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ORIGINAL ARTICLE

ATP-induced morphological changes in supporting cells of the developing cochlea

Nicolas X. Tritsch · Ying-Xin Zhang · Graham Ellis-Davies · Dwight E. Bergles

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Abstract The developing cochlea of mammals contains a large group of columnar-shaped cells, which together form a structure known as Kölliker's organ. Prior to the onset of hearing, these inner supporting cells periodically release adenosine 5′-triphosphate (ATP), which activates purinergic receptors in surrounding supporting cells, inner hair cells and the dendrites of primary auditory neurons. Recent studies indicate that purinergic signaling between inner supporting cells and inner hair cells initiates bursts of action potentials in auditory nerve fibers before the onset of hearing. ATP also induces prominent effects in inner supporting cells, including an increase in membrane conductance, a rise in intracellular Ca^{2+}, and dramatic changes in cell shape, although the importance of ATP signaling in non-sensory cells of the developing cochlea remains unknown. Here, we review current knowledge pertaining to purinergic signaling in supporting cells of Kölliker's organ and focus on the mechanisms by which ATP induces changes in their morphology. We show that these changes in cell shape are preceded by increases in cytoplasmic Ca^{2+}, and provide new evidence indicating that elevation of intracellular Ca^{2+} and IP_3 are sufficient to initiate shape changes. In addition, we discuss the possibility that these ATP-mediated morphological changes reflect crenation following the activation of Ca^{2+}-activated Cl^- channels, and speculate about the possible functions of these changes in cell morphology for maturation of the cochlea.

Keywords Cochlea · Supporting cell · ATP · Calcium · Chloride channel · Crenation · Development

N. X. Tritsch · Y.-X. Zhang · D. E. Bergles (✉)
The Solomon H. Snyder Department of Neuroscience,
Johns Hopkins University,
725 North Wolfe Street,
Baltimore, MD 21205, USA
e-mail: dbergles@jhmi.edu

G. Ellis-Davies
Department of Pharmacology and Physiology,
Drexel University College of Medicine,
245 North 15th Street,
Philadelphia, PA 19102, USA

D. E. Bergles
Department of Otolaryngology—Head and Neck Surgery,
Johns Hopkins University,
725 North Wolfe Street,
Baltimore, MD 21205, USA

Introduction

The developing cochlea contains a mass of columnar-shaped cells that lie immediately medial to inner hair cells. Together, these cells form what is known as Kölliker's organ or the Greater Epithelial Ridge (Fig. 1a) [1–3]. This pseudo-stratified epithelium forms shortly after the outgrowth of the cochlear duct from the otocyst, constitutes one of the first identifiable structures in the embryonic cochlea and remains prominent until the onset of hearing. During this period of cochlear development, the cells that populate Kölliker's organ, which we refer to here as inner supporting cells, progressively disappear in a medial-to-lateral and basal-to-apical gradient, most likely as a result of programmed cell death [4–6]. The inner supporting cells immediately adjacent to inner hair cells, known as border and phalangeal cells, are preserved in adults, while the rest of this region transforms into the inner sulcus. Other than participating in the formation of the overlying tectorial membrane by secreting glycoproteins such as otogelin and tectorin [7–9] and serving as progenitors for hair cells [3],

the function of this transient group of cells has remained largely unexplored. Our recent studies suggest that these cells initiate electrical activity in auditory nerve fibers before the onset of hearing [10, 11]. In cochleae isolated from pre-hearing rats, inner supporting cells within Kölliker's organ periodically secrete ATP, which activates purinergic (P2) receptors on neighboring inner hair cells to induce depolarization, Ca^{2+}-dependent release of glutamate from ribbon synapses, and ultimately bursts of action potentials in auditory nerve fibers (Fig. 1b) [10, 11]. This electrical activity is thought to propagate to neurons in auditory nuclei of the brain [12–15], where it has been proposed to participate in multiple aspects of neuronal development, including survival [16–18], maturation of membrane [19, 20], and synaptic properties [21, 22], and refinement of axonal projections [23–26]. Because inner supporting cells release ATP from birth until the onset of hearing in rats [11], and purinergic signaling between inner supporting cells and inner hair cells is necessary to trigger auditory nerve firing for most of the postnatal pre-hearing period [11], spontaneous purinergic signaling in the cochlea is likely to play a prominent role in the development of central auditory pathways before the onset of hearing.

Purinergic receptors are also expressed by outer hair cells, spiral ganglion neurons and various classes of supporting cells [27–29], suggesting that purinergic signaling may influence the development of the cochlea itself. In this review, we discuss how purinergic signaling affects inner supporting cells in the cochlea before the onset of hearing. We review the evidence that spontaneous ATP-mediated inward currents in inner supporting cells are accompanied by an increase in the concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) and changes in cell shape [10, 11], and provide new evidence indicating that a rise in $[Ca^{2+}]_i$ is sufficient to elicit these morphological changes. In addition, we discuss evidence in support of the hypothesis that ATP-evoked changes in the shape of inner supporting cells arise from crenation, the shrinking of cell membranes due to water loss, and highlight the potential functional significance of purinergic signaling in this transient group of supporting cells.

Purinergic receptors in inner supporting cells of the developing cochlea

Extracellular ATP acts on two main classes of purinergic receptors: fast-acting ionotropic (P2X) receptors, which are nonselective cation channels with significant permeability to Ca^{2+}, and slower-acting metabotropic (P2Y) receptors [30]. Binding of ATP to P2Y receptors, which predominantly couple to $G_{q/11}$, activates phospholipase C-β, leading to the production of diacylglycerol and inositol triphosphate (IP_3), IP_3-mediated Ca^{2+} release from intracellular stores and protein kinase C activation. These second messengers can affect many aspects of cellular physiology [30, 31], and increase the permeability of the cell membrane to various ions through modulation or direct gating of K^+, Ca^{2+}, Cl^-, and transient receptor potential (TRP) channels [32–35]. There are multiple isoforms of P2X and P2Y receptors, which differ in molecular structure, kinetics and sensitivity

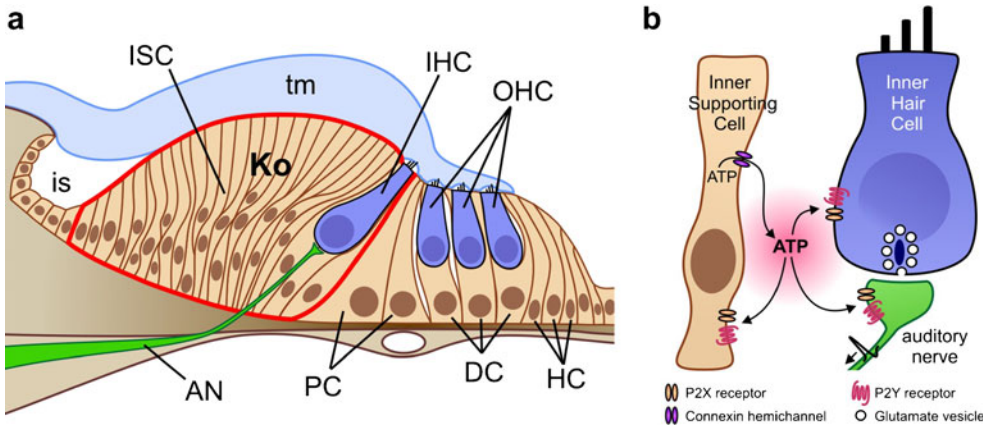

Fig. 1 Supporting cells within Kölliker's organ spontaneously release ATP before the onset of hearing. **a** Diagram of the immature organ of Corti in a postnatal pre-hearing rat cochlea, shown in cross section. *AN* auditory nerve, *DC* Deiters' cell, *HC* Hensen's cell, *IHC* inner hair cell, *is* inner sulcus, *ISC* inner supporting cell, *Ko* Kölliker's organ (*outlined in red*), *OHC* outer hair cell, *PC* pillar cell, *tm* tectorial membrane. Modified with permission from [10]. **b** Schematic of spontaneous purinergic signaling in the developing cochlea. Inner supporting cells in Kölliker's organ spontaneously release ATP into the extracellular space, possibly through connexin hemichannels, where it activates ionotropic (P2X) and metabotropic (P2Y) purinergic receptors in inner hair cells, auditory nerve fibers, and inner supporting cells. Connexin hemichannels and purinergic receptors are depicted on the basolateral membranes for clarity only; the subcellular loci of ATP release and signaling in the developing cochlea in vivo remain unknown. Although ATP signaling within inner hair cells has recently been shown to induce depolarization, Ca^{2+}-dependent release of glutamate from ribbon synapses, and ultimately bursts of action potentials in auditory nerve fibers, its effect on inner supporting cells is less clear

