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SHORT COMMUNICATION

Differences in molecular mechanisms of K+ clearance in the auditory sensory epithelium of birds and mammals

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ABSTRACT

Mechanoelectrical transduction in the vertebrate inner ear is a highly conserved mechanism that is dependent on K^+ influx into hair cells. Here, we investigated the molecular underpinnings of subsequent K^+ recycling in the chicken basilar papilla and compared them with those in the mammalian auditory sensory epithelium. As in mammals, the avian auditory hair cell uses KCNQ4, KCNMA1 and KCNMB1 in its K^+ efflux system. Expression of *KCNQ1* and *KCNE1* suggests an additional efflux apparatus in avian hair cells. Marked differences were observed for K^+ clearance. In mammals, KCC3, KCC4, Kir4.1 and CLC-K are present in supporting cells. Of these, only *CLC-K* is expressed in avian supporting cells. Instead, they possess NKCC1 to move K^+ across the membrane. This expression pattern suggests an avian clearance mechanism reminiscent of the well-established K^+ uptake apparatus present in inner ear secretory cells. Altogether, tetrapod hair cells show similar mechanisms and supporting cells show distinct molecular underpinnings of K^+ recycling.

KEY WORDS: Mechanotransduction, Avian, Evolution, Inner ear, Potassium, Slc12

INTRODUCTION

K^+ is the major charge ion in the vertebrate inner ear. Its apical influx into hair cells initiates mechanotransduction in both the auditory and vestibular system (Wangemann, 2002; Hibino and Kurachi, 2006). The influx is based on a high K^+ concentration in the endolymph of the scala media (Johnstone et al., 1963). Despite these conserved features, marked differences in detail exist across vertebrates such as the extent of the positive endocochlear potential in the endolymph. In therian mammals, it is typically +80 to +90 mV (Schmidt and Fernandez, 1962; Wangemann, 2006), and may even exceed +120 mV (Steel et al., 1987), whereas in birds it is normally +10 to +20 mV (Poje et al., 1995; Schmidt, 1963; Wilms et al., 2016), with the barn owl at the upper limit with +34 mV (Wilms et al., 2016), and in reptiles it is +2 to +7 mV (Schmidt and Fernandez, 1962). This difference is paralleled by a different organization of the secretory epithelia generating the endolymph. In mammals, the stria vasularis is situated in the lateral wall and consists of multiple layers of highly specialized cells (Hibino et al., 2010), whereas in birds, the secretory epithelium is single layered and overlies the basilar papilla (BP) and separates the scala media and scala vestibuli (Ishiyama et al., 1970).

Gene expression analysis of the secretory epithelia in mouse and chicken revealed a strong conservation of the molecular machinery for K^+ secretion into the endolymph (Wilms et al., 2016). Nine out of 11 genes were expressed in both tissues. K^+, however, must be not only secreted into the endolymph but also effluxed from hair cells, cleared from their extracellular space and then recycled to the stria vascularis (Mistrik and Ashmore, 2009; Hibino and Kurachi, 2006; Zdebik et al., 2009). Molecular and genetic analyses over the last two decades have identified many key players in the mammalian organ of Corti. K^+ efflux from hair cells is mediated by activation of voltage-gated KCNQ4 channels (Kubisch et al., 1999) and Ca^{2+}-activated K^+ BK channels (Lewis and Hudspeth, 1983; Navaratnam et al., 1997; Rosenblatt et al., 1997). They release K^+ into the extracellular space, where it is removed in order not to continuously depolarize hair cells (Mistrik and Ashmore, 2009; Hibino and Kurachi, 2006; Zdebik et al., 2009). Several pathways have been proposed that may act in parallel to achieve this clearance (Zdebik et al., 2009). Current measurements suggest that some K^+ diffuses extracellularly through the tunnel of Corti to reach the open perilymph space of the scala tympani (Zidanic and Brownell, 1990; Johnstone et al., 1989). Additionally, K^+ may be taken up by neighboring supporting cells such as Deiters' cells and Claudius' cells. K^+ is then passed through an epithelial tissue gap junction system coupling supporting cells to be relayed to the stria vascularis. This pathway is based on genetic analysis. Deiters' cells express the potassium chloride co-transporters KCC3 (Boettger et al., 2003) and KCC4 (Boettger et al., 2002) and the inwardly rectifying K^+ channel $K_{ir}4.1$ (Hibino et al., 1997). Mutations in any of the three genes cause deafness. The precise (patho-) mechanisms, however, have still to be elucidated (Hibino et al., 2004; Zdebik et al., 2009), as KCC3 (Boettger et al., 2003) and $K_{ir}4.1$ (Hibino et al., 2004) are also expressed in other areas of the cochlea. Further recycling of K^+ to the stria vascularis might then occur via an epithelial cell gap junction system (Kikuchi et al., 2000) involving connexins 26 and 30 (Kikuchi et al., 1994; Lautermann et al., 1998).

