ATP-mediated cell–cell signaling in the organ of Corti: the role of connexin channels

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Abstract Connexin 26 (Cx26) and connexin 30 (Cx30) form hemichannels that release ATP from the endolymphatic surface of cochlear supporting and epithelial cells and also form gap junction (GJ) channels that allow the concomitant intercellular diffusion of Ca^{2+} mobilizing second messengers. Released ATP in turn activates G-protein coupled P2Y_2 and P2Y_4 receptors, PLC-dependent generation of IP_3 , release of Ca^{2+} from intracellular stores, instigating the *regenerative* propagation of intercellular Ca^{2+} signals (ICS). The range of ICS propagation is sensitive to the concentration of extracellular divalent cations and activity of ectonucleotidases. Here, the expression patterns of Cx26 and Cx30 were characterized in postnatal cochlear tissues obtained from mice aged between P5 and P6. The expression gradient along the longitudinal

axis of the cochlea, decreasing from the basal to the apical cochlear turn (CT), was more pronounced in outer sulcus (OS) cells than in inner sulcus (IS) cells. GJ-mediated dye coupling was maximal in OS cells of the basal CT, inhibited by the nonselective connexin channel blocker carbenoxolone (CBX) and absent in hair cells. Photostimulating OS cells with caged inositol (3,4,5) tri-phosphate (IP_3) resulted in transfer of ICS in the lateral direction, from OS cells to IS cells across the hair cell region (HCR) of medial and basal CTs. ICS transfer in the opposite (medial) direction, from IS cells photostimulated with caged IP_3 to OS cells, occurred mostly in the basal CT. In addition, OS cells displayed impressive rhythmic activity with oscillations of cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) coordinated by the propagation of Ca^{2+} wavefronts sweeping repeatedly

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through the same tissue area along the coiling axis of the cochlea. Oscillations evoked by uncaging IP_3 or by applying ATP differed greatly, by as much as one order of magnitude, in frequency and waveform rise time. ICS evoked by direct application of ATP propagated along convoluted cellular paths in the OS, which often branched and changed dynamically over time. Potential implications of these findings are discussed in the context of developmental regulation and cochlear pathophysiology.

Keywords Cochlea · Hearing · DFNB1 · Mouse models · Development · Organotypic cultures · CELABs

Introduction

Cochlea, hair cells, and sound transduction The mammalian cochlea is a snail-shaped inner ear structure divided in three chambers, namely the *scala vestibuli*, the *scala tympani*, and the *scala media* [1]. The scala tympani is connected by the cochlear aqueduct to the subarachnoidal space of the cranial cavity, which is filled with cerebrospinal fluid. The scalae vestibuli and tympani are connected through an opening at the apical end of the cochlea, called the *helicotrema* and are both filled with *perilymph*, a fluid whose composition is similar to that of cerebrospinal fluid. The scala media is filled with *endolymph*, an unusual extracellular fluid containing 150 mM K^+ , 2 mM Na^+ and as little as 20 μM extracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_o$) [2].

Insulation of endolymph from perilymph is dependent on cells forming the tubular *cochlear duct* epithelium, which are connected by a network of tight and adherens junctions near their surface facing scala media. The epithelium comprises the *organ of Corti* (OoC), a sensory organ which rests on the *basilar membrane* and is responsible for *sound transduction* [3, 4]. The OoC has the form of an epithelial ridge encompassing highly specialized sensory inner hair cells (IHCs) and *outer hair cells* (OHCs), which are characterized by a mechanosensory organelle composed of a *stereociliary bundle*. In the medial direction from the OoC, the cochlear duct epithelium comprises *interdental* cells of the *spiral limbus*. In the lateral direction from the OoC, it comprises *spiral prominence* cells and marginal cells of the *stria vascularis* (SV). The rest of the duct wall is formed by the *Reissner's membrane*.

The SV is responsible for exporting K^+ to endolymph and generation of the *endocochlear potential* (EP) [5, 6]. The EP is an electrical potential difference between the endolymphatic and perilymphatic compartments of the cochlea, which appears around postnatal (P) day 5 (P5) and increases progressively to reach adult levels in excess of +80 mV by P18 [7]. Both the EP and the high

endolymphatic $[\text{K}^+]$ are key factors for the *mechanotransduction process* performed by the hair cells [8, 9], for the large potential difference between the endolymph and the cytoplasm of IHCs and OHCs drives K^+ through mechanically gated channels located in the second and third rows of stereocilia [10]. Hair cells then release K^+ through K^+ channels in their basolateral membrane [11]. IHCs are the genuine sensory cells and are presynaptic to *spiral ganglion neurons* (SGNs), the primary conveyors of auditory information to the central nervous system [12, 13]. OHCs provide the local mechanical amplification process, driven by the motor protein *prestin*, required for the high sensitivity and sharp frequency selectivity of mammalian hearing [14, 15].

Cochlear GJ networks All cells providing mechanical support to hair cells are designated as *supporting cells*. In the mature OoC, these include inner *phalangeal* cells, inner and outer *pillar* cells, outer *phalangeal* cells (also known as *Deiters' cells*), as well as *Hensen's*, *Böttcher's* and *Claudianus' cells*. The inner phalangeal cells completely surround the IHCs. The outer phalangeal cells form cups holding the synaptic poles of the OHCs and send fine process, or phalanges, to the *reticular lamina* (RL). This thin and stiff cytoplasmic plate is a mosaic formed by the apposing phalangeal process of outer pillar cells and outer phalangeal cells, both of which seal the endolymphatic poles of the hair cells, extending laterally from the innermost row of OHCs to the Hensen's cells. Thus, only stereociliary bundles of OHCs emerge above the RL [1].

Non-sensory cells in the cochlear duct form intercellular networks coupled by GJ channels [16–18], which mediate the transfer of ions, metabolites and second messengers between cells [19–21]. The *connective tissue* GJ network starts to develop around birth and comprises interdental cells and fibrocytes in the spiral limbus, fibrocytes of the spiral ligament, basal and intermediate cells of the SV. The *epithelial* GJ network forms around embryonic day 16 (E16) and connects all supporting cells in the OoC as well as adjacent epithelial cells [22]. In the hearing cochlea, the epithelial GJ network apparently subdivides further in two separate, medial and lateral, buffering compartments, which are thought to be individually dedicated to the homeostasis of IHCs and OHCs [23]. Furthermore, in the so-called potassium recycle *hypothesis*, the epithelial GJ network is presumed to intervene in the cycling of K^+ following mechanotransduction [24]. Indeed, several classes of supporting cells in the sensory epithelium express glial fibrillary acidic protein (GFAP) [25], a classic marker for astrocytes, and are thought to perform similar spatial buffering of K^+ [16, 18]. Although the widespread localization of GJ channels, K/Cl cotransporters [26], and aquaporins [27] could provide a passive

mechanism of K^+ buffering and osmotic homeostasis [23], it is worth emphasizing that currently *there is no direct evidence* for the cycling of K^+ ions through cochlear GJ channels.