for agonists and antagonists [36]; however, their overlapping expression patterns, their ability to assemble into heteromeric receptors containing different subunits and the dearth of subtype-specific antagonists has hindered the identification of the P2 receptor subtypes involved in many endogenous signaling events.

In situ hybridization and immunocytochemical localization studies have revealed that members of both P2X and P2Y receptor classes are expressed in the supporting cells of the developing organ of Corti (reviewed in [27, 28]). Among these, $P2X_2$ and $P2X_7$ were observed in inner supporting cells and Deiters' cells before the onset of hearing [37–39], $P2Y_4$ was detected in the supporting cells of Kölliker's organ [40] and $P2Y_1$, $P2Y_2$, and $P2Y_4$ were detected in outer sulcus supporting cells of pre-hearing rats [40, 41]. The expression of $P2Y_2$ and $P2Y_4$ receptors by Hensen's, Böttcher's and Claudius' cells in the outer sulcus of organotypic preparations was recently confirmed by showing that application of specific P2 receptor agonists induces a rise in $[Ca^{2+}]_i$ in these various classes of outer supporting cells [42]. In addition, whole-cell voltage-clamp recordings in acutely isolated pre-hearing apical cochlear turns revealed that ATP as well as UTP, a selective agonist of $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ receptors [35], evoke inward currents in inner supporting cells [11], indicating that they express functional P2X and P2Y receptors (Fig. 2). The onset of UTP-evoked responses was consistently delayed relative to those elicited by ATP, suggesting that the current induced by ATP consists of both a rapidly activating ligand-gated conductance as well as a conductance activated indirectly following metabotropic receptor activation. The ATP analog α,β-MeATP did not elicit currents in inner supporting cells, suggesting that these cells do not express $P2X_1$, $P2X_3$, and $P2X_{2/3}$ heteromers [10], and the $P2X_7$ receptor antagonist Brilliant Blue G did not decrease the amplitude or frequency of spontaneous electrical activity in inner supporting cells [10], implying that these receptors do not underlie endogenous purinergic signaling in Kölliker's organ. Together, these results suggest that inner supporting cells express both P2X and P2Y autoreceptors that may become activated in response to the release of ATP from these cells (Fig. 1b). As these receptors have different affinities for ATP [35, 43], the relative contribution of ionotropic and metabotropic receptors to these responses may depend on how far away a cell is from the site of ATP release. The concentration of ATP likely decreases with distance due to dilution and enzymatic degradation (see below), raising the possibility that responses generated in distant cells result primarily from activation of higher affinity P2Y receptors. Further studies will be required to define the full complement of P2 receptors expressed by inner supporting cells before the onset of hearing, as well as the composition of P2 autoreceptors activated in response to the spontaneous release of ATP.

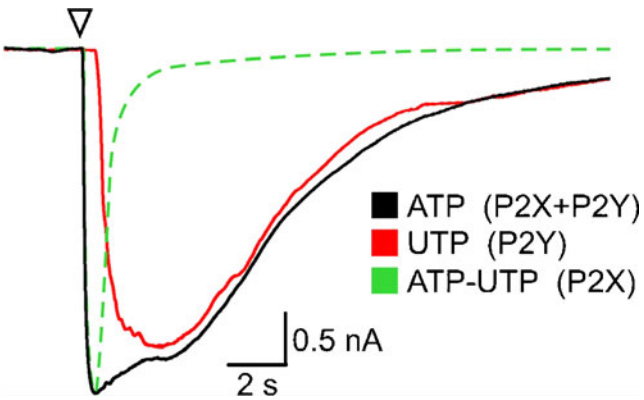

Fig. 2 ATP activates P2X and P2Y receptors in inner supporting cells. Inward currents recorded from an inner supporting cell in response to local application (100 ms, *arrowhead*) of ATP (100 μM, *black trace*) or the P2Y selective agonist UTP (100 μM, *red trace*) onto the apical membrane (membrane potential=−82 mV). UTP-evoked currents occurred with a considerable delay compared to ATP-mediated responses, suggesting that responses to ATP consist of both a rapidly-gating P2X conductance (*dashed green line*, obtained by subtracting the UTP-evoked response from the ATP-evoked response) and a second, delayed conductance triggered indirectly by P2Y receptor activation (*red trace*), probably through an IP_3/Ca^{2+}-dependent signaling cascade. Modified with permission from [10]

Although adenosine (P1) receptors are abundantly expressed in the mature cochlea [44], adenosine does not significantly contribute to the currents recorded from inner supporting cells in the developing cochlea. Decreasing adenosine-mediated signaling by either blocking P1 receptors [10] or preventing ATP hydrolysis (NXT and DEB unpublished observations) does not reduce spontaneous electrical activity in inner supporting cells, and exogenous application of adenosine fails to induce inward currents or morphological changes in these cells [10]. It is possible that adenosine modulates other aspects of this spontaneous activity, perhaps on a slower time scale than has been examined so far.

ATP-evoked intracellular Ca^{2+} transients in inner supporting cells

Activation of purinergic receptors in inner supporting cells triggers both an inward current and a rise in $[Ca^{2+}]_i$ [10, 11]. Time-lapse confocal imaging of cochleae loaded with the Ca^{2+} indicator fluo-4 revealed that spontaneously occurring Ca^{2+} transients initially appear in one to four inner supporting cells before propagating radially to neighboring inner supporting cells at 5 to 20 μm s^{-1}. The size of these events range from 100 to 5,000 μm^2 and Ca^{2+} remains elevated for 3 to 15 s [10, 11]. These Ca^{2+} transients resemble ATP-dependent Ca^{2+} waves that occur

in astrocytes, in that they propagate at similar speeds and rely on functional gap junctions [45, 46]. ATP release from inner supporting cells may be mediated by unpaired gap junctions or connexin hemichannels, as the inward currents induced by spontaneous ATP release are blocked by the gap junction antagonists octanol and carbenoxalone, and are potentiated by reducing extracellular Ca^{2+} [10], a condition that favors hemichannel gating [47]. Because the concentration of Ca^{2+} in the endolymph of the mature cochlea is very low [48], hemichannel-mediated ATP release from supporting cells has been proposed to occur preferentially from their apical surfaces into the endolymph [49]. However, the ionic composition of the endolymph in the mature cochlea (high in K^+ and low in Ca^{2+}) is not achieved until the end of the first postnatal week in mice [50] and rats [51], suggesting that spontaneous ATP release might not be biased toward the endolymphatic compartment in the developing cochlea in vivo. Moreover, although Ca^{2+} concentrations typically observed in the perilymph (~1.3 mM) decrease the open probability of connexin hemichannels [52], spontaneous hemichannel-mediated ATP release is readily observed from inner supporting cells in acutely isolated and cultured cochleae maintained in 1.3 mM extracellular Ca^{2+} [10, 11], indicating that some hemichannels within Kölliker's organ can open under these conditions. ATP release events occur spontaneously at seemingly random intervals and locations throughout Kölliker's organ (Fig. 3a), indicating that they are not likely to occur as a result of focal damage to the cochlear epithelium [41]; nevertheless, the factors responsible for

