Inner Ear Connexin Channels: Roles in Development and Maintenance of Cochlear Function

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Connexin 26 and connexin 30 are the prevailing isoforms in the epithelial and connective tissue gap junction systems of the developing and mature cochlea. The most frequently encountered variants of the genes that encode these connexins, which are transcriptionally coregulated, determine complete loss of protein function and are the predominant cause of prelingual hereditary deafness. Reducing connexin 26 expression by Cre/loxP recombination in the inner ear of adult mice results in a decreased endocochlear potential, increased hearing thresholds, and loss of >90% of outer hair cells, indicating that this connexin is essential for maintenance of cochlear function. In the developing cochlea, connexins are necessary for intercellular calcium signaling activity. Ribbon synapses and basolateral membrane currents fail to mature in inner hair cells of mice that are born with reduced connexin expression, even though hair cells do not express any connexin. In contrast, pannexin 1, an alternative mediator of intercellular signaling, is dispensable for hearing acquisition and auditory function.

In the sixties and seventies of the past century, gap junctions (GJs) were identified as planar arrays of intercellular conduits formed by the head-to-head docking, in the extracellular space, of two so-called connexons (Dewey and Barr 1962; Robertson 1963; Revel and Karnovsky 1967; Brightman and Reese 1969; Caspar et al. 1977; Makowski et al. 1977). Each connexon is a hexameric complex formed by circumferentially arranged connexin (Cx) protein subunits (Goodenough 1974), which delineate a pore in the plasma membrane. Thus, the coaxial arrangement of two docked connexons establishes

a communication pathway between the cytoplasm of the two adjoined cells (Fig. 1A,B).

Twenty connexin genes are expressed both in humans (identified by a “Gj” prefix) and in mice (identified by a “Gj” prefix) (Table 1). Note that two connexins are human only (CX25, CX59), and two are mouse only (Cx33, Cx23). All connexins are thought to share the same topology, with cytoplasmic amino and carboxyl termini, four transmembrane domains connected by two extracellular loops and a cytoplasmic loop (Beyer and Berthoud 2018). The amino acids forming these domains in CX26 are listed in Table 2.

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Figure 1. Computational models of connexin channels. (A,B) Small portion of a gap junction showing intercellular channels embedded in plasma membrane phospholipids. (A) View from the cytoplasmic side of one of the two adjacent membranes. (B) Transverse section in a plane orthogonal to the plasma membranes; four of the 12 protomers have been removed from the channel in the center to show its pore. (C) Model of a Cx26 hemichannel: one of the six protomers has been removed to show the channel pore (light blue); four connexins are rendered as solid surface and the remaining connexin is represented as a cartoon to highlight its 3D structure; transmembrane helix 2 (TM2) is colored in yellow. The computational and rendering methods used to generate this figure are described in Zonta et al. (2012, 2014) and Xu et al. (2017).

Connexins are posttranslationally oligomerized to form connexons before membrane insertion either within the endoplasmic reticulum (ER) or in the *trans*-Golgi network (Thomas et al. 2005; Aasen et al. 2018). Connexons can be homomeric (composed of six identical connexin protomers) or heteromeric (formed by two or more different connexin isoforms). Therefore, docked connexons can form (1) homomeric-homotypic, (2) heteromeric-homotypic, (3) homomeric-heterotypic, or (4) heteromeric-heterotypic GJ channels (Kumar and Gilula 1996). Thousands of GJ channels aggregate in a plaque (Solan and Lampe 2018) at an estimated density of 12,500 channels/ μm^2 (Forge et al. 2003a) driven by protein-protein interaction forces (Abney et al. 1987). In plaques purified from a HeLa cell line overexpressing Cx26, atomic force microscopy imaging revealed GJ channels assembled in a regular hexagonal lattice with a unit cell distance of 7.7 ± 0.5 nm (Muller et al. 2002).

Clustering in GJ plaques is considered a prerequisite for the opening of GJ channels (Bukauskas et al. 2000). Based on the available crystallographic data at 3.5 Å resolution, it has been estimated that the pore of the open CX26 GJ channel achieves a minimum diameter of ~ 14 Å (Maeda et al. 2009; Zonta et al. 2012, 2013). Size exclusion studies, using permeant molecules of various size, suggest that GJ channels formed by other connexin isoforms are characterized by similarly large pores, and consequently that GJs allow adjacent cells to exchange cytoplasmic molecules with a cut-off molecular mass up to ~ 1.4 kDa (Leybaert et al. 2017), a size that includes all small inorganic ions with their hydration shells (Volkov et al. 1997). The list of permeating molecules could potentially comprise >35,000 members of the Human Metabolome Database⁴ (Esseltine and Laird 2016). Permeability studies for glucose, ADP, ATP, adenosine, glutamate, and glutathione (Goldberg et al. 1999, 2002), secondary messengers (Niesen et al. 2000; Beltramello et al. 2005; Bedner et al. 2006; Hernandez et al. 2007; Kanaporis

⁴<http://www.hmdb.ca/structures/search/metabolites/mass>.

Table 1. List of human and mouse connexin genes

Human			Mouse		
OMIM ID	Gene symbol	Protein name	Protein name	Gene symbol	MGI ID
121014	<i>GJA1</i>	CX43	Cx43	<i>Gja1</i>	95713
121015	<i>GJA3</i>	CX46	Cx46	<i>Gja3</i>	95714
121012	<i>GJA4</i>	CX37	Cx37	<i>Gja4</i>	95715
121013	<i>GJA5</i>	CX40	Cx40	<i>Gja5</i>	95716
–	–	–	Cx33	<i>Gja6</i>	95717
600897	<i>GJA8</i>	CX50	Cx50	<i>Gja8</i>	99953
611923	<i>GJA9</i>	CX59	–	–	–
611924	<i>GJA10</i>	CX62	Cx57	<i>Gja10</i>	1339969
304040	<i>GJB1</i>	CX32	Cx32	<i>Gjb1</i>	95719
121011	<i>GJB2</i>	CX26	Cx26	<i>Gjb2</i>	95720
603324	<i>GJB3</i>	CX31	Cx31	<i>Gjb3</i>	95721
605425	<i>GJB4</i>	CX30.3	Cx30.3	<i>Gjb4</i>	95722
604493	<i>GJB5</i>	CX31.1	Cx31.1	<i>Gjb5</i>	95723
604418	<i>GJB6</i>	CX30	Cx30	<i>Gjb6</i>	107588
611921	<i>GJB7</i>	CX25	–	–	–
608655	<i>GJC1</i>	CX45	Cx45	<i>Gjc1</i>	95718
608803	<i>GJC2</i>	CX47	Cx47	<i>Gjc2</i>	2153060
611925	<i>GJC3</i>	CX30.2/31.3	Cx29	<i>Gjc3</i>	2153041
607058	<i>GJD2</i>	CX36	Cx36	<i>Gjd2</i>	1334209
607425	<i>GJD3</i>	CX31.9	Cx30.2	<i>Gjd3</i>	2384150
611922	<i>GJD4</i>	CX40.1	Cx39	<i>Gjd4</i>	2444990
–	–	–	Cx23	<i>Gje1</i>	1923993

et al. 2008; Carrer et al. 2018), short interfering RNA (siRNA), and microRNA (miRNA) (Valiunas et al. 2005; Zong et al. 2016) yielded up to 33-fold differences in the estimated unitary permeability of different channels for different molecules.