Connexins, deafness, and mouse models Most cell–cell channels in the cochlear GJ networks are composed of two types of integral transmembrane proteins subunits, namely Cx26 and Cx30 [28], which share 77% amino acid identity and may assemble to form heteromeric and heterotypic channels [29]. The genes encoding Cx26 (GJB2) and Cx30 (GJB6) are found within 50 kb of each other in the DFNB1 locus. DFNB1 mutations, which are almost as frequent as those causing cystic fibrosis, account for around 50% of genetic cases of severe to profound non syndromic hearing loss in various ethnic groups [30].

Mouse models with defective expression of Cx26 or Cx30 confirmed that they are essential for hearing. Thus, targeted ablation of Cx26 in the epithelial GJ network of Cx26^{OtogCre} mice [31] (obtained by crossing Cx26^{loxP/loxP} mice with Otog–Cre mice line expressing the Cre gene under the control of the murine Otog promoter) induced cell death accompanied by epithelial breaches shortly after the onset of hearing (which, in mice, occurs at P12 [32]), along with progressive and significant hearing loss ranging from 30 to 70 dB. The apoptotic process affected first the two supporting cells that surround the IHCs and later extended to OHCs and supporting cells around them. IHCs were preserved in adult Cx26^{OtogCre} mice, and cell death was not detected at any stage either in SGNs, in the fibrocytes of the spiral limbus and spiral ligament or in the SV. EP values developed normally up to P12–P13, thereafter decreased significantly in parallel with the appearance of epithelial breaches that compromised the integrity of the endolymphatic compartment. Endolymphatic K^+ concentration was also significantly lower in adult Cx26^{OtogCre} mice compared to controls. Likewise, expression of the Cx26R75W dominant negative mutation in mice, obtained by injection of the transgene into fertilized mouse eggs [33] (also called male pronucleus injection), resulted in deafness associated with significant histological abnormalities within the inner ear including degeneration, by postnatal week 7, of the OHCs. In a conditional (c) Cx26 null mouse model [34] (obtained by crossing Cx26loxP/loxP mice with R26cre-ERT mice in which Cre is activated in the presence of the synthetic estrogen 4-hydroxytamoxifen) the earliest cell death occurred around P14 in OHCs and their surrounding supporting cells in the middle CT. Hence, death rapidly spread to the basal CT so that all types of cells had disappeared from the OoC of cCx26 null mice a few months after birth. Unlike Cx26^{OtogCre} mice, in cCx26 null mice peripheral nerve fibers and soma of SGNs at corresponding cochlear locations were completely degen-

erated as well, whereas hair cells in the apical CT were relatively preserved [34].

In Cx30(–/–) mice [35] (obtained by deletion of the Cx30 coding region) the cochleae were morphologically indistinguishable from those of Cx30(+/-) and WT mice up to P17. However, Cx30(–/–) mice failed to develop any measurable EP, and adults had significantly decreased endolymphatic K^+ levels. Auditory thresholds worsened from 84 dB at P17–18 to more than 100 dB in adult Cx30(–/–) mice, hence hearing loss was more severe in Cx30(–/–) mice than in Cx26^{OtogCre} mice. Correspondingly, the apoptotic process in Cx30(–/–) mice, albeit delayed by about 4 days relative to Cx26^{OtogCre} mice, affected ultimately both IHCs and OHCs. Of note, transgenic expression of extra copies of the Cx26 gene from a modified bacterial artificial chromosome (BAC) in a Cx30(–/–) background restored cochlea development and hearing [36].

Permeability and selectivity of GJ channels Although mouse models confirmed that Cx26 and Cx30 are essential for auditory function, as well as survival and development of the OoC [31, 33–35], *the physiopathological mechanisms leading to deafness when connexins are absent or mutated remain unclear* [28]. Thus, deafness and lack of EP in Cx30(–/–) mice correlate with: (a) disruption of the endothelial barrier of the capillaries supplying the SV before EP onset; (b) significant down-regulation of Bhmt; and (c) local increase in homocysteine, a known factor of endothelial dysfunction [37], *with no obvious link to GJ channel function*. Likewise, the K^+ recycle hypothesis is challenged by the identification of Cx26 recessive deafness mutants, e.g., V84L [38], which retain partial channel function [39] and are *as permeable to K^+ as the WT channels*. Yet, the transfer of the Ca^{2+} -mobilizing second messenger IP₃ (and possibly other signaling molecules) is impaired between the cells expressing these mutant proteins [40].

Connexin hemichannels, ATP release, and cell–cell signaling While the exact function of inner ear connexins remains unclear [28], we note that Cx26 and Cx30 also form unpaired connexons [41], i.e., non-junctional connexin *hemichannels* [42, 43]. Using organotypic cultures of the cochlea from mice with defective expression of pannexin 1 (Px1), P2X₇ receptors, Cx30 or Cx26, Anselmi et al. [44] demonstrated that, in response to activation of a P2Y/PLC/IP₃/Ca²⁺ signaling cascade, hemichannels formed by these connexins release ATP from the endolymphatic surface of cochlear supporting and epithelial cells, whereas GJ channels allow the concomitant diffusion of Ca²⁺ mobilizing second messengers across these coupled cells. Nanomolar levels of ATP on the endolymphatic surface of the OoC, which have been linked to sound exposure [45],

Fig. 1 Schematic representation of ICS propagation across the network of GJ coupled cochlear supporting and epithelial cells. CBX, carbenoxolone, is a non-specific inhibitor of connexin hemichannels and GJ channels [116]; ARL67156, 6-*N,N*-diethyl-D- β -y-dibromomethylene adenosine tri-phosphate, originally named FPL 67156, is a commercially available inhibitor of ecto-ATPases [117]. Apyrase is an enzyme that catalyzes the hydrolysis of ATP to yield

AMP and inorganic phosphate [118, 119]. La^{3+} is a blocker of connexin hemichannels [75] that does not affect GJ channels when applied extracellularly to cochlear cultures [44]. P2YR, G-protein coupled P2Y receptor; IP_3R , receptor for inositol 1,4,5-trisphosphate (also commonly known as triphosphoinositol, abbreviated *InsP₃* or *IP₃*; reproduced with publisher's permission from Mammano and Anselmi [120])

in turn activate G-protein coupled P2Y_2 and P2Y_4 receptors (see also article by Huang et al., this volume), PLC-dependent generation of IP_3 and release of Ca^{2+} from intracellular stores, ensuing in the *regenerative* propagation of ICS across the networks of cochlear supporting and epithelial cells (Fig. 1) [40, 44, 46, 47] (see also articles by Lin-Chien Huang et al. and by Jonathan Gale, this volume).

The range of ICS propagation is sensitive to the concentration of divalent cations in the extracellular medium and is controlled by ATP degradation [46] due to ectonucleotidases [44], a heterologous family of enzymes involved in extracellular nucleotide hydrolysis that are abundantly expressed on the endolymphatic surface of the OoC [48, 49] (see also article by Mary G. O'Keeffe et al., this volume). By manipulating the bathing medium one can either reduce the rate of ATP degradation, by inhibiting ectonucleotidases with ARL67156, or else increase this rate, e.g. by supplementing soluble apyrase. ICS propagation is blocked altogether by cytoplasm acidification or by membrane permeable compounds, such as CBX, which inhibit both GJ channels and hemichannels. By contrast, inhibitors of the P2Y receptors such as suramin or blockers of hemichannels such as La^{3+} (both applied extracellularly) confine ICS propagation to cells directly coupled to the stimulated cells through GJ channels [40, 44].