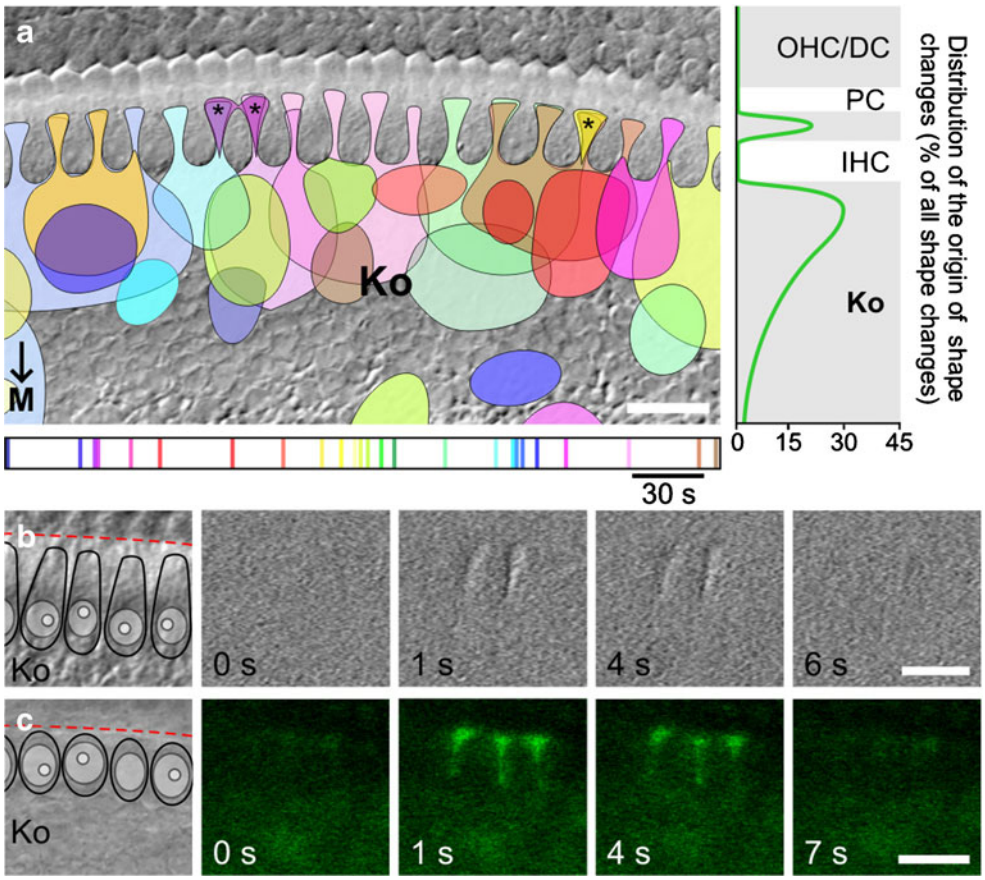

Fig. 3 Spontaneous ATP release occurs randomly throughout Kölliker's organ. **a** Map showing the location and maximum area of individual spontaneous ATP-mediated shape changes that occurred within Kölliker's organ during a 300-s sample period in a P8 cochlea. *Asterisks* indicate brief ATP-independent events that were limited to the processes of phalangeal and border cells. Modiolus (*M*) is indicated for orientation. *Ko* Kölliker's organ. *Scale bar* 20 μm. Relative time of occurrence of individual pseudocolored spontaneous shape changes is shown at bottom. *Right* distribution of locations where spontaneous shape changes originate along the modiolar-to-strial axis (plotted as a percentage of all imaged shape changes). Spontaneous morphological changes originate only in Kölliker's organ (with a bias towards inner hair cells) and in the distal processes of border and phalangeal cells, but were never observed in inner hair cells (*IHC*), outer hair cells (*OHC*), Deiters' cells (*DC*), or pillar cells (*PC*). Modified with permission from [10, 11]. **b** Time-lapse images of brief, spontaneously occurring ATP-independent shape changes that occurred in the processes of border and phalangeal cells surrounding inner hair cells (outlined in first DIC frame; subsequent images represent transmitted light difference signals to highlight changing pixels). **c** In cochleae loaded with the Ca^{2+} indicator fluo-4, these brief shape changes coincide with intracellular Ca^{2+} transients. Location of inner hair cells is depicted in the transmitted DIC image. Time-lapse sequences in **b** and **c** were obtained in different preparations. Scale for **b** and **c**: 15 μm

initiating ATP release in one or a few inner supporting cells and the subcellular location of ATP release sites remain unknown.

Once released into the extracellular space, ATP is likely to be degraded by the sequential action of ecto-nucleoside triphosphate diphosphohydrolases (NTPDases) and ecto-5′-nucleotidases, which hydrolyze extracellular ATP to AMP and AMP to adenosine, respectively [53]. NTDPase 2 is abundantly expressed by hair cells and supporting cells in the adult organ of Corti [54, 55], although it is not yet known what effects these extracellular enzymes have on the spatial and temporal profile of ATP diffusion in the developing cochlea.

P2 receptor activation in the developing cochlea has been studied most extensively in outer supporting cells. In these cells, exogenous ATP triggers a robust elevation of intracellular Ca^{2+} [10, 41, 56–58]. Although activation of both P2X and P2Y receptors can increase $[Ca^{2+}]_i$, ATP-evoked Ca^{2+} transients in these cells persist in the absence of extracellular Ca^{2+}, suggesting that most of the observed rise in $[Ca^{2+}]_i$ is due to release from internal stores following P2Y receptor activation [41, 42, 49, 57, 58]. Intercellular Ca^{2+} waves have also been observed in the outer sulcus of cultured cochleae upon mechanical stimulation or local ATP application [41, 42]. Like Ca^{2+} transients in Kölliker's organ, these Ca^{2+} waves are mediated by extracellular ATP and rely on connexin hemichannels for ATP release [49]. However, intercellular Ca^{2+} waves in outer supporting cells differ from the Ca^{2+} transients observed in Kölliker's organ, in that they do not appear to occur spontaneously during development. Propagation of Ca^{2+} waves through cells in the outer sulcus is thought to require regenerative mechanisms, such as Ca^{2+}-activated ATP release [41, 42]. In accordance with this hypothesis, photolysis of IP_3 in outer supporting cells elevates $[Ca^{2+}]_i$, triggers the release of ATP into the extracellular milieu and initiates a regenerative Ca^{2+} wave that propagates through these cells [49], a process similar to that responsible for Ca^{2+} wave propagation among astrocytes [46]. The presence of this regenerative process ensures that cells within the network experience similar electrical and biochemical signals, which may serve to synchronize their metabolism, influence gene expression [59] and alter the extent of gap junction coupling within the network [60, 61]. Whether the waves of $[Ca^{2+}]_i$ that occur in groups of inner supporting cells rely on a similar regenerative process for propagation is currently unknown.