Table 2. Cx26 structural domains and corresponding amino acid positions

Amino acid	Domain	Acronym
1–20	Amino terminus, cytoplasmic	NT
21–40	Transmembrane helix 1	TM1
41–75	Extracellular loop 1	ECL1
76–98	Transmembrane helix 2	TM2
99–131	Cytoplasmic loop	CL
132–154	Transmembrane helix 3	TM3
155–192	Extracellular loop 2	ECL2
193–215	Transmembrane helix 4	TM4
216–226	Carboxyl terminus, cytoplasmic	CT

Of note, undocked connexons can establish a communication pathway between cell cytoplasm and the extracellular milieu by operating as regular plasma membrane channels, referred to as connexin hemichannels (Fig. 1C) (Li et al. 1996). Open hemichannels lack ion selectivity and mediate autocrine/paracrine signaling by the release or uptake of small molecules including ATP (Leybaert et al. 2003), glutamate (Ye et al. 2003), lactate (Karagiannis et al. 2016), oxidized nicotinamide adenine dinucleotide (Bruzzone et al. 2001), prostaglandin E₂ (Cherian et al. 2005), reduced glutathione (Rana and Dringen 2007; Tong et al. 2015), which are critical for cell–cell communication (Wang et al. 2013; Saez and Leybaert 2014) and the regulation of inflammatory responses (Willebrords et al. 2016).

Permeation is regulated by numerous factors that control the opening and closing (i.e., gating) of GJ channels and connexin hemichannels, including voltage (Barrio et al. 1991; Verselis et al. 1994, 2000; Saez et al. 2005; Paulauskas et al.

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2012; Snipas et al. 2017), pH (Peracchia et al. 2000b; Palacios-Prado et al. 2009; Sanchez et al. 2014), redox potential (Retamal et al. 2007; Retamal 2014), CO₂ (Huckstepp et al. 2010; Meigh et al. 2013), nitric oxide, and S-nitrosylation (Retamal et al. 2006, 2009), phosphorylation (Kim et al. 1999; Moreno 2005), and other posttranslational modifications (D'Hondt et al. 2013; Aasen et al. 2018). Connexin hemichannels remain predominantly closed at negative membrane potential and/or in the presence of millimolar levels of extracellular divalent cations (Ca²⁺ and Mg²⁺) (Li et al. 1996; Pfahnl and Dahl 1999; Kamermans et al. 2001; Gomez-Hernandez et al. 2003; Verselis and Srinivas 2008; Bennett et al. 2016). Atomic force microscopy revealed that the pore at the extracellular mouth of Cx26 hemichannels changed diameter from 13 ± 3 Å, in the absence of Ca²⁺, to 5 ± 3 Å in 0.5 mM Ca²⁺ (Muller et al. 2002). A similar effect on pore size was imaged by atomic force microscopy in Cx43 hemichannels (Thimm et al. 2005).

GJ channels tend to close in response to an increase of the cytosolic free calcium concentration ([Ca²⁺]_c) in a variable range, from 300 nM to several μM, depending on the connexin isoforms (Rose and Loewenstein 1975; Peracchia 2004; Lurtz and Louis 2007). The Ca²⁺-dependent inhibition of different GJ channels might share a common mechanism mediated by calmodulin (Peracchia et al. 1983, 2000a; Zhou et al. 2009). In contrast, connexin hemichannels show a more complicated, bell-shaped dependence of their open probability (P_o) on [Ca²⁺]_c, as the P_o increases for [Ca²⁺]_c ≤ 500 nM and decays if [Ca²⁺]_c increases further (De Vuyst et al. 2006, 2009; Carrer et al. 2018). A pivotal role for calmodulin has been proposed also for this incompletely characterized gating phenomenon (Zhang et al. 2006; Hu et al. 2018).

CELL COUPLING IN THE MAMMALIAN COCHLEA

Intercellular junctions in the mammalian cochlea were initially characterized by electrophysiology (Santos-Sacchi and Dallos 1983; Oesterle and Dallos 1990; Santos-Sacchi 1991; Mam-

mano et al. 1996) and electron microscopy studies (Jahnke 1975; Reale et al. 1975; Gulley and Reese 1976; Iurato et al. 1976a,b; Hama and Saito 1977; Franke 1978, 1979; Nadol 1978; Forge 1984; Bagger-Sjoberg et al. 1987; Carlisle et al. 1990; Sakagami et al. 1993), leading to the identification of two distinct cellular networks, referred to as the epithelial GJ system (E-sys) and the connective tissue GJ system (C-sys), respectively (Kikuchi et al. 1995). In rodents, the E-sys forms around embryonic day 16, and encompasses supporting cells of the organ of Corti, the bordering epithelial cells of the inner sulcus and outer sulcus, the interdental cells of the spiral limbus and the root cells (Kikuchi et al. 1995) that extend their process into the spiral ligament (Jagger and Forge 2013). By postnatal (P) day 5 (P5, where P0 is date of birth), the mouse E-sys is already well developed, as shown by a highly sensitive cell-coupling assay based on the combination of patch-clamp and voltage imaging (Fig. 2). The C-sys, which starts to develop around P0, is further subdivided into two subsystems comprising, on the one hand, fibrocytes of the spiral limbus and, on the other hand, fibrocytes of the lateral wall, basal and intermediate cells of the stria vascularis, fibrocytes of the suprastrial zone, mesenchymal cells lining the scala vestibuli, and supralimbal dark cells (Kikuchi et al. 1995, 2000a,b; Forge et al. 1999).

GJ plaques are not present in marginal cells of the stria vascularis (Forge et al. 2003a), a conclusion supported by dye-coupling experiments (Kelly et al. 2011). Likewise, GJs were never found in the plasma membrane of sensory hair cells (Forge et al. 2003a), consistent with absence of recovery after photobleaching (Majumder et al. 2010) in “gap-FRAP” cell-coupling assays (Wade et al. 1986) and in accord also with the requirements imposed on the plasma membrane conductance for the generation of hair cell receptor potentials (Dallos et al. 1982; Dallos 1986; Palmer and Russell 1986; Cody and Russell 1987; Mammano and Nobili 1993; Nobili and Mammano 1996; Mistrik et al. 2009; Mistrik and Ashmore 2010; Johnson et al. 2011; see also Ashmore 2018; Holt and Corey 2018; Petit 2018).