In chicks, connexins 26 and 30 were shown to be expressed in supporting cells of the basilar papilla. In contrast, birds have lost the gene *Slc12a6* encoding KCC3 and amphibians and lizards are devoid of *Slc12a7* encoding KCC4 (Gagnon and Delpire, 2013; Hartmann et al., 2014), suggesting differences in the K^+ circulation system in tetrapod inner ears. To clarify this issue, we examined the expression of avian orthologs of the mammalian toolkit.

MATERIALS AND METHODS

Animals

Chickens, *Gallus gallus domesticus* (Linnaeus 1758), egg-layer breed, aged 12–17 days post-hatching were used. All protocols were in accordance with the German Animal Protection law and were approved by local animal care and use committees (Laves,

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Oldenburg, Germany). Protocols also followed the NIH guide for the care and use of laboratory animals.

Tissue fixation

Animals were injected with a lethal dose of sodium pentobarbital (Narkodorm, CP-Pharma GmbH, Burgdorf, Germany) and perfused transcardially with phosphate-buffered saline (PBS) containing (mmol l^{-1}): 130 NaCl, 7 Na_2HPO_4 and 3 NaH_2PO_4, pH 7.4, followed by fixation by 4% paraformaldehyde (PFA) in PBS. Animals were then decapitated and the head was stored in PFA at 4° C overnight. For dissection of the cochlea, the middle ear cavity was opened and bony structures were removed.

RNA isolation and reverse transcription

Total RNA was isolated from the brain and kidney by the guanidine thiocyanate method (Chomczynski and Sacchi, 1987). Reverse transcription of total RNA (10 µg) was performed using standard protocols as described previously (Nothwang et al., 2001; Wilms et al., 2016).