ATP-mediated morphological changes in inner supporting cells

In addition to activating a membrane conductance and an elevation of $[Ca^{2+}]_i$, ATP also induces striking morphological changes in inner supporting cells. When ATP is released from Kölliker's organ or is applied exogenously, inner supporting cells exhibit a substantial reduction in their diameter that can persist for tens to hundreds of seconds. Similar morphological changes can be evoked by UTP [11], indicating that P2Y receptor activation is sufficient to induce this apparent membrane contraction. The shrinkage of cells within Kölliker's organ also results in the appearance of voids or fluid pockets between adjacent inner supporting cells [10] (Fig. 4a). These events give rise to notable increases and decreases in light intensity when imaged using differential interference contrast (DIC) optics, due to changes in refractive index around cells. As all of the cells in Kölliker's organ undergo these changes when exposed to ATP, the spontaneous optical changes reveal when and where ATP was released along the organ of Corti (see Fig. 3a). Moreover, the maximum size of these events also provides a qualitative indication of the amount of ATP that was released. Alterations in cell shape are most evident along lateral membranes of inner supporting cells, but in certain locations adjacent cells remain tightly associated (Fig. 4a), suggesting that these events do not disrupt sites of cell adhesion or intercellular communication (e.g., gap junctional coupling). Consistent with this hypothesis, no appreciable changes in input resistance accompany ATP-mediated inward currents and shape changes (NXT and DEB, unpublished observations). The ability of ATP to elicit morphological changes in inner supporting cells is regulated with postnatal age, as spontaneous shape changes are only observed during a limited period of development starting a few days after birth until the onset of hearing in rats [11]. This developmental regulation does not stem from changes in purinergic receptor expression levels: while endogenously released ATP and exogenously applied nucleotides evoke large inward currents and intracellular Ca^{2+} transients in supporting cells throughout the postnatal pre-hearing period, they do not elicit membrane contraction at birth [11], suggesting that the cellular machinery required for membrane contraction is up-regulated with age.

The molecular mechanisms that mediate these morphological changes have not been determined, but several mechanisms can be envisioned: shape changes could be triggered by membrane depolarization, $[Ca^{2+}]_i$ elevation or signal transduction cascades activated by P2Y receptors. They might be mediated by voltage-dependent proteins analogous to prestin [62], or Ca^{2+}-dependent molecular motors such as myosin [63]. Alternatively, these pronounced changes in cell volume may be caused by the loss of water through osmosis following intracellular ion efflux.

In addition to the large ATP-evoked shape changes occurring in Kölliker's organ, small morphological changes can also be observed in the distal processes of phalangeal and border cells (see Fig. 3b) [11]. These events are brief and coincide spatially and temporally with short-lived

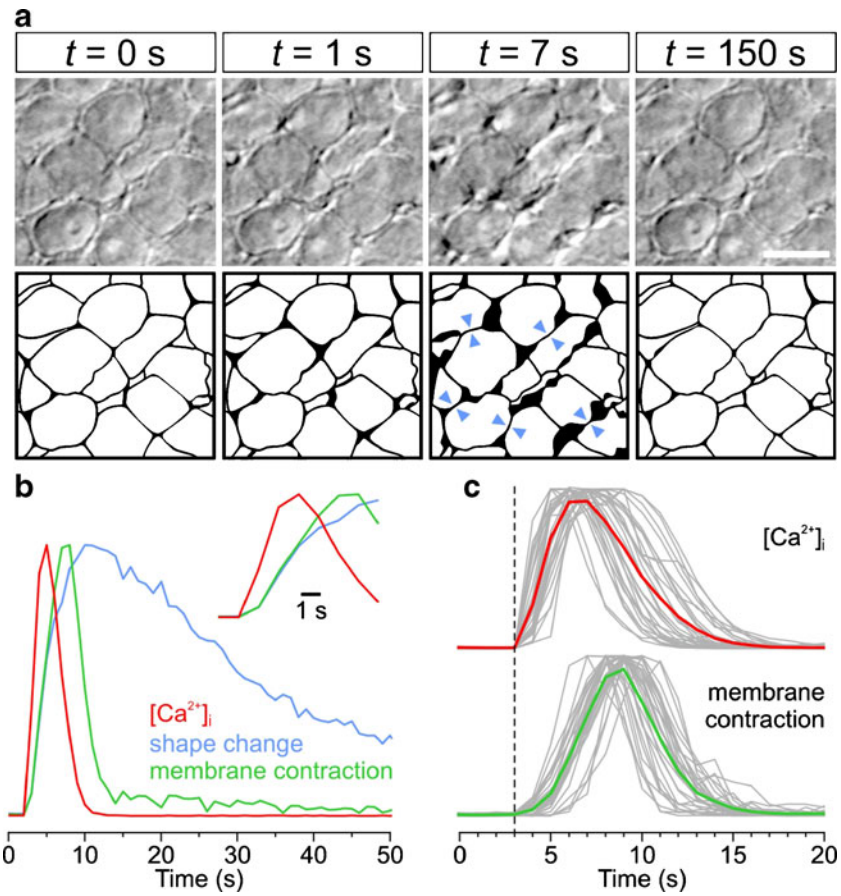

Fig. 4 Contraction of inner supporting cell membranes coincides with $[Ca^{2+}]_i$ elevation. **a** High magnification time-lapse DIC imaging (*top row*) of inner supporting cells during a spontaneous ATP release event at indicated times (*t*). *Bottom row* schematized representation of inner supporting cell somatas (*white*) and extracellular space (*black*) from images shown in top row. Changes in transmitted light properties were restricted to the outline of supporting cells and consist of a contraction of cell membranes and increase in extracellular space. While maximum contraction was observed at $t=7$ s, relaxation of these membranes to their original shape required more than 100 s. Note that many sites of cell contact (*blue arrowheads*) were maintained during these morphological changes. *Scale bar*=8 μm. Modified with permission from [10]. **b** Simultaneous imaging of morphological changes (*blue and green traces*) and $[Ca^{2+}]_i$ fluorescence (*red trace*). Traces were normalized to their peak for display purposes. The *blue and green traces* represent the number of pixels that changed in raw DIC images and subtracted movies, respectively (see the "Methods" section): the *blue trace* illustrates the actual time course of these slow shape changes, while the *green trace* emphasizes the duration of the contraction phase. *Inset* first 7 s following the initial rise in $[Ca^{2+}]_i$ shown on expanded time scale. **c** Overlay of simultaneously-imaged $[Ca^{2+}]_i$ fluorescence (*top*, *gray traces*) and membrane contraction (*bottom*, *gray traces*; obtained from subtracted movies) obtained from $n=29$ large ATP-release events in five cochleae (P8–9). Traces were normalized to their peak. Fluorescence traces were aligned to the first detectable increase in $[Ca^{2+}]_i$ (*black dashed line*). *Average traces shown in color*. Note that initial $[Ca^{2+}]_i$ elevations coincide with, or slightly precede membrane contraction

intracellular Ca^{2+} transients (Fig. 3c). However, these events are not affected by P2 receptor antagonists, indicating that they are distinct from ATP-mediated events in inner supporting cells. Although the mechanism implicated in the initiation of these events is unknown, it is possible they may arise from spontaneous release of Ca^{2+} from internal stores.

Although spontaneous purinergic signaling can depolarize inner supporting cells by up to 50 mV, membrane depolarization is not sufficient to evoke morphological changes in these cells, as neither elevation of extracellular K^+ to 30 mM nor injection of large current steps in inner supporting cells during whole-cell recordings initiates detectable shape changes (NXT and DEB, unpublished observations). Purinergic signaling itself is similarly unlikely to be required for shape changes because the brief morphological events observed in the distal processes of border and phalangeal cells do not require P2 receptor activation. To examine the relationship between cell shape and ATP-evoked Ca^{2+} transients, we simultaneously monitored changes in transmitted light using DIC imaging, and $[Ca^{2+}]_i$ using fluorescence imaging of the membrane-permeant Ca^{2+} indicator fluo-4 AM, in acutely isolated P8–10 cochleae. Nearly all morphological changes (98 %; $n=241$ spontaneous shape changes in 12 preparations) occurred at the same time as, or shortly following,

spontaneous Ca^{2+} transients, suggesting that supporting cells shrink following a rise in intracellular Ca^{2+} (Fig. 4b, c). By contrast, some Ca^{2+} transients (11 %) were not associated with morphological changes in supporting cells. Intracellular Ca^{2+} events that coincided with shape changes were on average threefold larger than those that did not elicit changes in cell shape (712±36 μm^2, n=252 vs. 239±16 μm^2, n=29; P<0.0001, two-sample t test), suggesting that there is a threshold for membrane contraction and that such events are more likely to be associated with large intracellular Ca^{2+} transients. In addition, the duration of active membrane contraction corresponds roughly to the duration of inward currents and the elevation of $[Ca^{2+}]_i$ (Fig. 4b, c and Ref. [10]).