Figure 2. Network connectivity in the epithelial gap junction system (E-sys) of the developing mouse cochlea. (A) Representative false color images showing the spatial distribution of fluorescence signals generated by the voltage sensitive dye Vf2.1.Cl (Miller et al. 2012) in organotypic cochlear cultures from P5 wild-type (WT) (*top left*), *Gjb6*^{T5M/T5M} (*top right*), and *Gjb6*^{-/-} (*bottom left*) mice; the *bottom right* image shows a wild-type culture in which GJ channels were blocked by 20 min incubation with carbenoxolone (CBX, 100 μM). (B) Data fit by a simple resistive network model that reflects the anatomy. For further details, see Ceriani and Mammano (2013).

Plaques containing $\sim 10^4$ channels are not infrequent among supporting cells of the organ of Corti, and some may contain in excess of 10^5 channels (Forge et al. 2003a), of which 1%–10%

are expected to be in the open state at any one time (Bukauskas et al. 2000; Palacios-Prado et al. 2009, 2010). Consistent with these estimates, the measured junctional currents in isolated pairs of

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supporting cells of the adult guinea pig organ of Corti, up to 1 μ S (Santos-Sacchi 1991), are compatible with a number of open channels per cell pair of the order of 10^4 (Ceriani and Mammano 2013), which permits the cell-to-cell diffusion of microinjected Lucifer yellow (Mammano et al. 1996; Jagger and Forge 2006), a fluorescent tracer vastly used for dye-coupling assays (Stewart 1978; Hanani 2012; Xu et al. 2017).

CELL COUPLING AND NUTRIENT SUPPLY TO THE ORGAN OF CORTI

The sensory epithelium of the mammalian cochlea is an essentially avascular structure. A single blood capillary, the vas spirale, runs in the tympanic layer of the basilar membrane just beneath the tunnel of Corti (Smith 1951; Kirikae et al. 1969). The rest of the blood supply relies on two networks of blood vessels: one serving the spiral limbus (Firbas et al. 1981) and the other serving the spiral ligament and stria vascularis (Ando and Takeuchi 1998; Cohen-Salmon et al. 2004, 2007). Spiral ligament vessels, which are embedded in the fibrocytes forming part of the C-sys, penetrate the stria vascularis in which they divide into numerous thin capillaries (Ando and Takeuchi 1998).

Blood glucose is exported via a facilitated transport system; in rats, its concentrations in perilymph of scala vestibuli, perilymph of scala tympani, and cerebrospinal fluid are ~50%, and in endolymph <10%, of that in plasma (Ferrary et al. 1987). Intercellular coupling via GJs has been proposed to aid transfer glucose and other metabolites to all cells of the cochlear sensory epithelium (Santos-Sacchi and Dallos 1983). Glucose diffusion through the network of cochlear GJ-coupled cells has been documented using 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG) (Chang et al. 2008), a fluorescent glucose analog that has been used to monitor glucose uptake in live cells (Zou et al. 2005). A similar role has been shown for astrocytes, in which GJ communication provides an activity-dependent intercellular pathway for the delivery of metabolites involved in energy production from blood vessels to distal neurons (Rouach et al. 2008).

CONNEXIN EXPRESSION IN THE MAMMALIAN COCHLEA

An in situ hybridization study reported strong and overlapping expression of Cx26, Cx30, Cx30.2, Cx31, Cx37, Cx43, Cx46 both in the organ of Corti and in the lateral wall of the mouse neonatal cochlea, whereas low expression was detected for Cx45 and Cx59 (Buniello et al. 2004).⁵ A cDNA microarray hybridization study identified a more limited number of four most prominent connexins expressed in the mouse cochlea, namely, Cx26, Cx29, Cx30, and Cx43 (Ahmad et al. 2003). A study based on immunofluorescence and electron microscopy in the adult cochlea of mice, guinea pigs, gerbils, and bats concluded that “Cx26 and Cx30 colocalized in supporting cells of the organ of Corti, in the basal cell region of the stria vascularis, and in type 1 fibrocytes of the spiral ligament. No other connexin was detected in these regions. Cx31 was localized among type 2 fibrocytes below the spiral prominence, a region where Cx30 was not expressed and Cx26 expression appeared to be low. Cx43 was detected only in the region of tension fibrocytes lining the inner aspect of the otic capsule” (Forge et al. 2003a).

Despite inconsistencies in the animal studies quoted above, there is widespread consensus that Cx26 and Cx30 are the prevailing connexins in both the E-sys and the C-sys of the developing and mature rodent cochlea (Kikuchi et al. 1995; Lautermann et al. 1998, 1999; Ahmad et al. 2003; Forge et al. 2003a,b; Zhao and Yu 2006). In mice, the coordinated regulation of Cx26 and Cx30 expression occurs as a result of signaling through the nuclear factor (NF)- κ B pathway and can be inhibited by expressing a stable form of the I κ B repressor protein that prevents the activation/translocation of NF- κ B (Ortolano et al. 2008). Not only the expression of Cx26 and Cx30 is coordinately regulated, but, in addition, it has been claimed that heteromeric channels formed by Cx26 and Cx30 are present in the organ of Corti and lateral wall tissues of the rodent co-

⁵Note that there is no entry for (mouse) Cx59 in Table 1, therefore it is unclear what the authors referred to.

chlea, and in the sensory epithelia of the vestibular system (Ahmad et al. 2003; Forge et al. 2003a,b). Studies performed with recombinant connexins indicate that Cx26 and Cx30 can indeed form functional heteromeric channels in cell lines (Sun et al. 2005; Yum et al. 2007). However, none of the immunofluorescence studies performed in rodents achieved the required spatial resolution to show convincingly that heteromeric Cx26/Cx30 connexons do exist in the cochlea. Actually, GJ plaques formed almost exclusively by Cx30 prevail in the Deiters' cell region of the P30 mouse cochlea (Jagger and Forge 2015). Furthermore, cell-specific distributions of Cx26 and Cx30 were reported in the guinea pig and rat cochlear lateral wall, in which Cx26 labeling was absent in the middle region just beneath the stria vascularis (Liu and Zhao 2008).

Recently, subdiffraction multicolor imaging (Gustafsson et al. 2008) of the lateral wall and sensory epithelium of the adult human cochlea achieved a resolution of ~100 nm in the focal plane (X-Y) and an axial (Z) resolution of ~400 nm (Liu et al. 2016, 2017). The investigators of those studies suggested that "CX26 and CX30 proteins are not coexpressed in the lateral wall of the human cochlea but, rather, form closely associated GJ plaques." In the organ of Corti, "CX26 subdomains appeared inside annular plaques" forming "hybrid plaques," supporting the notion that "diverse aggregates of GJ channels can populate the same GJ plaque or represent physically interacting plaques" (Liu et al. 2016, 2017). As shown by freeze-fracture replica immunogold labeling (Fujimoto 1995), a similar hybrid arrangement may characterize GJ plaques in rodent astrocytes, which express Cx26, Cx30, and Cx43 (Rash et al. 2001). In summary, the unresolved discrepancy between human and rodent studies prevents drawing a definitive conclusion regarding existence and relative importance of heteromeric versus homomeric GJ channels in the organ of Corti and lateral wall of the cochlea.