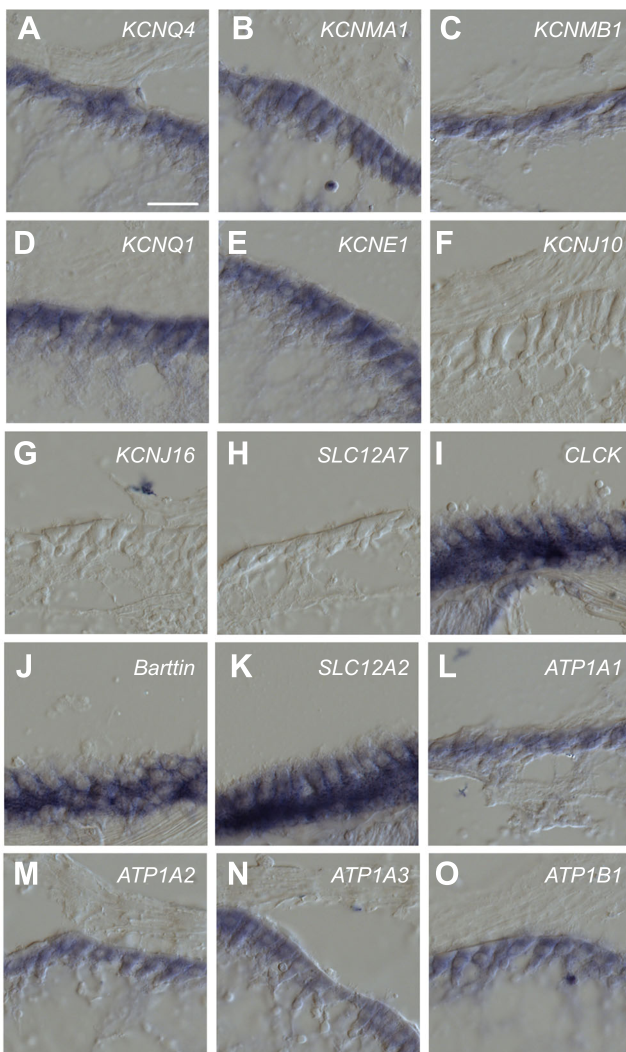

Fig. 1. RNA *in situ* hybridization on chicken basilar papilla. Cochlea cross-sections were hybridized with digoxigenin-labeled cRNA probes, as indicated in the images. Most probes led to a signal in the hair cells of the basilar papilla (A–E, I–O). Only hybridization with *KCNJ10*, *KCNJ16* and *SLC12A7* probes showed no signal, even after prolonged staining (F–H). Functionality of probes was shown previously (Wilms et al., 2016). *n*=4 for A–C, H; *n*=5 for D–G, I–O. Scale bar, 20 µm.

RNA *in situ* hybridization

Probes for *ATP1A1*, *ATP1A2*, *ATP1A3*, *ATP1B2*, *KCNJ10*, *KCNJ16*, *KCNMA1*, *KCNMB1*, *KCNQ1*, *KCNQ4*, *KCNE1*, *SLC12A2* and *SLC12A7* were generated from brain cDNA, and probes for *BSND* and *CLCK* were generated from kidney cDNA. Primers are given in Table S1. PCR products were cloned and transcribed by T7 or SP6 polymerases in the presence of digoxigenin-11-UTP (Roche, Mannheim, Germany) (Ehmann et al., 2013) to yield antisense and sense probes, respectively.

Cochleae were cryoprotected in 15% or 30% sucrose in PBS for ≥30 min. They were then cut into two pieces harboring approximately linear segments of basilar papilla each. The pieces were transferred to custom-made molds filled with TissueTek (VWR, Darmstadt, Germany), oriented for later cross-sectioning, and rapidly frozen on a metal platform cooled by liquid nitrogen. Cross-sections of 20 µm thickness were cut on a cryostat (HM 400, Microm, Waldorf, Germany or CM1950, Leica Biosystems) and sections were stored at −80°C until use. On-slide *in situ* hybridization was performed at 50–60°C overnight in hybridization buffer [50% formamide, 5× SSC, 2% blocker (Roche), 0.02% SDS, 0.1% *N*-lauryl sarcosine; Wilms et al., 2016; Pawlik et al., 2016]. Bound probes were detected with an anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche). SP6 (sense) probes served as negative controls and yielded no staining. Slides were documented using DIC optics on a Nikon Eclipse 90i microscope with a digital camera attached.

Immunohistochemistry

Cochlea sections were mounted on Superfrost Plus microscope slides (Thermo Fisher Scientific, Waltham, MA, USA), dried and subsequently immunoreacted according to standard protocols, using 3% bovine serum albumin+0.2% Triton X-100 as blocking solution. The primary antibody anti-Na^+/K^+-ATPase (A276, Sigma-Aldrich, Darmstadt, Germany) was applied at a concentration of 1:4000, followed by a secondary anti-mouse antibody (1:1000; Invitrogen, Carlsbad, CA, USA), coupled to Alexa Fluor 488. Control slides were treated in parallel, omitting the primary antibody. Finally, slides were coverslipped with Mowiol and documented using a TCS SP8 confocal laser scanning microscope (Leica, Wetzlar, Germany).

RESULTS AND DISCUSSION

To investigate the molecular basis of K^+ cycling in the avian basilar papilla, we characterized in the chicken the expression pattern of genes suggested to be involved in K^+ cycling in the auditory sensory epithelium of mammals: *KCNJ10*, *KCNQ4*, *KCNMA1* and *KCNMB1* encoding the K^+ channels $K_{ir}4.1$ and $K_v7.4$ and the Ca^{2+}-activated K^+ channel subunits α1 and β1, respectively, and *SLC12A7*, encoding KCC4. We also studied *CLC-K*, encoding a Cl^- channel, which is expressed in mature Deiters' cells. Note that the chicken genome has only one *CLC-K* gene, unlike mammals, which harbor two paralog genes in their genome (Wilms et al., 2016). Finally, we included several genes previously probed in the tegmentum vasculosum (Wilms et al., 2016), as some of them were apparently expressed in the basilar papilla. This set of genes comprised *ATP1A1*, *ATP1B1*, *ATP1A2*, *ATP1A3*, *ATP1B1*, *BSND*, *KCNJ16*, *KCNQ1*, *KCNE1* and *SLC12A2*. To study their expression, RNA *in situ* hybridization with digoxigenin-labeled antisense probes was performed on cross-sections of the cochlea

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isolated from chickens aged 12–17 days post-hatching (P12–P17). At this age, the chicken cochlea is mature (Runhaar et al., 1991).