Here we used an all-optical approach based on a combination of immunofluorescence, gap-FRAP (fluorescence recovery after photobleaching) assays, photostimulation with caged IP_3 , direct application of ATP and Ca^{2+} imaging, to further our understanding of purinergic signaling in organotypic cultures of the immature mouse cochlea.

Materials and methods

Reagents and drugs Membrane permeable AM ester derivatives of fluo-4 and calcein were obtained from Invitrogen/Molecular Probes (Milan, Italy). Membrane permeable caged inositol (3,4,5) tri-phosphate (caged- IP_3 -AM) was purchased from Alexis (San Diego, CA, USA). Carbenoxolone (CBX), pluronic F-127 and sulfinpyrazone were purchased from Sigma (Milan, Italy).

Cochlear organotypic cultures and transverse sections Cochleae were dissected from P5 mouse pups in ice-cold Hepes buffered (10 mM, pH 7.2) Hanks' balanced salt solutions (HBSS, Sigma, Milan, Italy) and placed onto coverslips coated with Cell Tak (Becton Dickinson, Milan, Italy). Cell Tak was diluted to a final concentration of

136 µg/ml. Cultures were incubated in Dulbecco's modified Eagle's medium DMEM/F12 (Invitrogen, Leek, The Netherlands), supplemented with FBS 5% and maintained at 37°C overnight.

For transverse sections, cochleae dissected from P6 mouse pups were fixed in 4% paraformaldehyde (PFA) and decalcified over night in 0.3 M ethylenediaminetetraacetic acid (EDTA). After three washes in phosphate buffer solution (PBS), cochleae were included in 3% agarose dissolved in PBS, and cut perpendicularly to the modiolar axis in 100 µm sections using a vibratome (VT 1000S, Leica) set at speed 6 (on a scale of 10) and frequency also 6 (on a scale of 10), and equipped with Gillette Platinum double-edge razor blades.

Immunohistochemistry and confocal imaging Cultures and slices were fixed in 4% PFA for 20 min at room temperature and rinsed in PBS containing 2% bovine serum albumin (rinse solution). Cultures were permeabilized for 1 h at room temperature with 0.1% Triton X-100, dissolved in rinse solution, whereas slices were permeabilized for 3 h. In both cultures and slices, Cx26 or Cx30 were immunolabeled by overnight incubation at 4°C with specific polyclonal antibodies diluted in rinse solution (2.5 µg/ml). For labeling of connexin hemichannels, slices were incubated overnight at 4°C with primary polyclonal antibodies directed against peptides corresponding to the extracellular loops of Cx26 (CELAb; a generous gift of Dr Guy Tran Van Nhieu, Institute Pasteur, Paris, France) [50]. Primary antibodies were omitted in negative controls. Cultures were then washed three times in PBS (5 min each time), whereas slices were washed for 1 h each time. For cultures, secondary antibodies (Alexa Fluor® 488 goat anti-rabbit IgG, Invitrogen, Cat. No. A-11008) were applied at 5 µg/ml for 1 h at room temperature, whilst F-Actin was stained by incubation with Texas Red-X phalloidin (Invitrogen, Cat. No. T7471) and nuclei were stained with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI, Invitrogen, Cat. No. D1306), both diluted in rinse solution (1:200). Invitrogen Cat. No. 51-2800 is a 13 amino acid synthetic peptide derived from the C-terminus of the mouse Connexin 26 protein. Invitrogen Cat. No. 71-2200 is a synthetic peptide derived from the unique C-terminus of the mouse Connexin 30 protein. This antibody is specific for the Connexin 30. Cross-reactivity with the highly related Connexin 26 protein or with other Connexin family members has not been detected.

For slices, incubation periods were extended to overnight. After washing a further three times in PBS, samples were mounted onto glass slides with mounting medium (FluorSave™ Reagent, Merk, Cat. No. 345789) and imaged using a confocal microscope (TCS SP5, Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany) equipped with an oil-immersion

objective (either 40× HCX PL APO 1.25 N.A. or 63× HCX PL APO 1.4 N.A., Leica). Alexa Fluor® 488 was excited by the 488 nm line of an air-cooled argon-ion laser (225 mW, Series 800, National Laser Company, UT, USA) and its fluorescence emission was collected in a spectral window between 495 and 540 nm. Texas Red was excited by the 561 nm line of a diode-pumped solid-state (DPSS) laser (10 mW, Model YLK 6110 T, LasNova 60 yellow series 60, LASOS Lasertechnik GmbH, Jena, Germany) and its emission was collected between 600 nm and 690 nm. DAPI was excited by the 405 nm line of a diode laser module (50 mW, Radius 405-50, Coherent Inc., CA, USA) and its emission was collected between 410 nm and 440 nm. Laser line intensities and detector gains were carefully adjusted to minimize signal bleed through outside the designated spectral windows.

Focal laser irradiation For focal irradiation of live cochlear cultures, which we used either for photostimulation with caged IP₃ or for photobleaching calcein, the output of a TTL-controlled semiconductor lased module (either 20 mW, 379 nm, part number FBB-375-020-FS-FS-1-1, RGLase LLC, CA, USA; or 50 mW, 405 nm, part number LGT 405-60, LG-Laser Technologies GmbH, Kleinostheim, Germany) was injected into a UV permissive fiber optic cable (either multimode step index 0.22 N.A., 105 µm core, part number AFS105/125YCUSTOM, Thorlabs GmbH, Dachau, Germany; or single mode 0.1 N.A., Mode Field Diameter (MFD) 3.2±0.5 µm, part number P1-405A-FC-2, Thorlabs). Fiber output was projected onto the specimen plane by an aspheric condenser lens (either 20 mm effective focal length, part number ACL2520, Thorlabs; or 5 mm effective focal length, part number HPUCO-23A-S-6.2AS, LG-Laser Technologies GmbH, Kleinostheim) and the re-collimated beam was directed onto a dichromatic mirror (either 400 or 440 dclp, Chroma) placed at 45° just above the objective lens of the microscope. By carefully adjusting the position of the fiber in front of the aspheric lens we projected a sharp image of the illuminated fiber core (spot) onto the specimen focal plane selected by the (infinity-corrected) objective lens. Under these conditions, the fiber optic diameter determined accurately the laser irradiated area, which encompassed one to few cells.