To determine if elevations in $[Ca^{2+}]_i$ mediate these morphological changes, we loaded the cytoplasm of inner supporting cells with a membrane-impermeant form of caged Ca^{2+} (DMNPE-4, 3 mM) through the recording electrode and monitored electrical and morphological responses evoked by brief (50 ms) flashes of ultraviolet (UV) light. Photolysis of caged Ca^{2+} triggered a brief and reversible reduction of cell diameter in the recorded cell as well as in adjacent inner supporting cells (n=8; Fig. 5a–d). As inner supporting cells are extensively coupled through gap junctions [64, 65], it is likely that DMNPE-4 diffused from the recorded cell into neighboring cells. Shape changes induced by Ca^{2+} uncaging were similar in their time course to spontaneous optical changes [10, 11]. In addition, similar morphological changes were evoked upon photo-liberation of caged IP_3 (6-NV-IP_3, 30–50 μM, 5 ms flashes, n=10) (data not shown), suggesting that Ca^{2+} release from intracellular stores is sufficient to mediate morphological changes. Importantly, shape changes were not observed when caged compounds were omitted from the pipette solution and 100-ms UV flashes were applied, excluding the possibility that these events resulted from photo-damage (Fig. 5e). Moreover, shape changes were restricted to inner supporting cells that were directly illuminated by the UV beam (dashed yellow circle in Fig. 5a), implying either that $[Ca^{2+}]_i$ elevation is not sufficient to evoke the release of ATP from inner supporting cells, or that the amount of ATP released from this manipulation is too small to significantly alter the shape of surrounding cells.

Photo-liberation of Ca^{2+} and IP_3 also produced large inward currents in inner supporting cells when maintained at their resting potential (approximately −90 mV; average amplitude: 2.2±0.6 nA, n=8 and 0.8±0.2 nA, n=10, respectively; Fig. 5d), suggesting that inner supporting cells express ion channels that are gated by intracellular Ca^{2+}. Thus, it is likely that this Ca^{2+}-activated conductance contributes to the inward current generated by the activation of P2 receptors, and provides an explanation for why P2Y receptor activation also induces an inward current (Fig. 2). Inner supporting cells cannot be effectively voltage-clamped due to extensive gap junctional coupling with surrounding supporting cells [66], precluding direct measurements of the reversal potential of this Ca^{2+}-activated conductance. This limitation could be overcome by repeating these experiments in the presence of the gap junction blockers such as octanol or carbenoxalone, which increase the membrane resistance of supporting cells [66, 67], if these agents do not inhibit any of the components required for this phenomenon. Nevertheless, given the high resting membrane potential of inner supporting cells, the inward currents induced by Ca^{2+}/IP_3 uncaging could reflect opening of Ca^{2+}-activated Cl^- channels [33] or Ca^{2+}-activated nonselective cation channels, such as TRP channels [68, 69]. The former is a particularly attractive candidate, because it reconciles both consequences of $[Ca^{2+}]_i$ elevation—the inward current (mediated by Cl^- efflux) and the reduction of cell diameter, due to the movement of water. Indeed, Ca^{2+}-activated Cl^- channels have previously been shown to contribute to ATP-mediated inward currents in outer supporting cells [61, 70] and have been implicated in the secretion of electrolytes and water by airway and intestinal epithelia, as well as exocrine glands [33, 71]. In these cells, activation of G protein-coupled receptors (P2Y receptors in the case of airway epithelia) induces Cl^- efflux across apical membranes, which subsequently drags water out of the cytoplasm by osmosis, leading to an overall reduction in cell size. The observed morphological changes in the supporting cells of the cochlear epithelium might therefore result from Cl^- efflux and crenation—the shrinkage of cell membranes due to osmosis. In accordance with this hypothesis, the frequency of spontaneous ATP-mediated shape changes in Kölliker's organ was decreased 87±5 % by the Cl^- channel antagonist 4,4′-diisothiocyano-2,2′-stilbene disulfonic acid (DIDS, 250 μM; n=6, P< 0.001, paired t test). Furthermore, a previous report suggested that inner supporting cells in cultured cochleae are capable of secreting water [72]. However, it is also possible that ATP-induced changes in conductance and cell shape are not inexorably linked. Indeed, outer supporting cells do not undergo detectable shape changes upon ATP stimulation [10, 11] (but see [73]) despite the activation of Ca^{2+}-activated Cl^- channels [61, 70]. An alternative possibility is that these morphological changes result from Ca^{2+}-dependent activation of contractile proteins in inner supporting cells.

Possible roles of ATP-induced changes in the shape of cochlear supporting cells

ATP-induced changes in the shape of inner supporting cells are present in cochleae isolated from both rats [10, 11] and