CONNEXIN MUTATIONS AND HEREDITARY HEARING IMPAIRMENT

Genetic studies have revealed the critical importance for human hearing of some of the connex-

ins expressed in the inner ear. In particular, variants of the *GJB2* (CX26) gene,⁶ which include more than 340 missense, nonsense, frame shift, insertions, and deletions,⁷ take the lion share in the spectrum of deafness related mutations.⁸ The overwhelming majority of them cause autosomal recessive nonsyndromic deafness 1a (DFNB1A; OMIM [Online Mendelian Inheritance in Man]:220290) (Kelsell et al. 1997; del Castillo and del Castillo 2017). These mutations, which account for ~50% of all autosomal recessive nonsyndromic hearing impairment cases,⁹ are responsible for hearing loss ranging from mild to profound (Kenna et al. 2010), and are broadly classified as truncating (e.g., premature stop codons, associated with a significantly greater degree of hearing impairment), and non-truncating (e.g., missense variants) (Snoeckx et al. 2005). The truncating variant 35delG (Zelante et al. 1997), also known as 30delG (Denoyelle et al. 1997), halts CX26 transcription at the level of the amino terminus (Table 2), causing complete loss of protein function (D'Andrea et al. 2002). In a study that included 82 families (51 Italian and 31 Spanish) with nonsyndromic congenital deafness of different degrees, 35delG accounted for 85% of *GJB2* variants (Estivill et al. 1998).

A restricted set of missense *GJB2* variants cause autosomal dominant nonsyndromic deafness 3a (DFNA3A; OMIM:601544) (Denoyelle et al. 1998). The corresponding mutant CX26 proteins are thought to exert dominant-negative effects on wild-type CX26 or CX30 (Forge et al. 2003b; Zhang et al. 2011), whereas a distinct set of dominantly inherited missense mutations apparently modify the activity of both wild-type CX26 and CX43 (Rouan et al. 2001; Shuja et al. 2016), causing syndromic hearing impairment associated with a spectrum of skin disorders, including Keratitis-ichthyosis-deafness syndrome (KID; OMIM:148210) (Richard et al. 2002),

⁶http://swissvar.expasy.org/cgi-bin/swissvar/result?global_textfield=gjb2&findProteins=search.

⁷<http://deafnessvariationdatabase.org/letter/g>, then select "GJB2".

⁸<https://www.ncbi.nlm.nih.gov/books/NBK1434>.

⁹<https://www.ncbi.nlm.nih.gov/books/NBK1272>.

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Hystrix-like ichthyosis with deafness (HID; OMIM:602540) (van Geel et al. 2002), Keratoderma, palmoplantar, with deafness (PPK-D; OMIM:148350) (Richard et al. 1998b), Vohwinkel syndrome (VS; OMIM:124500) (Maestrini et al. 1999), and Bart-Pumphrey syndrome (BPS; OMIM:149200) (Richard et al. 2004).

Variants of the *GJB3* (CX31) gene¹⁰ cause: (1) autosomal dominant deafness 2b (DFNA2B; OMIM¹¹:612644) (Xia et al. 1998); (2) digenic deafness (*GJB2/GJB3*; OMIM: 220290) (Liu et al. 2009); and (3) erythrokeratoderma variabilis et progressiva (OMIM:133200) (Richard et al. 1998a). Finally, variants of the *GJB6* (CX30)¹² gene, which is located just 30 kb upstream of *GJB2* in the same DFNB1 locus on chromosome 13 (del Castillo and del Castillo 2017) causes (1) autosomal recessive deafness 1b (DFNB1B; OMIM:612645) (del Castillo et al. 2002); (2) autosomal dominant deafness 3b (DFNA3B, OMIM:612643) (Grifa et al. 1999); (3) digenic deafness (*GJB2/GJB6*, OMIM:220290) (Lerer et al. 2001; Pallares-Ruiz et al. 2002; del Castillo et al. 2005); and (4) ectodermal dysplasia 2, clouston type (OMIM:129500) (Lamartine et al. 2000).

The existence of a digenic pattern of inheritance is supported by the discovery of large deletions that truncate the *GJB6* gene leaving *GJB2* intact (Lerer et al. 2001; del Castillo et al. 2002, 2003, 2005; Pallares-Ruiz et al. 2002). One of these variants, del(*GJB6*-D13S1830), accounts for hearing impairment in 30% to 70% of affected *GJB2* heterozygotes in some populations (del Castillo et al. 2005). Thus, it can be safely concluded that mutations in the genes that encode CX26 and CX30 are the predominant cause of prelingual hereditary deafness, and the most frequently encountered variants cause complete loss of protein function.

The connection between hearing loss and dermatological manifestations is not surprising,

as a number of inner ear connexins are expressed also in skin keratinocytes (Butterweck et al. 1994; Goliger and Paul 1994; Salomon et al. 1994; Di et al. 2001). However, variants of *GJA1* (CX43)¹³ and *GJB4* (CX30.3)¹⁴ cause skin disease without hearing loss (Lilly et al. 2016). Likewise, *GJB2* somatic mutations of the KID type that occur only in keratinocytes cause Porokeratotic eccrine ostial and dermal duct nevus (PEODDN) without affecting hearing (Easton et al. 2012; Lazic et al. 2012). It has been suggested that diverse connexin mutations associated with distinct epidermal phenotypes consistently give rise to novel gain of function activities, including changes in hemichannel activity (Retamal et al. 2015; Lilly et al. 2016). This conclusion is supported by in vitro studies with connexin gene variants that cause cell death in expression systems, including some autosomal dominant syndromic mutants of *GJB2* (Stong et al. 2006; Lee and White 2009; Sanchez et al. 2010, 2013, 2014, 2016; Mhaske et al. 2013; Press et al. 2017b; Xu et al. 2017), *GJB3* (Di et al. 2002; He et al. 2005), and *GJB6* (Essenfelder et al. 2004; Berger et al. 2014). Mouse models for human syndromic deafness associated with skin disorders (Mese et al. 2011; Schutz et al. 2011; Bosen et al. 2014; Press et al. 2017a) lend further support to this view (Garcia et al. 2016; Srinivas et al. 2018).

In the attempt to find a rationale for the pervasiveness of *GJB2* mutations, it has been suggested that reduced CX26 expression in human skin may confer a selective advantage as heterozygous carriers of the *GJB2* 35delG variant show significantly increased epidermal thickness, which may enhance protection against pathogen invasion or trauma compared with noncarriers (D'Adamo et al. 2009). In the same vein, in vitro models of infection showed “a significant reduction in both cellular invasion by *Shigella flexneri* and adherence by enteropathogenic *Escherichia coli* in human intestinal cell lines following treatment with CX26

¹⁰http://swissvar.expasy.org/cgi-bin/swissvar/result?global_textfield=gjb3&findProteins=search.

¹¹Online Mendelian Inheritance in Man, <https://www.omim.org/about>.

¹²http://swissvar.expasy.org/cgi-bin/swissvar/result?global_textfield=gjb6&findProteins=search.