Calcein and FRAP For staining with calcein, live cultures were incubated for 10 min at 37°C in EXM (an extracellular medium containing 138 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 0.3 mM NaH₂PO₄, 0.4 mM KH₂PO₄, 10 mM Hepes–NaOH and 6 mM D-glucose, pH 7.2, 320 mOsm) supplemented with 5 µM calcein-AM, 250 µM sulfinpyrazone and 0.01 w/v pluronic F-127. For recording, cultures were transferred on the stage of an upright fluorescence microscope (BX51, Olympus) and perfused in EXM for

20 min at 2 ml/min to allow for de-esterification, and thereafter maintained in still EXM at room temperature. Calcein fluorescence was excited and detected using a U-MGFHQ filter cube (Olympus) incorporating a BP460–480 excitation filter, a DM485HQ dichromatic mirror and a BA495–540HQ barrier (emission) filter. Cultures were imaged with a 60× water immersion objective (0.90 NA, Olympus Lumplan FL) and fluorescence emission was monitored with a cooled CCD camera (Sensicam QE, PCO AG, Kelheim, Germany). In all experiments, the effects of photobleaching due to sample illumination in the 460–480 nm window were controlled by carefully selecting the most appropriate inter-frame interval (4 s) while controlling light exposure (50 ms) with a mechanical shutter triggered by the frame-valid (FVAL) signal of the CCD camera. Baseline fluorescence in the 495–540 nm emission window was recorded for 2 min, followed by focal laser irradiation at 405 nm to bleach intracellular calcein [44, 51, 52]. Laser irradiation intervals were adjusted so as to cause 50% photobleaching of the mean baseline fluorescence, which required 0.5 s for OS cells and 1.2 s for IS cells. Fluorescence recovery after photobleaching (FRAP) was monitored for up to 10 min. Image sequences were acquired using software developed in the laboratory, stored on disk and processed off-line using the Matlab 7.0 software package (The MathWorks, Inc., Natick, MA, USA) as described in Refs. [44, 53]. In particular, for the analysis of

FRAP experiments we delineated a region of interest (ROI) inside the bleached (*b*) area, plus a ROI in a proximal unbleached (*u*) area, and we computed ratios of fluorescence intensities (f_b/f_u) at each time point.

Ca²⁺ imaging Cochlear cultures were incubated for 60 min at 37° in EXM supplemented with fluo-4AM (16 μM) and caged IP₃ AM (5 μM). The incubation medium contained pluronic F-127 (0.01%, w/v), and sulfinpyrazone (250 μM) to prevent dye sequestration and secretion [54]. For recording, cultures were transferred to the stage of an upright fluorescence microscope (BX51, Olympus) and perfused in EXM for 20 min at 2 ml/min to allow for de-esterification. Fluo-4 fluorescence was excited at 470 nm by a fast-switching monochromator (Polychrome V, Till Photonics, Martinsried, Germany) and directed onto the sample through a 505 dxr dichromatic mirror (Chroma, Rockingham, VT, USA). Fluorescence emission was selected by a HQ520/40 M filter (Chroma, Rockingham, VT), centered around a 520 nm wavelength, to form fluorescence images on a scientific-grade CCD camera (SensiCam; PCO AG) using a 20× water immersion objective (NA 0.95, LumPlan FL, Olympus). For uncaging experiments, baseline (pre-stimulus) fluorescence emission (f_0) was recorded for 2 s, thereafter a UV laser pulse of 200 ms was applied to release IP₃ and fluorescence emission was monitored for up to 21 sec. Since intense UV illumination can damage cells and trigger ICS also in the

Fig. 2 Cx26 immunoreactivity. *Top*: maximal projection rendering of two consecutive midmodiolar confocal optical sections, taken at 1 μm intervals in the indicated CTs, from cochlear preparations of P6 mice. Cx26 expression was probed by a Cx26 selective antibody (green), nuclei were stained with DAPI (blue) and actin filaments with Texas Red conjugated phalloidin (red). *Scale bars* 30 μm. *Bottom* Cx26

immunolabeling of P5 cochlear cultures, processed after 1 day in vitro. Images are maximal projection rendering of confocal optical sections selected from a z stack of 20 images taken at 0.5 μm intervals. *OoC* organ of Corti, *IS* inner sulcus, *HCR* hair cell region, *OS* outer sulcus. *Scale bars* 30 μm

Fig. 3 Cx30 immunoreactivity. *Top* maximal projection rendering of two consecutive midmodiolar confocal optical sections, taken at 1- μ m intervals in the indicated cochlear turns, from cochlear preparations of P6 mice. Cx30 expression was probed by a Cx30 selective antibody (green), nuclei were stained with DAPI (blue) and actin filaments with Texas Red conjugated phalloidin (red). Scale bars, 30 μ m. *Bottom*

Cx30 immunolabeling of P5 cochlear cultures, processed after 1 day in vitro. Images are maximal projection rendering of confocal optical sections selected from a *z* stack of 20 images taken at 0.5 μ m intervals. *OoC* organ of Corti, *IS* inner sulcus, *HCR* hair cell region, *OS* outer sulcus. Scale bars 30 μ m

absence of caged IP_3 [46], we performed a series preliminary control experiments to find the minimal UV dose sufficient for uncaging IP_3 , and determined that, under our experimental conditions, it corresponded to the above-mentioned laser pulse duration of 200 ms. To establish a safety margin, we additionally verified that no Ca^{2+} signal was evoked by UV pulses of the same intensity and duration up to 2 s if caged IP_3 was omitted from the loading solution. Image sequences were acquired using software developed in the laboratory, stored on disk and processed off-line using the Matlab 7.0 software package (The MathWorks, Inc., Natick, MA, USA) as described in Refs. [47, 55]. In particular, signals were measured as relative changes of fluorescence emission

intensity ($\Delta f/f_0$), where f_0 is initial (pre-stimulus) fluorescence, f is fluorescence at post-stimulus time t and $\Delta f = f - f_0$ [56].

Ratiometric Ca^{2+} imaging For ATP stimulation experiments organ cultures were incubated for 30 min at 37° in EXM with fura-2 AM (16 μ M) Ca^{2+} indicator, with pluronic F-127 and sulfinpyrazone as for the fluo-4 AM-based Ca^{2+} imaging. Fura-2 fluorescence was excited alternatively at 360 nm and 380 nm by a fast-switching monochromator (Polychrome V, Till Photonics, Martinsried, Germany) and directed onto the sample through a dichromatic mirror (470 dcrx, Chroma, Rockingham, VT,

Fig. 4 Cx26 hemichannels at the endolymphatic surface of the OoC. Three-dimensional reconstruction of the OoC in the basal CT obtained by the maximal projection rendering of 14 confocal optical sections, taken at 1.5 μ m intervals in a slice cut from a P6 mouse cochlea. Cx26

hemichannel expression was probed by CELAbs (green) and actin filaments were stained with Texas Red conjugated phalloidin (red). *IS* inner sulcus, *HCR* hair cell region, *OS* outer sulcus, *RL* reticular lamina, *OHCs* outer hair cells, *IHCs* inner hair cells. Scale bar 10 μ m

Fig. 5 Probing GJ channel function by gap-FRAP assays. **a** Fluorescence images of P5 organotypic cultures from basal CTs imaged after 1 day in vitro (*top*) immediately after the delivery of a photobleaching laser pulse and (*bottom*) 3 min into the recovery period. Regions of interest (ROIs), delineated inside the bleached area (b) and in a proximal unbleached area (u) to compute fluorescence intensity ratios (f_b/f_u), are shown superimposed on the *top* images. *IS* inner sulcus, *HCR* hair cell region. Scale bar in upper left panel is 30 μ m and applies to all images in this figure. **b** Plots of f_b/f_u vs. time in the apical CT (red traces), middle CT (blue traces), and basal CT (green traces) in IS cells (*left*) and OS cells (*right*). Downward arrowheads mark time of laser pulse delivery. Solid lines are averages of $n=3$ cultures, dashed lines indicate 1 S.D. confidence intervals

USA). Fluorescence emission was selected at around 510 nm using an interference filter (D510/40 m, Chroma, Rockingham, VT). Signals were measured as dye emission ratio changes, $\Delta R = R - R_0$, where R is emission intensity excited at 360 nm divided by the intensity excited at 380 nm and R_0 indicates pre-stimulus ratio [56].

