sensitivity differences among cochlear-nerve/hair-cell synapses. J Neurosci 31:801–808. https://doi.org/10.1523/JNEUROSCI.3389-10.2011

95. Wu JS, Young ED, Glowatzki E (2016) Maturation of spontane- ous firing properties after hearing onset in rat auditory nerve fib- ers: spontaneous rates, refractoriness, and interfiber correlations. J Neurosci 36:10584–10597. https://doi.org/10.1523/JNEUROSCI.1187-16.2016

96. Siebald C, Vincent PFY, Bottom RT, Sun S, Reijntjes DOJ, Manca M, Glowatzki E, Müller U (2023) Molecular signatures define subtypes of auditory afferents with distinct peripheral pro- jection patterns and physiological properties. Proc Natl Acad Sci USA 120:e2217033120. https://doi.org/10.1073/pnas.2217033120

97. Liberman MC (1982) Single-neuron labeling in the cat auditory nerve. Science 216:1239–1241. https://doi.org/10.1126/science.7079757

98. Winter IM, Robertson D, Yates GK (1990) Diversity of charac- teristic frequency rate-intensity functions in guinea pig auditory nerve fibres. Hear Res 45:191–202. https://doi.org/10.1016/0378-5955(90)90120-e

99. Petitpre C, Faure L, Uhl P, Fontanet P, Filova I, Pavlinkova G, Adameyko I, Hadjab S, Lallemend F (2022) Single-cell RNA- sequencing analysis of the developing mouse inner ear identifies molecular logic of auditory neuron diversification. Nat Commun 13:3878. https://doi.org/10.1038/s41467-022-31580-1

100. Sanders TR, Kelley MW (2022) Specification of neuronal sub- types in the spiral ganglion begins prior to birth in the mouse. Proc Natl Acad Sci U S A 119:e2203935119. https://doi.org/10.1073/pnas.2203935119

101. Shrestha BR, Chia C, Wu L, Kujawa SG, Liberman MC, Goodrich LV (2018) Sensory neuron diversity in the inner ear is shaped by activity. Cell 174:1229–1246. https://doi.org/10.1016/j.cell.2018.07.007

102. Markowitz AL, Kalluri R (2020) Gradients in the biophysical properties of neonatal auditory neurons align with synaptic con- tact position and the intensity coding map of inner hair cells. Elife 9:e55378. https://doi.org/10.7554/eLife.55378

103. Berglund AM, Ryugo DK (1987) Hair cell innervation by spiral ganglion neurons in the mouse. J Comp Neurol 255:560–570. https://doi.org/10.1002/cne.902550408

104. Huang LC, Thorne PR, Housley GD, Montgomery JM (2007) Spatiotemporal definition of neurite outgrowth, refinement and retraction in the developing mouse cochlea. Development 134:2925–2933. https://doi.org/10.1242/dev.001925

105. Echteler SM, Magardino T, Rontal M (2005) Spatiotemporal patterns of neuronal programmed cell death during postnatal development of the gerbil cochlea. Brain Res Dev Brain Res 157:192–200. https://doi.org/10.1016/j.devbrainres.2005.04.004

106. Weisz CJ, Glowatzki E, Fuchs PA (2009) The postsynaptic func- tion of type II cochlear afferents. Nature 461:1126–1129. https://doi.org/10.1038/nature08487

107. Liu C, Glowatzki E, Fuchs PA (2015) Unmyelinated type II afferent neurons report cochlear damage. Proc Natl Acad Sci 112:14723–14727. https://doi.org/10.1073/pnas.1515228112

108. Weisz CJ, Glowatzki E, Fuchs PA (2014) Excitability of type II cochlear afferents. J Neurosci 34:2365–2373. https://doi.org/10.1523/JNEUROSCI.3428-13.2014

109. Bachman JL, Kitcher SR, Vattino LG, Beaulac HJ, Chaves MG, Hernandez Rivera I, Katz E, Wedemeyer C, Weisz CJC (2025) GABAergic synapses between auditory efferent neurons and type II spiral ganglion afferent neurons in the mouse cochlea. Proc Natl Acad Sci 122:e2409921122. https://doi.org/10.1073/pnas.240992112

110. Johnson SL, Kuhn S, Franz C, Ingham N, Furness DN, Knipper M, Steel KP, Adelman JP, Holley MC, Marcotti W (2013) Presynaptic maturation in auditory hair cells requires a critical period of sensory-independent spiking activity. Proc Natl Acad Sci 110:8720–8725. https://doi.org/10.1073/pnas.1219578110