¹³http://swissvar.expasy.org/cgi-bin/swissvar/result?global_textfield=gja1&findProteins=search.

¹⁴http://swissvar.expasy.org/cgi-bin/swissvar/result?global_textfield=gjb4&findProteins=search.

siRNA,” suggesting that heterozygous carriers may benefit also from increased gastrointestinal protection (Simpson et al. 2013).

MOUSE MODELS FOR HUMAN NONSYNDROMIC DEAFNESS

Embryonic lethality affects Cx26 homozygous knockout mice ($Gjb2^{tm1Kwi}/Gjb2^{tm1Kwi}$; MGI [Mouse Genome Informatics]¹⁵:2155739; EM [European Mouse Mutant Archive]¹⁶:00229) because of impaired transplacental uptake of glucose (Gabriel et al. 1998). On the contrary, Cx30 homozygous knockout-LacZ mice ($Gjb6^{tm1Kwi}/Gjb6^{tm1Kwi}$; MGI:2447863; EM:00323), abbreviated as $Gjb6^{-/-}$, are viable and fertile, show a severe constitutive hearing impairment by auditory brainstem recordings (ABRs), completely lack the endocochlear potential (Teubner et al. 2003), show defects of the endothelial barrier in capillaries of the stria vascularis (Cohen-Salmon et al. 2007), and impaired transfer of the fluorescent D-glucose derivative 2-NBDG (Chang et al. 2008).

In $Gjb6^{-/-}$ mice, the Cx30 coding exon of *Gjb6* was both removed and replaced by the *lacZ* reporter gene and a *neo* resistance cassette. This may interfere with expression of *Gjb2* nearby, as Cx26 levels were severely reduced in the developing cochlea of $Gjb6^{-/-}$ mice (Ortolano et al. 2008). Likewise, “gene deletion of Cx30 led to the near total elimination of immunofluorescence labeling for Cx26 in all leptomeningeal tissues covering brain surfaces as well as in astrocytes of brain parenchyma” in these mice (Lynn et al. 2011).

A different Cx30 global knockout strain ($Gjb6^{tm1.1Mcsa}/Gjb6^{tm1.1Mcsa}$; MGI:5486677), abbreviated as $Gjb6^{\Delta/\Delta}$, in which *Gjb6* was deleted without insertion of *lacZ* and *neo* showed normal hearing thresholds. Residual expression of inner ear Cx26 was ~50% in $Gjb6^{\Delta/\Delta}$ mice, but only ~10% in $Gjb6^{-/-}$ mice, compared with $Gjb6^{+/+}$ controls (Boulay et al.

2013). Mice in which extra copies of *Gjb2* were transgenically expressed from a modified bacterial artificial chromosome in a $Gjb6^{-/-}$ background ($Gjb6^{tm1Kwi}/Gjb6^{tm1Kwi}$ Tg(*Gjb2*)1Xili/0; MGI: 3713363) had normal hearing sensitivity (Ahmad et al. 2007). Altogether, these experiments have prompted the conclusion that (1) Cx30 is dispensable for auditory function, and (2) the hearing loss in $Gjb6^{-/-}$ mice depends only on reduction of Cx26 expression in the inner ear, below a certain critical level (<50%) (Boulay et al. 2013).

The essential role played by Cx26 for mouse hearing was shown by cross-breeding mice with floxed *Gjb2* exon 2 ($Gjb2^{tm1Ugds}/Gjb2^{tm1Ugds}$; MGI:2183509; EM:00245), abbreviated as $Gjb2^{loxP/loxP}$, and *Otog-Cre* mice (Tg(*Otog-cre*)1Ugds; MGI:2183511) (Cohen-Salmon et al. 2002). In $Gjb2^{loxP/loxP}$ *Otog-Cre* offspring (MGI: 3588875), abbreviated as $Gjb2^{OtogCre}$, Cx26 immunoreactivity was undetectable from birth onward in cells of the E-sys, but still present in fibrocytes of the C-sys. Similar hearing thresholds were measured in control ($Gjb2^{loxP/loxP}$) mice and heterozygous $Gjb2^{loxP/+}$ *Otog-Cre* mice, whereas thresholds were significantly elevated in (homozygous) $Gjb2^{OtogCre}$ mice, in which endocochlear potential was reduced¹⁷ (Cohen-Salmon et al. 2002).

Subsequent loss of the *Otog-Cre* line promoted a search for alternative Cre-expressing lines. Cross-breeding $Gjb2^{loxP/loxP}$ mice and *Sox10-Cre* mice (Tg(*Sox10-cre*)1Wdr; MGI: 3586900) yielded $Gjb2^{loxP/loxP}$ *Sox10-Cre* offspring, abbreviated as $tGjb2^{-/-}$ (MGI:5297177; EM:11478)¹⁸, that lacked Cx26 immunoreactivity in cells of the E-sys (Anselmi et al. 2008). Immunoreactivity for both Cx26 and Cx30 was still present in the cochlear lateral wall of adult $tGjb2^{-/-}$ mice, in which in vivo electrophysiological measurements revealed congenital profound hearing impairment, accompanied by a major reduction of the endocochlear potential

¹⁵Mouse Genome Informatics, <http://www.informatics.jax.org>.

¹⁶European Mouse Mutant Archive (EMMA/INFRAFRONTIERS), <https://www.infrafrontier.eu>.

¹⁷[http://www.informatics.jax.org/allele/genoview/MGI: 3588875](http://www.informatics.jax.org/allele/genoview/MGI:3588875).

¹⁸“t” stands for *targeted*; these mice were previously named $Cx26^{Sox10Cre}$, whereas their correct nomenclature is: $Gjb2^{tm1Ugds}/Gjb2^{tm1Ugds}$ Tg(*Sox10-cre*)1Wdr/0.

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Table 3. List of main mouse models mentioned in text

Abbreviation(s)	Allele composition	Identifier
<i>Gjb2</i> ^{loxP/loxP}	<i>Gjb2</i> ^{tm1Ugds} / <i>Gjb2</i> ^{tm1Ugds}	MGI:2183509; EM:00245
<i>Otog-Cre</i>	Tg(<i>Otog-cre</i>)1Ugds	MGI:2183511
<i>Sox10-Cre</i>	Tg(<i>Sox10-cre</i>)1Wdr	MGI:3586900
<i>Gjb2</i> ^{OtogCre}	<i>Gjb2</i> ^{tm1Ugds} / <i>Gjb2</i> ^{tm1Ugds} Tg(<i>Otog-cre</i>)1Ugds/0	MGI: 3588875
<i>tGjb2</i> ^{-/-} (<i>Cx26</i> ^{Sox10Cre})	<i>Gjb2</i> ^{tm1Ugds} / <i>Gjb2</i> ^{tm1Ugds} Tg(<i>Sox10-cre</i>)1Wdr/0	MGI:5297177; EM:11478
<i>Gjb6</i> ^{-/-}	<i>Gjb6</i> ^{tm1Kwi} / <i>Gjb6</i> ^{tm1Kwi}	MGI:2447863; EM:00323
<i>Gjb6</i> ^{Δ/Δ}	<i>Gjb6</i> ^{tm1.1Mcsa} / <i>Gjb6</i> ^{tm1.1Mcsa}	MGI:5486677
<i>Gjb6</i> ^{T5M/T5M}	<i>Gjb6</i> ^{tm1.1Fama} / <i>Gjb6</i> ^{tm1.1Fama}	MGI:4848154; EM:05214

(Crispino et al. 2011). Table 3 provides a summary of the abbreviations used to identify mouse models most frequently mentioned in this review.