Results

Overview We focused on a region medial to the IHCs which, at this developmental stage, is occupied by a transient structure known as Kölliker's organ or greater

epithelial ridge [23, 57]. In keeping with the definitions used in our previous publications, cells medial to the IHCs and populating Kölliker's organ are broadly referred to as IS cells. We also investigated supporting and epithelial cells

Fig. 6 The FRAP process does not take place in IHCs bathed in normal EXM, and is inhibited in IS and OS cells exposed to CBX. **a** Fluorescence images of a P5 cochlear culture from the middle CT imaged after 1 day in vitro (*top*) immediately after the delivery of a photobleaching laser pulse to an IHC and (*bottom*) 9 min into the recovery period. Regions of interest (ROIs), delineated inside the bleached area (b) and in a proximal unbleached area (u) to compute fluorescence intensity ratios (f_b/f_u), are shown superimposed on the *top* image. *IS* inner sulcus, *HCR* hair cell region. Scale bar in upper panel is 30 μ m and applies to both images in this figure. **b** Plots of f_b/f_u vs. time for the IHC shown in **a** (orange trace), together with a representative IS cell (cyan trace) and OS cell (purple trace) from cultures exposed to 100 μ M CBX. Downward arrowheads mark time of laser pulse delivery

lateral to OHCs, hereafter referred to as OS cells, comprised between OHCs and the cochlear lateral wall. All experiments were performed on inner ear tissues from C57BL/6 mice aged between P5 and P6. Live cell imaging experiments were performed at room temperature (22–24°C). Unless otherwise stated, results are expressed as mean $\pm$ standard deviation (S.D.).

Inner ear connexin expression It has been previously documented that development of the cochlear GJ system precedes the functional maturation of the murine inner ear [22], which takes place between the second and third postnatal week [58]. To better characterize connexin expression in our experimental model, we performed immunolabeling of Cx26 and Cx30 with selective antibodies. Labeling was similar, but not identical, for Cx26 (Fig. 2) and Cx30 (Fig. 3).

For both connexins, expression was evident in several sub-compartments of the cochlear duct even at this early post natal stage that precedes by one week the onset of hearing [32]), in keeping with previous findings (see for example Refs. [23, 59–65]) and with the notion that intercellular connections by GJ proteins are crucial for maturation of different tissues [66]. In particular, our confocal images highlight the expression of connexins at point of contacts between supporting and epithelial cells of the OoC on both sides of the HCR (which comprises the RL) as well as in basal and intermediate cells of the SV. Furthermore, both Cx26 and Cx30 showed marked immunoreaction in the spiral limbus and the spiral ligament. Immunolabeling intensity decreased markedly in OS cells along the *longitudinal* axis, from the *basal* to the *apical* end of the cochlea. However, this expression gradient was less evident in IS cells, which showed intense immunolabeling

Fig. 7 Ca^{2+} responses evoked by focal photostimulation of OS cells with caged IP_3 in the apical (left panels), middle (middle panels) and basal (right panels) CTs of P5 cochlear cultures; HCR hair cell region (delineated by dashed lines in the top panels); IS inner sulcus, OS outer sulcus. Shown are fluo-4 relative fluorescence changes ($\Delta f/f_0$) encoded as shown by their respective color scale bars. Times of image capture, relative to stimulus offset, are shown at left. Scale bar in upper right panel is 50 μm and applies to all pseudocolor images in this figure

in all CTs. IHCs and OHCs showed no sign of immunoreactivity to Cx26 or Cx30 antibodies, consistent with the notion that sensory cells are not coupled by GJ to any other cell in the OoC.

Using antibodies against Cx26 extracellular loop peptides (CELAbS) [50], we detected immunofluorescence signals corresponding to unpaired connexons (i.e., connexin hemichannels) in the cell plasma membrane. In particular, CELAbS decorated the endolymphatic surface of the epithelium, except the hair cells, from IS cells across the RL to OS cells, with maximal intensity in the basal CT (Fig. 4).

Data in Figs. 2, 3, and 4 are representative of samples obtained from $n \geq 3$ different mice for each condition, identically processed according to the protocols described in the “Materials and methods” section.

Probing GJ channel function by gap-FRAP assays To determine the efficacy of cell–cell coupling, we performed gap-FRAP assays [51] in P5 cochlear cultures after loading them with the acetoxymethyl (AM) ester of calcein. This polyanionic fluorescein derivative exhibits fluorescence essentially independent of pH between 6.5 and 12, has about six negative and two positive charges at pH 7 (net charge -4 , MW 622) and permeates through GJ channels in the immature OoC [44]. To minimize dye loss through connexin hemichannels at the surface of the epithelium (Fig. 4), these recordings were obtained from cultures bathed in extracellular medium (EXM) containing 2 mM CaCl_2 [67, 68] (for composition, see the “Materials and methods” section). Following delivery of a 405-nm laser pulse to a restricted tissue area, the

Fig. 8 Ca^{2+} responses evoked by focal photostimulation of IS cells with caged IP_3 in the apical (left panels), middle (middle panels), and basal (right panels) CTs of P5 cochlear cultures; HCR hair cell region (delineated by dashed lines in the top panels); OS outer sulcus, IS inner sulcus. Shown are fluo-4 relative fluorescence changes ($\Delta f/f_0$) encoded as shown by their respective color scale bars. Times of image capture, relative to stimulus offset, are shown at left. Scale bar in upper right panel is 50 μm and applies to all pseudocolor images in this figure

intracellular calcein fluorescence was partially restored via diffusion of the indicator dye through GJ channels from adjacent unbleached IS cells (Fig. 5).

Solid lines in Fig. 5b are averages of results obtained from $n=3$ cultures at each location, dashed lines indicate 1 S.D. confidence intervals. Incomplete recovery of fluorescence intensity is ascribed to the fraction of the calcein pool which is not available for intercellular transfer (immobile fraction), due to trapping into subcellular organelles and/or binding to subcellular structures [52]. No recovery of fluorescence intensity occurred after photobleaching calcein in IHCs (Fig. 6a), consistent with lack of immunolabeling with connexin antibodies in

cochlear sensory cells (Figs. 2 and 3). Likewise, FRAP in IS and OS cells was inhibited by pre-incubating cochlear cultures for 20 min in EXM supplemented with 100 μ M CBX (Fig. 6b). Note that light absorption is an exponential function of depth within the tissue. This phenomenon is responsible for the small residual downward peak, visible in all three traces presented in Fig. 6b, which is due to dye remix within the cytoplasm of the photobleached cells.