We first focused on the efflux system in chicken hair cells by probing *KCNQ4*, *KCNMA1* and *KCNMB1*, encoding $K_v7.4$ and the Ca^{2+}-activated K^+ channel BK α1 and β1 subunits, expressed in mammalian hair cells. Additionally, we included *KCNQ1* and *KCNE1*, encoding $K_v7.1$ (also known as K_vLQT1) and its modulatory subunit KCNE1 (also known as IsK channel). All five genes were clearly expressed in the hair cells, whereas supporting cells were unlabeled (Fig. 1A–E). As *KCNQ1* and *KCNE1* are specifically expressed in the mammalian stria vascularis, we included *KCNJ10* and *KCNJ16* as two further strial K^+ channel-encoding genes. However, neither gene was expressed in the basilar papilla (Fig. 1F,G).

To probe genes involved in K^+ clearance and buffering by supporting cells, the expression of *SLC12A7* and *CLC-K* was analyzed. No signal for *SLC12A7* was observed in the basilar papilla even after prolonged incubation, whereas *CLC-K* was clearly expressed in supporting cells underneath the hair cells of the basilar papilla (Fig. 1H,I). In the mammalian stria vascularis, CLC-K associates with Barttin (Estévez et al., 2001). We therefore also probed the expression of *BSND*, encoding Barttin. Indeed, *BSND* was also expressed in supporting cells (Fig. 1J). We furthermore analyzed *SLC12A2*, encoding the $Na^+/K^+/Cl^-$ co-transporter NKCC1, as we had noted its expression in the inner ear in a previous study (Wilms et al., 2016). The probe clearly labeled supporting cells (Fig. 1K).

Finally, we studied four genes, encoding Na^+/K^+-ATPase subunits, i.e. *ATP1A1*, *ATP1A2*, *ATP1A3* and *ATP1B1*, as they might also be involved in K^+ cycling and clearance in the inner ear (Schuth et al., 2014). All four genes were clearly expressed in hair cells (Fig. 1L–O). To probe whether Na^+/K^+-ATPase subunits are also present in the plasma membrane of supporting cells, we performed immunohistochemistry with an anti-Na^+/K^+-ATPase antibody. We preferred this technique over RNA *in situ* hybridization because it avoided false-positive signals due to diffusion of the dye during the long incubation required to detect low-abundance gene expression. The antibody strongly labeled the base of hair cells, but immunoreactivity was also observed in the surrounding supporting cells (Fig. 2).