The sensory epithelium of the developing cochlea is subdivided into two embryologically distinct regions, the greater epithelial ridge (GER) and the lesser epithelial ridge (LER), which are separated by a pair of immature inner and outer pillar cells (Eggston and Wolff 1947; Lim and Anniko 1985; Lim and Rueda 1992). The GER encompasses the nascent inner sulcus, the tall columnar cells of the so-called Kölliker's organ (Hinojosa 1977), the inner hair cells (IHCs), and their abutting supporting cells (Bryant et al. 2002; Kelley 2007; see also Montcouquiol and Kelley 2018). Postnatal maturation of pillar cells leads to the opening of the tunnel of Corti and a progressive increase in the distance between the IHCs and the outer hair cells (OHCs). In the basal turn of the cochlea, the tunnel of Corti and Nuel's space were open at P6 in control mice (C57BL6/N), but failed to open in *tGjb2*^{-/-} mice, indicative of developmental defects. Consistent with this conclusion, *Cx30* expression was developmentally delayed in *tGjb2*^{-/-} mice, and almost undetectable in cells of the epithelial network during the first postnatal week (Crispino et al. 2011).

Irreversible cell loss in the organ of Corti was detected either before or around the onset of hearing (P12) in both *Gjb2*^{OtogCre} and *tGjb2*^{-/-} mice. In the latter, degeneration of the sensory epithelium affected both sensory and nonsensory cells of the basal turn at P30 (Crispino et al. 2011). In *GJB2*^{OtogCre} mice, detection of

apoptosis by the TUNEL assay, and immunofluorescence detection of activated caspase 3, showed labeling of the two supporting cells flanking the IHCs (i.e., border cell and inner phalangeal cell) from P14 onward. Labeling extended to OHCs and their supporting cells (Deiters' and Hensen's cells) by P16 onward (Cohen-Salmon et al. 2002).

The deafness phenotype was more severe in *tGjb2*^{-/-} mice than in *Gjb2*^{OtogCre} mice, most likely a result of the different Cre drivers used to generate these mice. Indeed, an even more dramatic hearing loss and cellular degeneration phenotype affected *Gjb2*^{loxP/loxP}R26cre-ER^T offspring (EM:11488), in which Cre expression was activated globally by a single injection, on embryonic day 19, of the synthetic estrogen 4-hydroxytamoxifen (Sun et al. 2009). Two more lines were generated by cross-breeding *Gjb2*^{loxP/loxP} mice with *Foxg1-cre* and *Pax2-cre* drivers, respectively, and both displayed sign of developmental disturbance, as well as dramatic loss of the OHCs and surrounding supporting cells around P13 (Wang et al. 2009). However, a note of caution is mandatory. Although *Sox10-cre* mice are viable and have normal hearing thresholds, homozygous *Foxg1-cre* mice die perinatally (Tian et al. 2012). Furthermore, some Cre driver lines used for conditional gene deletion/expression in the inner ear (Cox et al. 2012) were recently reported with an early-onset progressive hearing loss, which was absent in their wild-type littermates (Matern et al. 2017), suggesting that the inner ear may be particularly vulnerable to expression of the bacterial Cre recombinase.

Pending demonstration that Cre-related toxicity effects (Loonstra et al. 2001) can be ex-

cluded for the mouse models described above, it seems reasonable to conclude that (1) ablation of Cx26 in the cochlea is associated with down-regulation of Cx30; (2) affects hair cell survival despite the fact that hair cells do not express any connexin; and (3) plays a critical role in organ of Corti development and acquisition of hearing. This conclusion is in reasonable agreement with reports of biallelic carriers of pathogenic *GJB2* variants, whose hearing thresholds were normal at birth but deteriorated during the first year of life (Orzan and Murgia 2007; Gopal Rao et al. 2008; Minami et al. 2013).

CRITICAL ROLE FOR ATP- AND CONNEXIN-DEPENDENT INTERCELLULAR Ca^{2+} SIGNALING IN THE DEVELOPING COCHLEA

Extracellular adenosine triphosphate (ATP_e) is a neurohumoral agent (Housley et al. 2009) that modulates the function of both sensory and nonsensory cells in the inner ear (Ashmore and Ohmori 1990; Dulon et al. 1991, 1993; Housley and Ashmore 1992; Housley et al. 1992, 2009, 2013; Sugawara et al. 1996; Mammano et al. 1999; Lagostena and Mammano 2001; Lagostena et al. 2001; Frolenkov et al. 2003; Frolenkov 2006; Yan et al. 2013). In the developing cochlea, ATP_e triggers $[\text{Ca}^{2+}]_c$ oscillations and propagation of intercellular Ca^{2+} waves that convey crucial biochemical information to every district of the cochlear sensory epithelium (Gale et al. 2004; Beltramello et al. 2005; Zhang et al. 2005; Piazza et al. 2007; Tritsch et al. 2007). Purinergic signaling is terminated by ectonucleotidases, expressed at the endolymphatic surface of the organ of Corti (Housley et al. 2002; Vlajkovic et al. 2004), which degrade ATP_e (Zimmermann et al. 2012). Ca^{2+} wave propagation requires ATP_e binding to $\text{G}_{q/11}$ -coupled P_2Y receptors that activate a canonical intracellular signal transduction cascade, leading to diacylglycerol and inositol-1,4,5-trisphosphate (IP_3) generation from phospholipase C_β (PLC_β)-dependent hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) (Okamura et al. 2001; Abbracchio et al. 2006; Kadamur and Ross 2013). Intracellular diffusion

of IP_3 and subsequent binding to IP_3 receptors triggers Ca^{2+} release from the ER (Berridge 2009), raising the $[\text{Ca}^{2+}]_c$ to peak levels of ~ 500 nM (Beltramello et al. 2005; Ceriani et al. 2016b). ATP is found at millimolar concentration in the cytoplasm of most cells (Imamura et al. 2009), and the Ca^{2+} -dependent increase in the open probability of connexin hemichannels (De Vuyst et al. 2006, 2009; Zhang et al. 2006; Leybaert et al. 2017; Carrer et al. 2018; Hu et al. 2018) favors the release of ATP from cytosol to extracellular milieu (Anselmi et al. 2008; Majumder et al. 2010). Thus, ATP -dependent ATP release enables the regenerative cell-to-cell propagation of Ca^{2+} signals across the network of cochlear nonsensory cells by exploiting a cascade of biochemical reactions governed by critical phenomena that control also the frequency of intracellular $[\text{Ca}^{2+}]_c$ oscillation (Ceriani et al. 2016b).