Thus, our experiments clearly indicate that both IS cells and OS cells are dye-coupled in all CTs of P5 cochlear organotypic cultures, albeit the immobile fraction is substantially larger in the IS.

Fig. 9 Ca^{2+} dynamics in basal turn OS cells of a P5 cochlear culture after delivery of three UV flashes to uncage IP_3 in different cells of the same view field. **a** fluo-4 relative fluorescence changes ($\Delta f/f_0$) obtained by maximal intensity projection of 200 consecutive frames acquired in a 20 s interval and encoded as shown by the color scale bar. **b** $[\text{Ca}^{2+}]_i$ oscillation profiles: each trace represents $\Delta f/f_0$ changes vs. time in one of the 23 labeled cells along a linear microcircuit **a**. **c** and **d** are oscillation amplitude and frequency histograms, respectively, derived from data in **b**. **e** Scatter plot of oscillation frequency vs. amplitude obtained by pooling data in **c,d**. **f** Matrix of correlation coefficients (normalized covariance matrix) derived from the 23 traces in **b**; correlation coefficient values are encoded according to the scale bar shown

Ca²⁺ signaling The OoC of the developing cochlea is the site of intense Ca²⁺ signaling activity [69]. IP₃ is a key intermediate that mediates ATP-dependent Ca²⁺-responses of cochlear sensory [70] and non-sensory cells [40, 47, 71, 72] (Fig. 1). To trigger and visualize Ca²⁺ signals associated with the IP₃ signal transduction cascade, we co-loaded cochlear cultures with the AM esters of (1) a photolabile IP₃ precursor (caged IP₃) and (2) fluo-4, a visible light-excitable intracellular Ca²⁺ sensor [56]. We tested ICS propagation while bathing cochlear cultures in ECM, a medium obtained by replacing 2 mM Ca²⁺ in EXM with an endolymph-like concentration of Ca²⁺ (20 μ M). We photostimulated a small number of supporting cells by focusing ultraviolet (UV) laser light (375 nm, 200 ms) within a well-defined area in the OoC. Signals were quantified as $\Delta f/f_0$, where Δf is fluorescence emission change relative to initial fluorescence intensity f_0 . Focal IP₃ uncaging elicited ICS that spread to unirradiated cells, as previously shown [44]. Here, we further determined that the same UV dose elicited different Ca²⁺ responses in different CTs, also depending on the site of irradiation. Thus, photostimulating supporting OS cells in the *middle* and *basal* CT elicited propagation of ICS in the medial direction across the HCR and consequent activation of Ca²⁺ responses in IS cells adjacent to the IHCs (Fig. 7 and corresponding animation in [Online Resource 1](#)). Ca²⁺ responses in IS cells were detected with delays ranging from 1.8 s to 3.7 s following delivery of the UV stimulus to OS cells (2.9 s $\pm$ 0.9 s, $n=4$). The transfer of ICS from OS to IS cells happened rarely in the *apical* CTs compared to medial and basal CTs, where we observed it more consistently, which correlates well with immunofluorescence data (Figs. 2 and 3).

Focusing the UV laser spot on IS cells in the proximity of the IHCs triggered ICS that remained confined to the IS area in both *apical* and *middle* CTs. Instead, transfer of Ca²⁺ signals in the lateral direction, from IS to OS cells, occurred in response to photostimulation of IS cells in *basal* CTs (Fig. 8).

These results are consistent with the higher connexin immunoreactivity of OS cells in the basal CT, at this stage. Ca²⁺ responses in OS cells were detected with delays ranging from 1.1 to 3.9 s relative to the delivery of the UV stimulus to IS cells (2.7 s $\pm$ 1.1 s, $n=7$). In these conditions, we also observed the propagation of ICS through the OS region along the *longitudinal* axis, both towards the apex and toward the base of the cochlea ([Online Resource 2](#)) and occasionally traversing the same subset of cells more than once.

We also investigated the effects of stimulating repeatedly the same OS area, but avoiding multiple irradiations of the same set of cells with UV light. Three laser pulses delivered at ~4-min intervals in the OS of the basal CT triggered prominent activity in a sub-population identified as Claudius' cells (Fig. 9). Under these conditions, Ca²⁺

transients in a given cell (due to the opening of IP₃ receptor channels and subsequent release of Ca²⁺ from intracellular stores [47]) peaked repeatedly, yielding remarkably regular *self-sustained* [Ca²⁺]_i oscillations (Fig. 9b) with amplitudes mostly comprised between 0.14 and 0.24 $\Delta f/f_0$ (Fig. 9c). Analysis of inter-peak intervals yielded [Ca²⁺]_i oscillation frequencies comprised in a remarkably narrow range from 0.18 to 0.22 Hz (Fig. 9d,e). The spatial counterpart of [Ca²⁺]_i oscillations were striking ICS that propagated across these cells (see animation in [Online Resource 3](#)). ICS traveled from cell to cell along curvilinear paths, henceforth designated as "ICS microcircuits", repeatedly and with impressive regularity, thus imparting a high degree of coherence to fluorescence signals emitted by adjacent cells (Fig. 9f). The speed of ICS propagation along a microcircuit was not constant and ranged between 24 μ m/s to 34 μ m/s.

By contrast, the pattern of Ca²⁺ transients evoked by uncaging IP₃ in IS cells remained unchanged in the limited time frame of these recordings (~20 s), and similar to the

Fig. 10 Ca²⁺ responses of middle turn IS cells in a P5 cochlear culture evoked by the last in a series of three UV flashes delivered to uncage IP₃ in different cells of the same view field. **a** [Ca²⁺]_i profiles: each trace represents $\Delta f/f_0$ fluo-4 changes vs. time in one of 40 cells. **b** Peak amplitude histogram derived from data in **a**

Fig. 11 Ca^{2+} responses evoked by prolonged bath application of ATP (100 nM, 20 min) to apical turn OS cells in a P5 culture. **a** Images of fura-2 relative fluorescence ratio changes (ΔR), encoded as shown by the color scale bar, obtained by maximal intensity projection of 20 consecutive frames acquired at the start of ATP application (*left*) and in between two successive volleys of $[\text{Ca}^{2+}]_i$ oscillations. *IS* inner sulcus, *HCR* hair cell region. *Scale bar* 25 μm . **b** (*left*) Fura-2 traces from six randomly selected cells in each one of the three OS zones delineated in **a**; *arrowheads* mark inter-burst Ca^{2+} elevations. (*right*) Rate histograms derived from an ensemble of 100 cells in each zone represent the probability of detecting a $[\text{Ca}^{2+}]_i$ oscillation peak at the corresponding time; **c** normalized covariance matrices for 15 randomly selected cells of zone 3 computed in the two time intervals marked as T_1 (volley onset) and T_2 (volley offset) in **b**

one depicted in Fig. 10, irrespective of the number of UV flashes (one to three) delivered to this area.