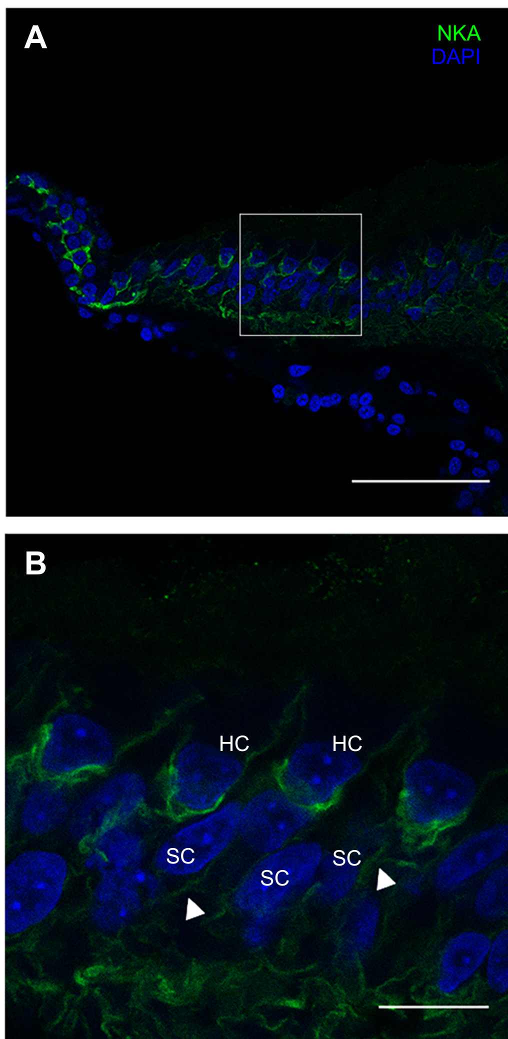

Fig. 2. Immunohistochemical analysis of Na^+/K^+-ATPase subunits. Cochlear cross-sections were labeled with an anti-Na^+/K^+-ATPase antibody (NKA). Strong immunoreactivity was observed at the base of hair cells, consistent with the strong RNA *in situ* hybridization signals. Additionally, immunoreactivity was detected in the membrane of supporting cells (arrowheads). The boxed region in A is shown at greater magnification in B. *n*=4. Scale bar: A, 50 µm; B, 10 µm. HC, hair cell; SC, supporting cell.

In sum, our data reveal similarities but also striking differences in K^+ recycling between mammals and birds. In mammals, K^+ efflux from hair cells is mediated by $K_v7.4$ and the BK channels. Both are present in avian hair cells, but we observed a third efflux apparatus, consisting of the $K_v7.1$ and KCNE1 channel proteins. These two proteins mediate K^+ secretion into the endolymph in the mammalian stria vascularis (Sakagami et al., 1991) and the tegmentum vasculosum (Wilms et al., 2016). The reason for their recruitment is currently unknown as the driving force of K^+ into avian hair cells is reduced compared with that in mammals, because of the lower endocochlear potential in birds. Consequently, the intracellular accumulation of K^+ in avian hair cells should be less. Intriguingly, we noted expression of all four tested Na^+/K^+-ATPase subunits in the avian hair cells. The inwardly directed K^+ transport of this pump counteracts the efflux of this ion. This might contribute to the necessity for an additional K^+ efflux system in the basilar papilla. In contrast, the pump is only weakly expressed in the mammalian organ of Corti. The rat cochlea weakly expresses α2 in outer hair cells and α1 in pillar cells (ten Cate et al., 1994), and does not express α3, β1 or β2. Similarly, mice showed weak expression of α1 in the organ of Corti, whereas α2, α3, β1 and β2 were absent (Erichsen et al., 1996). The absence of the ATP-driven pump in mammalian hair cells is thought to reflect the need to avoid metabolic energy. A high metabolism would likely require vascularization close to hair cells, changing cochlea micromechanics. Furthermore, blood flow-associated vibrations may add noise to the system (Zdebik et al., 2009). Especially during loud sound stimulation, the absence of an ATP pump might also prevent hyperpolarization, which could produce an effect analogous to photoreceptor adaptation.

Of note, hair cells in the rat vestibular system express α1 (Schuth et al., 2014) and hair cells of the fish inner ear are also positive for Na^+/K^+-ATPase (Clemens Grisham et al., 2013), indicating an ancient requirement for the Na^+/K^+-ATPase in hair cells.

Efflux of K^+ from hair cells must be accompanied by rapid ion removal from the extracellular space in order not to continuously depolarize hair cells. Our data demonstrate striking differences between mammals and birds. In chickens, supporting cells express NKCC1, Barttin and CLC-K, and the Na^+/K^+-ATPase. NKCC1 transports 1 Na^+, 1 K^+ and 2 Cl^- per cycle (Gamba et al., 1994) and exhibits important features required for an efficient K^+ buffer. The