In the LER, nonsensory cells develop spontaneous $[\text{Ca}^{2+}]_c$ oscillations when exposed to ARL 67156 (Anselmi et al. 2008), an ectonucleotidase inhibitor (Levesque et al. 2007), indicating that ATP is rapidly degraded by ectonucleotidases in the absence of the inhibitor. In the GER, spontaneous $[\text{Ca}^{2+}]_c$ transients arise periodically (suggesting that ectonucleotidase activity is constitutively reduced compared with the LER) and propagate as Ca^{2+} waves that reach the IHCs (Tritsch et al. 2007).

Flufenamic acid, a nonspecific blocker of connexin channels (Verselis and Srinivas 2013; Willebrords et al. 2017) blocks both Ca^{2+} waves in the LER (Anselmi et al. 2008), and spontaneous Ca^{2+} signaling activity in the GER (Schutz et al. 2010; Mammano and Bortolozzi 2018). The latter is also inhibited by a monoclonal antibody that binds the EC1 loop of Cx26 and blocks hemichannels, but not GJ channels (Xu et al. 2017). Other pharmacological interference experiments (Piazza et al. 2007; Rodriguez et al. 2012) with (1) thapsigargin, a noncompetitive inhibitor of the Ca^{2+} -ATPase (SERCA) that causes the complete and irreversible depletion of Ca^{2+} from the ER, (2) the PLC inhibitor U73122, and (3) the IP_3R antagonist 2-aminoethoxydiphenyl borate (2-APB), indicate that ATP -dependent Ca^{2+} signaling in the

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GER and the LER are both mediated by the $\text{PIP}_2 \rightarrow \text{PLC} \rightarrow \text{IP}_3$ signaling cascade described above. This conclusion is also supported by Ca^{2+} imaging in the LER and the GER of mice with reduced expression of phosphatidylinositol-4-phosphate 5-kinase, type 1 gamma (*Pip5k1c*^{+/-}; MGI:3054978) (Rodriguez et al. 2012), a key enzyme that generates PIP_2 by phosphorylation of $\text{PI}(4)\text{P}$ (Di Paolo et al. 2004).

Ca^{2+} waves failed to propagate in the developing sensory epithelium of *Gjb6*^{-/-} and *tGjb2*^{-/-} mice (Anselmi et al. 2008; Ortolano et al. 2008; Rodriguez et al. 2012), both of which

failed to acquire hearing (Teubner et al. 2003; Crispino et al. 2011), whereas waves propagated normally in mice that were global knockouts for molecular components of alternative ATP release conduits (Pellegatti et al. 2005; Suadicani et al. 2006; Huang et al. 2007), such as *P2rx7*^{-/-} mice (MGI:3606250) and *Panx1*^{-/-} mice (EM:11476; MGI:5297171), both of which had hearing thresholds indistinguishable from those of their wild-type (+/+) siblings (Fig. 3) (see also Zorzi et al. 2017).

Bovine adeno-associated virus (BAAV) has a shown high tropism for nonsensory cells of

Figure 3. Ca^{2+} signals evoked by 100 msec focal photostimulation with caged IP_3 fail to propagate in connexin deficient cochlear cultures but propagate normally in *P2rx7*^{-/-} and *Panx1*^{-/-} cultures. False-color images show Ca^{2+} signals in cochlear supporting and epithelial cells from P3-P6 mouse organotypic cultures coloaded with the AM esters of fura red and caged IP_3 . The corresponding audiograms were measured by auditory brainstem recordings (ABRs) in adult mice (P30-P45). For further details, see Anselmi et al. (2008) and Zorzi et al. (2017).

rodent inner ear (Di Pasquale et al. 2005; Di Pasquale and Chiorini 2006; Shibata et al. 2009; Sheffield et al. 2011). Viral transduction with BAAV vectors encoding Cx30 restored Cx30 expression as well as Ca^{2+} wave propagation in cochlear organotypic cultures from P5 *Gjb6*^{-/-} mice (Ortolano et al. 2008). In vivo transduction with BAAV vectors encoding Cx30 via canalostomy in P4 *Gjb6*^{Δ/Δ} mice promoted formation of Cx30 GJs and improved slightly but significantly hearing thresholds (measured around P30) of injected mice compared with noninjected controls (Crispino et al. 2017). In contrast, hearing thresholds were elevated by ~15 dB sound pressure level (SPL) around P30 in wild-type (C57BL6) mice that had received a single shot of flufenamic acid via canalostomy at P4, whereas mice injected with artificial endolymph (vehicle) were not affected (Rodriguez et al. 2012). Similarly, elevated thresholds were also measured in a mouse model for a DFNA3 mutation (*Gjb6*^{T5M/T5M}; MGI:4848154; EM:05214), in which spontaneous Ca^{2+} signaling was slightly albeit significantly reduced in the GER at P5 compared with *Gjb6*^{+/+} controls (Schutz et al. 2010).

The propagation of intercellular Ca^{2+} waves is accompanied by crenation of GER tall columnar cells (Tritsch et al. 2007), which is thought to depend on osmotic cell shrinkage by TMEM16A Ca^{2+} -activated chloride channel opening. Release of chloride through these channels, which are highly expressed by supporting cells adjacent to IHCs, also forces K^{+} efflux that has been proposed to depolarize transiently the IHCs (Wang et al. 2015). Depolarization increases the frequency with which prehearing IHCs spontaneously generate Ca^{2+} action potentials (APs) (Kros et al. 1998; Beutner and Moser 2001) that trigger exocytosis (Glowatzki and Fuchs 2000). Optical detection of spontaneous AP activity by confocal Ca^{2+} imaging in cochlear organotypic cultures has been shown (Ceriani et al. 2016a). The biophysical properties and distribution of K^{+} currents change dramatically during postnatal development in IHCs, which gradually lose the ability to fire Ca^{2+} APs (Kros et al. 1998; Marcotti et al. 2003) and produce instead graded receptor po-

tentials in response to hair bundle stimulation (Beurg et al. 2006; Wu et al. 2017).

Most afferent nerve fibers innervate a single IHC by P12, guaranteeing that the place-frequency tonotopic map forms independently of sound-evoked neural activity (Sher 1971; Rubel and Fritzsch 2002; Echterler et al. 2005; Bulankina and Moser 2012). Thus, genetically encoded guidance is primarily responsible for initial targeting and segregation of inputs in auditory nuclei, whereas patterned spontaneous activity in immature IHCs is thought to provide essential clues for postnatal refinements in spiral ganglion connectivity before hearing onset (Tritsch et al. 2010; Clause et al. 2014).