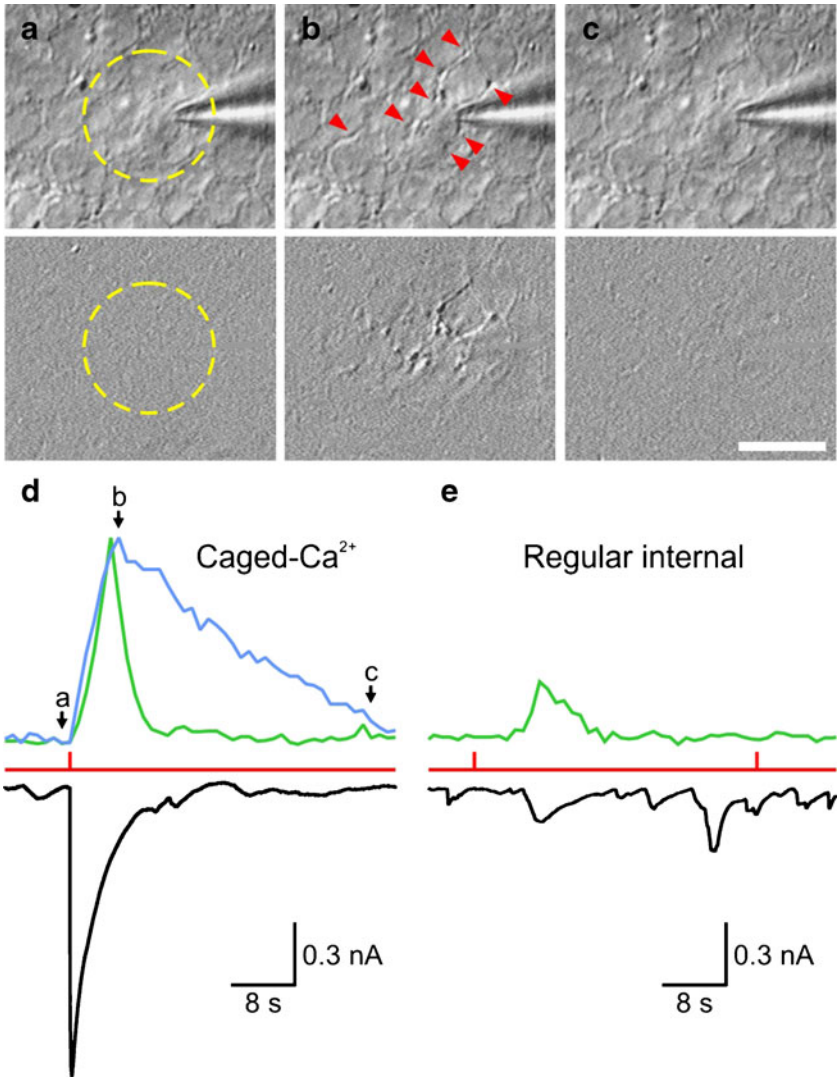

Fig. 5 Intracellular Ca^{2+} elevation is sufficient to evoke morphological changes in inner supporting cells. **a–c** Flash photolysis of caged Ca^{2+} initiates membrane contraction in both the recorded cell and surrounding inner supporting cells (*red arrowheads*). *Top* DIC images 1 s before (**a**), 6 (**b**) and 37 s (**c**) after UV-mediated Ca^{2+} uncaging. Morphological changes in surrounding supporting cells were restricted to the portion of the preparation exposed to UV light (*dashed yellow circle*) and result either from direct photo-liberation of caged Ca^{2+} that diffused from the patched cell through gap junctions or passive diffusion of uncaged Ca^{2+} through gap junctions. *Scale bar* =15 μm. *Bottom* same images after subtraction of a control frame to highlight changing pixels (shape changes). **d** Overlaid membrane currents (*black trace*) and morphological changes (normalized number of changing pixels in raw DIC images and subtracted movies plotted in *blue* and *green*, respectively) evoked upon UV-mediated Ca^{2+} uncaging (50 ms, *red tick mark*) in inner supporting cell shown in frames **a–c**. **e** same as **d** for a supporting cell patched with internal solution devoid of caged compounds. Two 100-ms-long UV flashes were presented

mice (NXT and DEB, unpublished observation), and are preserved in organotypic culture for more than 1 week [10]. Moreover, these events occur during most of the postnatal pre-hearing period [11], suggesting that they are involved in some fundamental aspect of cochlear development; yet, the absence of detailed insight into the mechanisms that mediate such prominent alterations, and the means with which to selectively manipulate this behavior, precludes any definitive conclusion as to their function. If these morphological changes result from membrane crenation due to the net movement of water into the extracellular space, Kölliker's organ may participate in the formation of the endolymph (scala media) or perilymph (scala tympani) as the cochlea increases in size during postnatal development [74]. Through the secretion of electrolytes, inner supporting cells might also contribute to the maturation of the ionic composition of cochlear fluids during the first postnatal week [50]. Alternatively, changes in the shape of inner supporting cells might fulfill a mechanical role. For example, the coordinated action of extracellular ATP on

large groups of inner supporting cells results in the contraction of Kölliker's organ, which could facilitate detachment of the overlaying tectorial membrane before the onset of hearing [2, 75]. These movements can also displace IHCs (NXT and DEB, unpublished observation), which could lead to activation of mechanotransduction channels in hair bundles [76]. The currents evoked by such events would likely contribute to, and perhaps also prolong, ATP-mediated depolarization of inner hair cells and the resulting bursts of activity in auditory nerve fibers during development.

Conclusions

The columnar-shaped supporting cells that make up Kölliker's organ in the developing cochlea spontaneously release ATP into the extracellular space. The resulting activation of purinergic receptors in inner hair cells induces depolarization, Ca^{2+} influx, glutamate release and ultimately bursts of action potentials in primary auditory afferents [10, 11]. This supporting cell-derived activity may play important roles in development of the auditory system by promoting the survival and maturation of auditory neurons and by refining axonal projections in auditory nuclei [16–26]. However, purinergic receptors are expressed by many other cell types within the developing cochlea, suggesting that ATP-mediated signaling also may influence the maturation of this peripheral sensory organ. One target of this signaling appears to be the inner supporting cells themselves, as ATP elicits inward currents, intracellular Ca^{2+} transients and pronounced morphological changes in these cells as a result of activation of purinergic autoreceptors (Fig. 6). These ATP-initiated morphological changes are exhibited only by inner supporting cells during a defined period of development [11], pointing to a unique role for Kölliker's organ in cochlear development. As these changes in cell shape appear to result from crenation, the resulting movement of water into the extracellular space may contribute to the formation of fluid-filled endolymphatic and perilymphatic compartments. A greater understanding of the molecular underpinnings of these responses may help us determine the various roles that purinergic signaling in Kölliker's organ plays in the development of the cochlea during the pre-hearing period.

Methods

All experiments were performed according to protocols approved by the Animal Care and Use Committee at Johns Hopkins University. Apical cochlear turns of P7–10 Sprague–Dawley rats were isolated, imaged and recorded

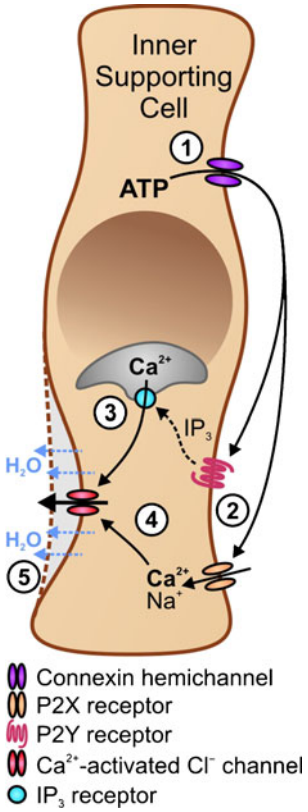

Fig. 6 Model to explain the mechanisms responsible for ATP-evoked morphological changes in inner supporting cells of the developing cochlea. (*1*) ATP is released in the extracellular space, perhaps through the opening of individual gap junction hemichannels. The molecular elements that initiate hemichannel gating are currently unknown. (*2*) Extracellular ATP activates P2X and P2Y receptors on inner supporting cells in an autocrine and paracrine (not shown) manner. Activation of ionotropic P2X receptors leads to a net influx of Na^+ and Ca^{2+} into the cytoplasm. P2Y receptor signaling leads to the production of IP_3. (*3*) IP_3 induces the release of Ca^{2+} from internal stores. (*4*) $[Ca^{2+}]_i$ elevation gates Ca^{2+}-activated Cl^- channels, leading to a net efflux of Cl^- from inner supporting cells resulting in an inward current. (*5*) Cl^- efflux draws water out of the cytoplasm by osmosis, leading to membrane crenation. Connexin hemichannels and purinergic receptors are depicted on the basolateral membranes for clarity only; the subcellular loci of ATP release and signaling in the developing cochlea in vivo remain unknown

from as described previously [10, 11] with the following exceptions. Intracellular Ca^{2+} levels were imaged in acutely isolated cochleae incubated for 30–45 min at room temperature in HEPES-buffered saline containing 2.5 μM fluo-4 AM (Invitrogen), 0.01% (*w*/*v*) pluronic acid F-127 (Invitrogen), and 250 μM sulfinpyrazone (Sigma) using a LSM 510 confocal microscope (Zeiss), and 488 nm laser illumination. Supporting cell morphology was simultaneously monitored using scanning DIC imaging using a photomultiplier tube placed below the sample. Images were acquired at 1 frame per second Changes in cell shape were visualized by subtracting DIC images collected 5 s apart, which approximates the first derivative of light transmit-

tance over time at individual pixels, and quantified by applying a thresholding function to highlight changing pixels. Intracellular Ca^{2+} transients and transmittance changes were normalized to maximum pixel counts at the peak of each spontaneous event (see Fig. 4c). For intracellular Ca^{2+} and IP_3 uncaging, inner supporting cells were recorded in whole-cell mode with glass electrodes (2–4 MΩ) containing (in mM) either 120 K-gluconate, 20 HEPES, 3 DMNPE-4 (a photolabile EGTA derivative [77]), 2.5 $CaCl_2$, 2 MgATP, 0.2 NaGTP, pH 7.3 or 134 K-methanesulfonate, 20 HEPES, 2 $MgCl_2$, 2 Na_2ATP, 0.2 NaGTP, 0.03–0.05 6-NV-IP_3 (caged IP_3 [78]), pH 7.3. Photolysis was performed as described previously [79] using 5–50 ms-long pulses of ~110 mW UV light generated by an argon laser, which was focused to a ~20-μm spot. To control for photo-damage-induced shape changes, supporting cells were recorded from using a K-methanesulfonate-based internal solution containing 0.2 mM EGTA instead of the caged compounds, and were exposed to 100-ms-long UV flashes. DIDS was purchased from Sigma and applied by addition to the superfusing saline.