Finally, we report the effects of directly stimulating cochlear cultures with extracellular ATP. For this type of experiments, which did not require the use of UV light to photolyse caged compounds, tissues were loaded with the (AM ester of) the UV excitable ratiometric Ca^{2+} sensor fura-2

(see the “Materials and methods” section). Prolonged delivery of ATP (100 nM, up to 20 min) through the ECM perfusion evoked volleys of $[\text{Ca}^{2+}]_i$ oscillations in most OS cells (Fig. 11).

Based on similarity of responses, we grouped OS cells in three relatively homogeneous zones (Fig. 11a, *left*). The duration of $[\text{Ca}^{2+}]_i$ oscillations was zone-dependent

Fig. 12 Averages of Ca^{2+} response characteristics for OS cells stimulated with 100 nM ATP, computed over $n=100$ cells in each zone. Waveforms in top row are spline interpolations (thick lines) to mean Ca^{2+} transient data (symbols) bounded by 1 S.D. confidence intervals (thin lines)

(Fig. 11b). Longest volleys (>6 min) were detected in zone 3 cells (identified as Claudius' cells in Fig. 9). During the first two minutes from the onset of ATP delivery, the majority of OS cells exhibited highly correlated $[\text{Ca}^{2+}]_i$ oscillations (Fig. 11c, left). Correlation decreased progressively over time (Fig. 11c, right), thereafter cells fell into a quiescent phase almost abruptly. Silent periods between volleys were interrupted by sudden bursts of $[\text{Ca}^{2+}]_i$ elevations that propagated rapidly and radially from a trigger cell to a limited cluster of neighbors both in zone 2 and in zone 3 (Fig. 11a, right; marked by arrowheads in

Fig. 11b). Thus, during prolonged exposure to ATP at sub-micromolar concentration, Ca^{2+} signaling in OS cells toggled between volley and burst mode of operation. Waveforms, amplitude, and frequency histograms of $[\text{Ca}^{2+}]_i$ oscillations evoked by ATP stimulation are shown in Fig. 12, which summarizes data pooled from $n=100$ cells in each zone.

Note that frequency distributions in Fig. 12 do not overlap with that in Fig. 9. Indeed, the slow $[\text{Ca}^{2+}]_i$ oscillations evoked in Claudius' cells (zone 3) by 100 nM ATP (Fig. 13a) differed substantially from the self-

Fig. 13 Averages of Ca^{2+} transient waveforms in zone 3: **a** evoked by 100 nM ATP (data in Fig. 12) or **b** self-sustained after photostimulation with caged IP_3 (data in Fig. 9b). Shown are spline interpolations (thick lines) to average Ca^{2+} transient data (symbols) bounded by 1 S.D. confidence intervals (thin lines). **c** Mean traces from **a** and **b** are shown superimposed and normalized to peak amplitudes. Note different time scales

sustained oscillations triggered by the uncaging of IP_3 characterized by approximately threefold higher mean frequency (Table 1), skewed waveforms (Fig. 13b) and approximately tenfold faster rise times (Fig. 13c).

The high degree of correlation observed at the onset of ATP-evoked $[\text{Ca}^{2+}]_i$ oscillations in the OS is consistent with most cells reacting nearly simultaneously to the

arrival of ATP through the perfusion. Progressive loss of coherence simply reflects the relentless accumulation of phase differences among individual cells, consistent with the broad frequency distributions in Fig. 12. However, correlation did not vanish altogether (Fig. 11c), suggesting that a fraction of the OS population remained partially synchronized through intercellular coupling [73]. That this is the case is shown by ICS propagation along microcircuits, which often branched and evolved dynamically over time (Fig. 14a), in contrast to the more stable microcircuits delineated by the uncaging of IP_3 (Online Resource 3).

Short OS microcircuits, confined to distances of about 120 μm , were commonly observed, however also longer ones, up to 450 μm , were occasionally detected. ICS propagated with variable speed along a given microcircuit (Fig. 14b). However, mean values were similar throughout the OS region (Fig. 14c) and significantly lower than corresponding values for ICS propagation speed along microcircuits following photostimulation with caged IP_3 (Table 1).

These observations were consistent across $n \geq 3$ experiments for each condition.

Discussion

Summary of results

In the present study, we first characterized the expression patterns of Cx26 and Cx30 in tissue samples from mice aged between P5 and P6 using antibodies selective for these two connexins (Figs. 2, 3 and 4). We report a decrease of immunoreactivity along the longitudinal axis from basal to apical CTs. This observation accords well with the fact that: (a) functional maturation of the cochlea requires differentiation and proper organization of non-sensory cells in the OoC and cochlear lateral wall; (b) acquisition and maturation of mechanoelectrical transduction in hair cells occur in a gradient from the base to the apex of the cochlea (with IHCs differentiating prior to OHCs) [74].

The connexin expression gradient was more pronounced in OS cells, whereas IS cells displayed a more uniform

Table 1 Average properties of Claudius' cell responses (mean $\pm$ S.D.)

	IP_3 uncaging	100nM ATP
$[\text{Ca}^{2+}]_i$ oscillations		
Frequency (Hz)	0.221 ± 0.021	0.058 ± 0.036
Inter-peak interval (s)	4.9 ± 0.5	17.2 ± 10.6
Peaks/min	12 ± 1 ($n=23$ cells in 1 culture)	3.5 ± 2.1 ($n=100$ cells in 1 culture)
ICS microcircuits		
propagation speed ($\mu\text{m/s}$)	31.1 ± 5.6 ($n=41$ cells in 3 cultures)	8.8 ± 1.6 ($n=47$ cells in 4 cultures)

Fig. 14 ICS microcircuits. **a** Examples of microcircuits delineated in zone 1 (left) and zones 2–3 (right) during ATP application (100 nM); scale bars 10 μm . **b** three-dimensional pseudo-line-scan plots obtained by plotting color-coded fura-2 normalized ratio signals from each pixel along the microcircuit line vs. time. **c** Averages of ICS propagation speed in $n=3$ cultures; error bars represent SD

connexin distribution. This is consistent with the notion that, at this early postnatal stage, this region hosts a relatively uniform population of tall columnar cells forming Kölliker's organ. These transient cells are replaced by cuboidal cells as Kölliker's organ is retracted and the inner

sulcus proper becomes clearly defined in the mature OoC [23, 57]. In accord with these semi-quantitative observations, our gap-FRAP assays (Fig. 5), show faster FRAP in basal OS cells, indicative of a higher degree of GJ coupling in this region. Dye coupling was absent in IHCs and inhibited in IS and OS cells exposed to CBX (Fig. 6).

Next, we investigated the effects of photostimulating either OS or IS cells with caged IP_3 . Transfer of ICS in the lateral direction, from OS cells to IS cells across the HCR, was mostly evident in the medial and basal CTs (Fig. 7 and Online Resource 1). We detected ICS transfer in the opposite direction, from IS cells to OS cells, mostly in the basal CT (Fig. 8 and Online Resource 2). La^{3+} , which blocks connexin hemichannels [75] but does not affect GJ channels when applied extracellularly to cochlear cultures (Fig. 1), prevents ICS transfer across the HCR [44], implying that diffusion of released ATP is the principal effector of cell–cell signaling across the HCR.