These modifications are paralleled by functional maturation of synapses formed by IHCs with afferent fibers (Johnson et al. 2013a,b; see also Marcotti and Safieddine 2018). IHC afferent synapses present electron-dense body, surrounded by tethered vesicles (Safieddine et al. 2012; Wichmann and Moser 2015), which changes progressively the shape from spherical to an elongated “ribbon”-like structure during the first two postnatal weeks (Sobkowicz et al. 1982). Ribbon synapses failed to mature in IHCs of both *Gjb2*^{OtogCre} mice and *Gjb6*^{-/-} mice (Cohen-Salmon et al. 2002; Johnson et al. 2017). Recall that Cx30 is absent, and Cx26 is massively down-regulated during cochlear postnatal development in *Gjb6*^{-/-} mice (Ortolano et al. 2008; Boulay et al. 2013). In addition, ATP-dependent Ca^{2+} activity was impaired in *Gjb6*^{-/-} cochlear organotypic cultures (Fig. 4) (Anselmi et al. 2008; Ortolano et al. 2008; Rodriguez et al. 2012). Patch clamp recordings in *Gjb6*^{-/-} IHCs revealed a comprehensive brake in IHC development, as basolateral membrane currents retained a prehearing phenotype (Fig. 5) (Johnson et al. 2017). Maturation of IHCs was partially prevented also in *tGjb2*^{-/-} mice, in which Cx26 was deleted from the E-sys but retained, with Cx30, in the C-sys, and was almost normal in *Gjb6*^{Δ/Δ} mice, which lack Cx30 altogether but retain ~50% Cx26 in the cochlea (Johnson et al. 2017). Analysis of the unrelated Cx26 conditional KO mouse *Gjb2*^{loxP/loxP} *Prox1-CreER*^{T2}, suggests that connexin expression in the organ of Corti may be critical also for OHC functional

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Figure 4. Spontaneous Ca^{2+} transients in the greater epithelial ridge (GER) of postnatal cochlear cultures. (A) Representative false color image of fura-2 fluorescence ratio changes (ΔR), encoded as shown by the color scale bar, obtained as maximal projection rendering of all frames recorded in a basal turn culture from a control P5 mouse (C57BL6/N) imaged for 4 min at 8 frame/sec; 20 regions of interests (ROIs) are shown superimposed on different Ca^{2+} hot spots. Scale bar, 100 μm . (B) Fura-2 traces generated as pixel averages from the 20 color-matched ROIs shown in the left panel. (C,D) Frequency histograms of spontaneous Ca^{2+} transients (events) in cultures from P5 wild-type (C) and littermate $Gjb6^{-/-}$ mice (D); in each graph, the solid black line (right ordinates) is the time integral of the corresponding frequency histogram and, as such, it tracks the mean number of events in the cell population from the onset of the recording. For further details, see Rodriguez et al. (2012).

Figure 5. Membrane currents, exocytosis, and ribbon morphology in inner hair cells (IHCs) of *Gjb6*^{-/-} mice. (A,B) Potassium currents recorded from P18 IHCs of wild-type (A) and littermate *Gjb6*^{-/-} (B) mice using depolarizing voltage steps in 10 mV nominal increments from the holding potential of -84 mV to the various test potentials shown by some of the traces. The adult-type currents ($I_{K,f}$ and $I_{K,n}$) were only present in IHCs from wild-type mice (A). IHCs from *Gjb6*^{-/-} mice retained the currents characteristic of immature cells ($I_{K,s}$ and I_{K1}). (C) Calcium current, I_{Ca} , and corresponding change in membrane capacitance, ΔC_m , recorded from adult control and *Gjb6*^{-/-} IHCs obtained in response to 50 msec voltage steps, in 10 mV increments, from -81 mV. For clarity, only maximal responses at -11 mV are shown in panel C. (D) Average peak I_{Ca} (bottom panel) and ΔC_m (top panel) curve from control (P17–25, $n = 15$) and *Gjb6*^{-/-} (P18–24, $n = 5$) IHCs. (E,F) Typical cross-sectional profiles of synaptic ribbons obtained from a control (E) and a *Gjb6*^{-/-} (F) IHC. Note that some of the synaptic vesicles are missing around the ribbon of the *Gjb6*^{-/-} IHC (arrow). Scale bar, 200 nm. For further details, see Johnson et al. (2017).

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maturation (Zhu et al. 2013). Taken together, these results provide compelling evidence that the delicate and tightly intertwined relationship between Cx26/Cx30 expression and ATP-dependent intercellular Ca^{2+} signaling in the post-natal cochlea plays a fundamental role for hearing acquisition.

CONNEXINS AND MAINTENANCE OF COCHLEAR FUNCTION

In vivo injection of a BAAV vector encoding a bacterial Cre recombinase via canalostomy in adult *Gjb2*^{loxP/loxP} mice promoted Cre/LoxP recombination in nonsensory cells of the cochlear duct, resulting in decreased Cx26 expression (particularly in fibrocytes of the spiral ligament), decreased endocochlear potential, increased hearing thresholds (by as much as 60 dB SPL at 20 kHz), and >90% loss of OHCs. No effect was observed after delivery of this vector to control C57BL6/N mice, therefore these results indicate that Cx26 expression is essential also for maintenance of cochlear function in the adult cochlea (Crispino et al. 2017). As mentioned previously, reduced GJ coupling may limit the transfer of nutrients, and glucose in particular, from distant blood vessels to the avascular sensory epithelium. In turn, nutrient deficiency impacts on ATP production, leading to reactive oxygen species (ROS) overload and apoptosis (Zhao et al. 2017), whereas massive loss of cytosolic ATP triggers necrosis in several cell types (Leist et al. 1997). This offers, in particular, a mechanistic explanation for the massive loss of OHCs because of their elevated mitochondrial metabolism (Jensen-Smith et al. 2012), which makes them particularly vulnerable to depletion of intracellular ATP levels. The role played by insufficient expression of Cx26 in redox imbalance, dysregulation of the Nrf2 pathway and etiopathogenesis of presbycusis has been further explored in a recent article (Fetoni et al. 2018).

CONCLUDING REMARKS

Genetic studies in humans have uncovered a critical for hearing role played by inner ear con-

nexins, particularly CX26, CX30, and CX31. Mouse models have confirmed that Cx26 is essential for hearing. Indeed, ablation of Cx26 in the cochlea is associated with down-regulation of Cx30 and affects not only hair cell survival but also the normal development of the organ of Corti. However, studies of connexin channel composition have provided conflicting results in humans and mice.

Although the exact function of inner ear connexin remains incompletely undefined, there is compelling evidence that the networks of GJ channels they form supply the virtually avascular sensory epithelium with glucose and possibly other key metabolites. In addition, connexin hemichannels play a fundamental paracrine function in the developing cochlea, in conjunction with extracellular P2Y receptors and intracellular IP₃ receptors, enabling the propagation of intercellular Ca^{2+} waves, influencing hair cell maturation and wiring of the organ of Corti. A recent review has also explored the potential role played by these connexin-dependent Ca^{2+} -signaling mechanisms in sculpting the auditory sensory epithelium into its terminally functional shape through autophagy and apoptosis (Mammano and Bortolozzi 2018).

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