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References

1. Retzius G (1884) Das Gehororgan der Wirbelthiere. II. Das Gehororgan der Reptilien, der Vogel und Saugethiere. Samson & Wallin, Stockholm
2. Hinojosa R (1977) A note on development of Corti's organ. Acta Otolaryngol 84:238–251
3. Kelley MW (2007) Cellular commitment and differentiation in the organ of Corti. Int J Dev Biol 51:571–583
4. Knipper M, Gestwa L, Ten Cate WJ, Lautermann J, Brugger H, Maier H, Zimmermann U, Rohbock K, Kopschall I, Wiechers B, Zenner HP (1999) Distinct thyroid hormone-dependent expression of TrKB and p75NGFR in nonneuronal cells during the critical TH-dependent period of the cochlea. J Neurobiol 38:338–356
5. Kamiya K, Takahashi K, Kitamura K, Momoi T, Yoshikawa Y (2001) Mitosis and apoptosis in postnatal auditory system of the C3H/He strain. Brain Res 901:296–302
6. Takahashi K, Kamiya K, Urase K, Suga M, Takizawa T, Mori H, Yoshikawa Y, Ichimura K, Kuida K, Momoi T (2001) Caspase-3-deficiency induces hyperplasia of supporting cells and degeneration of sensory cells resulting in the hearing loss. Brain Res 894:359–367
7. Lim DJ (1987) Development of the tectorial membrane. Hear Res 28:9–21
8. El-Amraoui A, Cohen-Salmon M, Petit C, Simmler MC (2001) Spatiotemporal expression of otogelin in the developing and adult mouse inner ear. Hear Res 158:151–159
9. Rau A, Legan PK, Richardson GP (1999) Tectorin mRNA expression is spatially and temporally restricted during mouse inner ear development. J Comp Neurol 405:271–280
10. 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
11. Tritsch NX, Bergles DE (2010) Developmental regulation of spontaneous activity in the mammalian cochlea. J Neurosci 30:1539–1550
12. Lippe WR (1994) Rhythmic spontaneous activity in the developing avian auditory system. J Neurosci 14:1486–1495
13. Jones TA, Jones SM, Paggett KC (2001) Primordial rhythmic bursting in embryonic cochlear ganglion cells. J Neurosci 21:8129–8135
14. Jones TA, Leake PA, Snyder RL, Stakhovskaya O, Bonham B (2007) Spontaneous discharge patterns in cochlear spiral ganglion cells before the onset of hearing in cats. J Neurophysiol 98:1898–1908
15. 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
16. 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
17. Glueckert R, Wietzorrek G, Kammen-Jolly K, Scholtz A, Stephan K, Striessnig J, Schrott-Fischer A (2003) Role of class D L-type Ca2+ channels for cochlear morphology. Hear Res 178:95–105
18. Seal RP, Akil O, Yi E, Weber CM, Grant L, Yoo J, Clause A, Kandler K, Noebels JL, Glowatzki E, Lustig LR, Edwards RH (2008) Sensorineural deafness and seizures in mice lacking vesicular glutamate transporter 3. Neuron 57:263–275
19. Leao RN, Sun H, Svahn K, Berntson A, Youssoufian M, Paolini AG, Fyffe RE, Walmsley B (2006) Topographic organization in the auditory brainstem of juvenile mice is disrupted in congenital deafness. J Physiol 571:563–578
20. Walmsley B, Berntson A, Leao RN, Fyffe RE (2006) Activity-dependent regulation of synaptic strength and neuronal excitability in central auditory pathways. J Physiol 572:313–321
21. Erazo-Fischer E, Striessnig J, Taschenberger H (2007) The role of physiological afferent nerve activity during in vivo maturation of the calyx of held synapse. J Neurosci 27:1725–1737
22. McKay SM, Oleskevich S (2007) The role of spontaneous activity in development of the endbulb of held synapse. Hear Res 230:53–63
23. Leake PA, Snyder RL, Hradek GT (2002) Postnatal refinement of auditory nerve projections to the cochlear nucleus in cats. J Comp Neurol 448:6–27
24. Friauf E, Lohmann C (1999) Development of auditory brainstem circuitry. Activity-dependent and activity-independent processes. Cell Tissue Res 297:187–195
25. Kandler K (2004) Activity-dependent organization of inhibitory circuits: lessons from the auditory system. Curr Opin Neurobiol 14:96–104
26. Kandler K, Clause A, Noh J (2009) Tonotopic reorganization of developing auditory brainstem circuits. Nat Neurosci 12:711–717
27. Housley GD, Bringmann A, Reichenbach A (2009) Purinergic signaling in special senses. Trends Neurosci 32:128–141
28. Housley GD, Marcotti W, Navaratnam D, Yamoah EN (2006) Hair cells—beyond the transducer. J Membr Biol 209:89–118
29. Lee JH, Marcus DC (2008) Purinergic signaling in the inner ear. Hear Res 235:1–7
30. Abbracchio MP, Burnstock G, Verkhratsky A, Zimmermann H (2009) Purinergic signalling in the nervous system: an overview. Trends Neurosci 32:19–29
31. Erb L, Liao Z, Seye CI, Weisman GA (2006) P2 receptors: intracellular signaling. Pflugers Arch 452:552–562
32. Hille B (1994) Modulation of ion-channel function by G-protein-coupled receptors. Trends Neurosci 17:531–536