111. Lee J, Kawai K, Holt JR, Géléoc GS (2021) Sensory transduction is required for normal development and maturation of cochlear inner hair cell synapses. Elife 10:e69433. https://doi.org/10.7554/eLife.69433

112. Carlton AJ, Jeng JY, Grandi FC, De Faveri F, Ceriani F, De Tomasi L, Underhill A, Johnson SL, Legan PK, Kros CJ, Richardson GP, Mustapha M, Marcotti W (2023) A critical period of prehearing spontaneous Ca2+ spiking is required for hair-bundle maintenance in inner hair cells. EMBO J 42:e112118. https://doi.org/10.15252/embj.2022112118

113. Kersbergen CJ, Bergles DE (2024) Priming central sound processing circuits through induction of spontaneous activity in the cochlea before hearing onset. Trends Neurosci 47:522–537. https://doi.org/10.1016/j.tins.2024.04.007

114. Johnson SL, Adelman JP, Marcotti W (2007) Genetic deletion of SK2 channels in mouse inner hair cells prevents the developmental linearization in the Ca2+ dependence of exocytosis. J Physiol 583:631–646. https://doi.org/10.1113/jphysiol.2007.136630

115. Marcotti W, Erven A, Johnson SL, Steel KP, Kros CJ (2006) Tmc1 is necessary for normal functional maturation and survival of inner and outer hair cells in the mouse cochlea. J Physiol 574:677–698. https://doi.org/10.1113/jphysiol.2005.095661

116. Lee J, Kawai K, Holt JR, Géléoc GS (2021) Sensory transduction is required for normal development and maturation of cochlear inner hair cell synapses. Elife 10:e69433. https://doi.org/10.7554/eLife.69433

117. Hussain S, Sedlacek M, Cui R, Zhang-Hooks W, Bergles DE, Shin JB, Kindt KS, Kachar B (2025) Spontaneous calcium transients in hair cell stereocilia. Sci Rep 15(1):33660. https://doi.org/10.1038/s41598-025-17976-1

118. Beurg M, Fettiplace R, Nam J-H, Ricci AJ (2009) Localization of inner hair cell mechanotransducer channels using high-speed calcium imaging. Nat Neurosci 12:553–558. https://doi.org/10.1038/nn.2295

119. Vélez-Ortega AC, Freeman MJ, Indzhykulian AA, Grossheim JM, Frolenkov GI (2017) Mechanotransduction current is essential for stability of the transducing stereocilia in mammalian auditory hair cells. Elife 6:e24661. https://doi.org/10.7554/eLife.24661

120. Corns LF, Johnson SL, Roberts T, Ranatunga KM, Hendry A, Ceriani F, Safieddine S, Steel KP, Forge A, Petit C, Furness DN, Kros CJ, Marcotti W (2018) Mechanotransduction is required for establishing and maintaining mature inner hair cells and regulating efferent innervation. Nat Commun 9:4015. https://doi.org/10.1038/s41467-018-06307-w

121. Babola TA, Li S, Gribizis A, Lee BJ, Issa JB, Wang HC, Crair MC, Bergles DE (2018) Homeostatic control of spontaneous activity in the developing auditory system. Neuron 99:511-524.e5. https://doi.org/10.1016/j.neuron.2018.07.004

122. Hoffpauir BK, Grimes JL, Mathers PH, Spirou GA (2006) Synaptogenesis of the calyx of held: rapid onset of function and one-to-one morphological innervation. J Neurosci 26:5511–5523. https://doi.org/10.1523/JNEUROSCI.5525-05.2006

123. Kandler K, Friauf E (1995) Development of electrical membrane properties and discharge characteristics of superior olivary complex neurons in fetal and postnatal rats. Eur J Neurosci 7:1773–1790. https://doi.org/10.1111/j.1460-9568.1995.tb00697.x

124. Kim J, Ricci AJ (2023) A chemo-mechanical cochleostomy preserves hearing for the in vivo functional imaging of cochlear cells. Nat Protoc 18:1137–1154. https://doi.org/10.1038/s41596-022-00786-4

125. Kim J, Ricci AJ (2022) In vivo real-time imaging reveals megalin as the aminoglycoside gentamicin transporter into cochlea whose inhibition is otoprotective. Proc Natl Acad Sci U S A 119:e2117946119. https://doi.org/10.1073/pnas.2117946119

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.