Following stimulation with caged IP_3 , OS cells in the basal CT, particularly Claudius' cells, displayed an impressive rhythmic activity characterized by a high degree of temporal and spatial coherence (Fig. 9). High frequency $[\text{Ca}^{2+}]_i$ oscillations in Claudius' cells were coordinated by the rapid propagation of ICS, at precise time intervals of ~ 5 s, along curvilinear microcircuits with primary orientation parallel to the longitudinal axis of the cochlea (Online Resource 3). This activity was not mirrored in IS cells (Fig. 10).

In OS cells, exogenously applied ATP (100 nM) triggered volleys of low frequency $[\text{Ca}^{2+}]_i$ oscillations separated by inactive periods, which were occasionally interrupted by the occurrence of Ca^{2+} bursts within localized cell clusters (Figs. 11, 12, and 13). Under these conditions, OS cells propagated slow ICS signals along curvilinear microcircuits (Fig. 14), maintaining a limited degree of coherence in the activity of participating cells (Fig. 11c).

Mechanisms and significance

The mechanisms underlying the spreading of ICS in this preparation have been analyzed in detail and shown to entail release of ATP from connexin hemichannels at the endolymphatic surface of the epithelium, as well as diffusion of Ca^{2+} -mobilizing second messengers across GJ channels (Fig. 1). Also specific isoforms of P2YRs may play a significant role in shaping the spatio-temporal aspects of Ca^{2+} signaling in the network of participating cells [76]. Other key players are ectonucleotidases [44, 47] and mitochondria [77], which function as spatial Ca^{2+} buffers. ICS spread from cell to cell in a wave-like pattern depends also on positive feedback in the $\text{IP}_3/\text{Ca}^{2+}$ cascade and involves an active diffusion reaction scheme [78–81]. As such, it may be crucially dependent upon the properties of the messenger's amplifier mechanism and rate of

messenger permeation across connexin channels [19, 82–85]. Even modest selectivity at cellular junctions could have a major impact on the strength, character and location of the transmitted signal. This could account for the preferential propagation of ICS along curvilinear microcircuits exemplified in [Online Resources 3](#), Figs. 9 and 14 due, for example, to incomplete formation of the connexin network in these immature tissues.

The concept that each connexin channel and hemichannel is unique in terms of permeability and selectivity is supported by a large number of studies and suggests that permeability to larger metabolites, rather than small inorganic ions, plays an important role in development, physiology and etiology of connexin-related diseases [20, 21, 85]. The effect of single-point mutations can be subtle, discriminating between molecules having the same net charge but different charge distribution; however the molecular mechanisms remain unclear. The recent publication of the structure of the Cx26 at 3.5 Å resolution [86] will undoubtedly pave the way to the study, at atomic level, of connexin function and dysfunction.

What could be the significance of ICS exchange and strictly related $[Ca^{2+}]_i$ oscillations? Although no precise answers are available, it is known that P2X₂ and transiently expressed P2X₃ ionotropic channels on the endolymphatic surface of IHCs activate in response to extracellular ATP [11, 69], which is released from supporting cells into the endolymph through hemichannels formed by Cx26 and Cx30 [44], causing nearby IHCs to fire bursts of Ca^{2+} action potentials in the developing OoC [87, 88]. We note in passing that a possible interpretation for (a) limited FRAP and (b) absence of ICS propagation along linear microcircuits in IS cells, despite remarkable immunoreactivity to connexin antibodies, is that immunostaining in the IS is due mostly to unpaired hemichannels. This accords well also with the report that ATP release occurs spontaneously in the IS but not in the OS (at least in apical cochlear coils) according to Refs. [87, 88]. The contribution of P2 receptors (i.e., both P2Y and P2X receptors) in the signaling between IS cells and IHC is investigated and discussed further in this special edition in a companion article by Tritsch et al.

ATP-evoked firing activity promotes the release of the afferent neurotransmitter glutamate at the synaptic pole of the IHC [12, 13], which in turn triggers discrete bursts of Ca^{2+} action potentials in primary auditory neurons [89]. Furthermore, although immature OHCs are not known to fire spontaneous action potentials [11] and the role of OHC afferent signaling remains obscure [90], the endolymphatic surface of the OHC is a primary region for purinergic influence, where ATP activates Ca^{2+} signaling through the combined action of P2X and P2Y receptors [11, 70]. ICS and $[Ca^{2+}]_i$ oscillations of are of great interest also in

relation to the responses evoked by damaging stimuli delivered to hair cells [46, 91].

The complex scenarios summarized in Fig. 9, 11, 12 and 13 suggest that, in the developing OoC, Ca^{2+} oscillation amplitude and frequency are influenced by a cohort of regulatory and feedback mechanisms, which may include activation and inhibition of the IP₃ receptor at different Ca^{2+} levels [92], as well as regulation of both PLC and G proteins by Ca^{2+} itself [93]. It is critically important for sustained $[Ca^{2+}]_i$ oscillations that: (a) PLC-β is a GTPase-activating protein for G_q capable of accelerating steady state GTP-ase activity up to 60-fold; (b) activated α subunits of G_q proteins interact with PLC isozymes at their C2 domains, a class of Ca^{2+} binding modules found in a number of signal transduction proteins [94]. Thus P2YRs and PLC-β may coordinately regulate the amplitude of the Ca^{2+} signal and the rate of signal termination [93].

In non-excitable cells, $[Ca^{2+}]_i$ oscillations control the dynamics of gene expression through transcription factors [95–99]. A widely held hypothesis is that information is encoded mainly by the frequency of $[Ca^{2+}]_i$ oscillations [100–104]; however, a possible role of amplitudes in signal transduction has been discussed [95, 105, 106]. It has also been argued that amplitude modulation and frequency modulation regulate distinct targets differentially [107]. In the immature organ of Corti, around P5, $[Ca^{2+}]_i$ oscillations evoked by ATP regulate connexin expression through NFκB [53] (nuclear factor kappa-light-chain-enhancer of activated B cells), a thoroughly investigated Ca^{2+} -dependent transcription factor [99]. A binding site for NFκB has been identified in the promoter region of *GJB2* [108, 109] and NFκB is reported to regulate the expression of at least one other connexin (Cx43, Ref. [110]). These findings [53] offer crucial insight into the observations that (a) some deafness associated DFNB1 alleles are characterized by hereditary significant reduction of both GJB2 and GJB6 [111], and (b) that GJB6 deletions in *trans* with recessive mutations of GJB2 cause deafness in humans [112–114]. A recent publication identified also a distant *cis*-regulatory region that controls the expression of both GJB2 and GJB6 [115].

Conclusions

Although the picture is far from complete, what is beginning to emerge is an intriguing set of interactions between cochlear sensory hair cells and their supporting cells, with ATP playing a key signaling role. On-going studies will reveal more of the implications of cell–cell communication in the inner ear in the near future.

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