33. Hartzell C, Putzier I, Arreola J (2005) Calcium-activated chloride channels. Annu Rev Physiol 67:719–758
34. Venkatachalam K, Montell C (2007) TRP channels. Annu Rev Biochem 76:387–417
35. Abbracchio MP, Burnstock G, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, Knight GE, Fumagalli M, Gachet C, Jacobson KA, Weisman GA (2006) International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. Pharmacol Rev 58:281–341
36. Burnstock G (2007) Purine and pyrimidine receptors. Cell Mol Life Sci 64:1471–1483
37. Housley GD, Luo L, Ryan AF (1998) Localization of mRNA encoding the P2X2 receptor subunit of the adenosine 5'-triphosphate-gated ion channel in the adult and developing rat inner ear by in situ hybridization. J Comp Neurol 393:403–414
38. Jarlebark LE, Housley GD, Thorne PR (2000) Immunohistochemical localization of adenosine 5'-triphosphate-gated ion channel P2X(2) receptor subunits in adult and developing rat cochlea. J Comp Neurol 421:289–301
39. Nikolic P, Housley GD, Thorne PR (2003) Expression of the P2X7 receptor subunit of the adenosine 5'-triphosphate-gated ion channel in the developing and adult rat cochlea. Audiol Neurootol 8:28–37
40. Huang LC, Thorne PR, Vlajkovic SM, Housley GD (2010) Differential expression of P2Y receptors in the rat cochlea during development. This issue of Purinergic Signalling
41. Gale JE, Piazza V, Ciubotaru CD, Mammano F (2004) A mechanism for sensing noise damage in the inner ear. Curr Biol 14:526–529
42. Piazza V, Ciubotaru CD, Gale JE, Mammano F (2007) Purinergic signaling and intercellular Ca2+ wave propagation in the organ of Corti. Cell Calcium 41:77–86
43. Khakh BS, Burnstock G, Kennedy C, King BF, North RA, Seguela P, Voigt M, Humphrey PP (2001) International union of pharmacology. XXIV. Current status of the nomenclature and properties of P2X receptors and their subunits. Pharmacol Rev 53:107–118
44. Vlajkovic SM, Abi S, Wang CJ, Housley GD, Thorne PR (2007) Differential distribution of adenosine receptors in rat cochlea. Cell Tissue Res 328:461–471
45. Giaume C, Venance L (1998) Intercellular calcium signaling and gap junctional communication in astrocytes. Glia 24:50–64
46. Fiacco TA, McCarthy KD (2006) Astrocyte calcium elevations: properties, propagation, and effects on brain signaling. Glia 54:676–690
47. Gomez-Hernandez JM, de Miguel M, Larrosa B, Gonzalez D, Barrio LC (2003) Molecular basis of calcium regulation in connexin-32 hemichannels. Proc Natl Acad Sci U S A 100:16030–16035
48. Wangemann P, Schacht J (1996) Homeostasic mechanisms in the cochlea. In: Dallos P, Popper AN, Fay R (eds) Springer handbook of auditory research: the cochlea. Springer, Berlin, pp 130–185
49. 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
50. Anniko M, Wroblewski R (1986) Ionic environment of cochlear hair cells. Hear Res 22:279–293
51. Bosher SK, Warren RL (1971) A study of the electrochemistry and osmotic relationships of the cochlear fluids in the neonatal rat at the time of the development of the endocochlear potential. J Physiol 212:739–761
52. Verselis VK, Trelles MP, Rubinos C, Bargiello TA, Srinivas M (2009) Loop gating of connexin hemichannels involves movement of pore-lining residues in the first extracellular loop domain. J Biol Chem 284:4484–4493
53. Schetinger MR, Morsch VM, Bonan CD, Wyse AT (2007) NTPDase and 5'-nucleotidase activities in physiological and disease conditions: new perspectives for human health. Biofactors 31:77–98
54. Vlajkovic SM, Thorne PR, Sevigny J, Robson SC, Housley GD (2002) NTPDase1 and NTPDase2 immunolocalization in mouse cochlea: implications for regulation of p2 receptor signaling. J Histochem Cytochem 50:1435–1442
55. Vlajkovic SM, Thorne PR, Sevigny J, Robson SC, Housley GD (2002) Distribution of ectonucleoside triphosphate diphosphohydrolases 1 and 2 in rat cochlea. Hear Res 170:127–138
56. Dulon D, Moataz R, Mollard P (1993) Characterization of Ca2+ signals generated by extracellular nucleotides in supporting cells of the organ of Corti. Cell Calcium 14:245–254
57. Ashmore JF, Ohmori H (1990) Control of intracellular calcium by ATP in isolated outer hair cells of the guinea-pig cochlea. J Physiol 428:109–131
58. Lagostena L, Mammano F (2001) Intracellular calcium dynamics and membrane conductance changes evoked by Deiters' cell purinoceptor activation in the organ of Corti. Cell Calcium 29:191–198
59. Dolmetsch RE, Xu K, Lewis RS (1998) Calcium oscillations increase the efficiency and specificity of gene expression. Nature 392:933–936
60. Sato Y, Santos-Sacchi J (1994) Cell coupling in the supporting cells of Corti's organ: sensitivity to intracellular H + and Ca2+. Hear Res 80:21–24
61. Lagostena L, Ashmore JF, Kachar B, Mammano F (2001) Purinergic control of intercellular communication between Hensen's cells of the guinea-pig cochlea. J Physiol 531:693–706
62. Dallos P, Fakler B (2002) Prestin, a new type of motor protein. Nat Rev Mol Cell Biol 3:104–111
63. Huxley HE (2000) Past, present and future experiments on muscle. Philos Trans R Soc Lond B Biol Sci 355:539–543
64. Forge A, Becker D, Casalotti S, Edwards J, Marziano N, Nevill G (2003) Gap junctions in the inner ear: comparison of distribution patterns in different vertebrates and assessment of connexin composition in mammals. J Comp Neurol 467:207–231
65. Jagger DJ, Forge A (2006) Compartmentalized and signal-selective gap junctional coupling in the hearing cochlea. J Neurosci 26:1260–1268
66. Glowatzki E, Cheng N, Hiel H, Yi E, Tanaka K, Ellis-Davies GC, Rothstein JD, Bergles DE (2006) The glutamate-aspartate transporter GLAST mediates glutamate uptake at inner hair cell afferent synapses in the mammalian cochlea. J Neurosci 26:7659–7664
67. Mammano F, Goodfellow SJ, Fountain E (1996) Electrophysiological properties of Hensen's cells investigated in situ. NeuroReport 7:537–542
68. Yan HD, Villalobos C, Andrade R (2009) TRPC channels mediate a muscarinic receptor-induced afterdepolarization in cerebral cortex. J Neurosci 29:10038–10046
69. Fleig A, Penner R (2004) The TRPM ion channel subfamily: molecular, biophysical and functional features. Trends Pharmacol Sci 25:633–639
70. Sugasawa M, Erostegui C, Blanchet C, Dulon D (1996) ATP activates a cation conductance and Ca(2+)-dependent Cl- conductance in Hensen cells of guinea pig cochlea. Am J Physiol 271: C1817–C1827
71. Kidd JF, Thorn P (2000) Intracellular Ca2+ and Cl- channel activation in secretory cells. Annu Rev Physiol 62:493–513
72. Sobkowicz HM, Loftus JM, Slapnick SM (1993) Tissue culture of the organ of Corti. Acta Otolaryngol Suppl 502:3–36
73. Dulon D, Blanchet C, Laffon E (1994) Photo-released intracellular Ca2+ evokes reversible mechanical responses in supporting cells

of the guinea-pig organ of Corti. Biochem Biophys Res Commun 201:1263–1269
74. Mu MY, Chardin S, Avan P, Romand R (1997) Ontogenesis of rat cochlea. A quantitative study of the organ of Corti. Brain Res Dev Brain Res 99:29–37
75. Lim DJ, Rueda J (1992) Structural development of the cochlea. In: Romand R (ed) Development of auditory and vestibular systems 2. Elsevier, Amsterdam, pp 33–58
76. Geleoc GS, Holt JR (2003) Developmental acquisition of sensory transduction in hair cells of the mouse inner ear. Nat Neurosci 6:1019–1020
77. Ellis-Davies GC, Barsotti RJ (2006) Tuning caged calcium: photolabile analogues of EGTA with improved optical and chelation properties. Cell Calcium 39:75–83
78. Kantevari S, Hoang CJ, Ogrodnik J, Egger M, Niggli E, Ellis-Davies GC (2006) Synthesis and two-photon photolysis of 6-(ortho-nitro-veratryl)-caged IP3 in living cells. Chembiochem 7:174–180
79. Huang YH, Sinha SR, Fedoryak OD, Ellis-Davies GC, Bergles DE (2005) Synthesis and characterization of 4-methoxy-7-nitro-indolinyl-D-aspartate, a caged compound for selective activation of glutamate transporters and *N*-methyl-D-aspartate receptors in brain tissue. Biochemistry 44:3316–3326