transporter experiences a strong net inwardly directed driving force and has a high affinity for external K^+ (Payne et al., 1995), making it a very potent apparatus to remove K^+ from the surroundings of hair cells. Additional K^+ might be removed via Na^+/K^+-ATPases, which will also accumulate extracellular Na^+ for driving NKCC1. The co-transported Cl^- can then be effluxed by the Cl^- channel formed by CLC-K and Barttin. This is reminiscent of inner ear secretory cells, i.e. marginal cells of the stria vascularis and vestibular dark cells, which operate with an identical system for K^+ uptake (Hibino and Kurachi, 2006; Wangemann, 2006) (Fig. 3). Thus, K^+ clearance in supporting cells is based on a system that has already been shown to be well suited for K^+ uptake in secretory epithelia in the inner ear (Hibino and Kurachi, 2006; Wangemann, 2006; Zdebik et al., 2009) (Fig. 3). In contrast, the logic of the mammalian clearance system is less evident. Mice lacking KCC3 or KCC4 are deaf (Boettger et al., 2002, 2003). The expression of the two transporters in supporting cells strongly suggests their involvement in K^+ clearance and recycling (Fig. 3). However, their precise role is still debated (Wangemann, 2006; Zdebik et al., 2009; Lang et al., 2007), as the direction and magnitude of net K^+/Cl^- co-transport by these two electroneutral transporters will be dictated by the sum of the K^+ and Cl^- chemical potential differences. Both K^+/Cl^- transporters usually operate in an outwardly directed transport mode (Gamba, 2005) and reversal of transport direction would require a low intracellular Cl^- concentration (Payne, 1997). This has not been demonstrated so far in supporting cells. A shared feature of the avian and mammalian supporting cells is the presence of CLC-K (chicken: this study; mammals: Qu et al., 2006). We demonstrated in addition the expression of *BSND*, encoding the accessory subunit barttin. This gene, however, has not been analyzed in the mature mammalian cochlea so far. Independent of the precise mode of operation of K^+ clearance in the mammalian cochlea, the different expression patterns of *SLC12* members between mammals and birds reveal the evolution of different molecular underpinnings in K^+ clearance across vertebrate inner ears.

In conclusion, the variable degree of shared and distinct molecular underpinnings across different features of the vertebrate auditory system will provide ample possibilities to study evolutionary processes and constraints therein, making the auditory system a rewarding research area in this field.

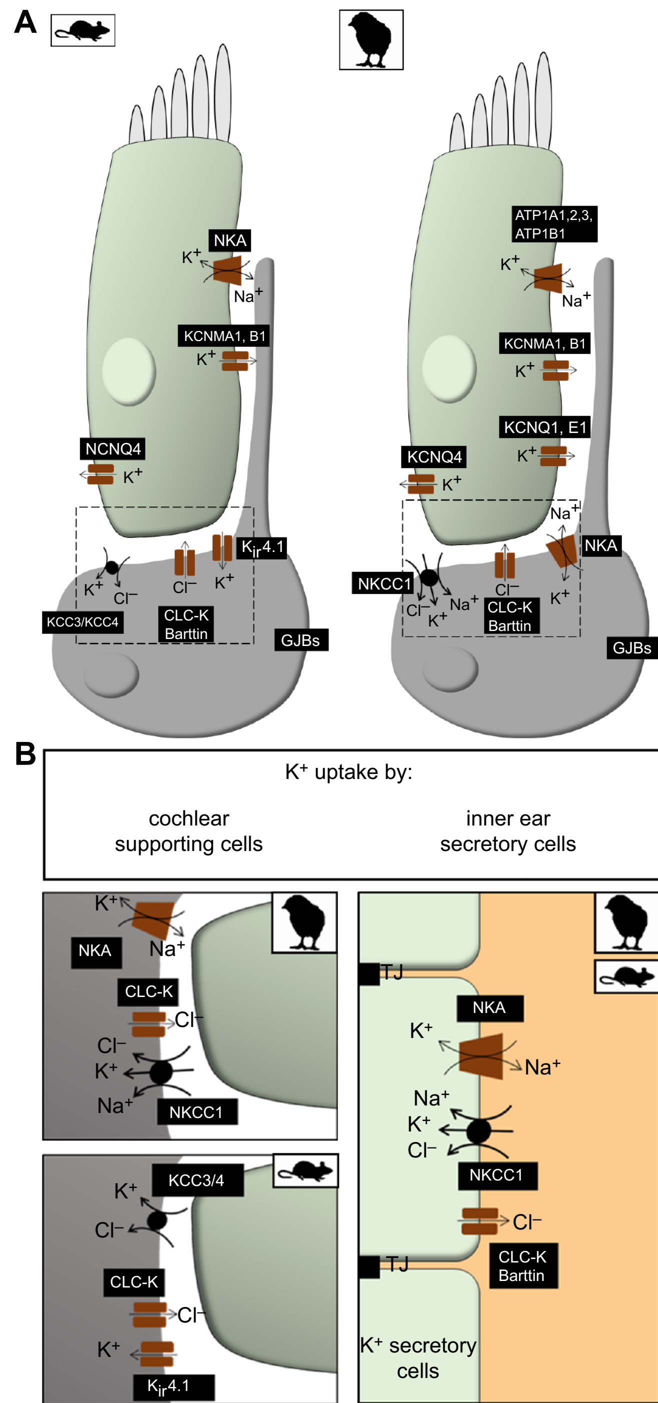

Fig. 3. Schematic model of the molecular repertoire in hair cells, supporting cells and dark cells of mouse and chicken. (A) Proteins responsible for K^+ efflux from the mammalian hair cells are also present in chicken hair cells. The latter additionally contain KCNQ1 and KCNE1. (B) In contrast, the K^+ uptake molecules in chicken supporting cells strongly differ from those in corresponding mammalian cells by the presence of NKCC1 instead of KCC3 and KCC4. The molecular repertoire of avian supporting cells thus resembles the K^+ uptake mechanism of inner ear secretory cells, i.e. the endolymph-producing dark cells in the vestibular system of both chicken and mouse and the mammalian marginal cells in the stria vascularis.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: H.G.N.; Investigation: V.W., C.S.; Resources: H.G.N.; Writing - original draft: H.G.N.; Writing - review & editing: V.W.; Supervision: H.G.N.; Project administration: H.G.N.; Funding acquisition: H.G.N.

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Table S1. Primers and GenBank accession numbers

Gene	Genbank accession number	Primer
gg*ATP1a1*	NM_205521	5’- GGGTAAGACTCCCATTGC -3’ 5’- CTGAAACACAGCACGGTTG -3’
gg*ATP1a2*	NM_205476	5’- AGTATTCCCCCGCTGCCACC -3’ 5’- ATGACGACAGCGGCCAGCACC -3’
gg*ATP1a3*	NM_205475	5’- TGGGGGACAAAGGGGAGAAG -3’ 5’- TGACCACGGCGGCCAAAACG -3’
gg*ATP1b1*	NM_205520	5’- TGGCCCCTAAGTATTGCTGC -3’ 5’- TAGCCTGACCCAGGTCTCTC -3’
gg*Barttin*	XM_003641691.2	5’- ATGGCCGAGGAGAAGACGTTTC -3’ 5’- CTGTGCTGATGCCTGGATCTG -3’
gg*CLC-k*	XM_425749.4	5’- GGCTTTGCCAACAGCATCAC -3’ 5’- CATGAAGGCACCACAAGTAG -3‘
gg*GJB2*	XM_425641	5’- TCCTGTTCATCTTCCGTATC -3’ 5’- GTAGGCCGAGACACGAAAC -3’
gg*GJB6*	NM_204931	5’- TGTGTCCAGCATTTGCATTC -3’ 5’- TATCTGCACGAAGGCACTAG -3’
gg*KCNE1*	XR_210189	5’- CAACCTAGCCAGGTCAAAGC -3’ 5’- GCCCAAGCCAAGAAGTCAAC -3’
gg*KCNJ10*	XM_003643494	5’- CTTCATCACCGGGACCTTC -3’ 5’- AAATCCGCCACGTACTTCC -3’
gg*KCNJ16*	XM_004946241	5’- AGATGAGATGACTGTTTTGG -3’ 5’- CTCATCAGCTAGTGGTTGAC -3’
gg*KCNQ1*	XM_421022	5’- GTGGAGGACAAGGTTACGC -3’ 5’- TCTGAGCCATAATGAGTTGC -3’
ggKCNQ4	XM_015297701	5’- TGCAGTCCAT CGAGCACAAG -3’ 5’- TAATGCAGAGAGGGACGCAG -3’
gg*KCNMA1*	NM_204224	5’- ATCATTCTGC TGTGGAGGAC -3’ 5’- CATAGCTTGTCATTGGCTGC -3’
gg*KCNMB1*	NM_204602	5’- AAAAG CTGGTGACTGCACAG -3’ 5’- GACATTGGTCTGCATTCCTC -3’
gg*SLC12A7*	NM_001006371	5’- ACCAGAGTTT GGTGGAGCAG -3’ 5’- GGAGCCACAAGTTTGTAGTC -3’
gg*SLC12A2*	XM_004949378	5’- ATGGAGGGAAAGCAGCAG -3’ 5’- CTTCCTCTCCATTTGCATAGC -3’

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