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Review

The enigmatic root cell – Emerging roles contributing to fluid homeostasis within the cochlear outer sulcus

Daniel J. Jagger*, Andrew Forge

Centre for Auditory Research, UCL Ear Institute, University College London, 332 Gray's Inn Road, London WC1X 8EE, UK

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ABSTRACT

Despite their curious morphology prompting numerous hypotheses of their normal function, the root cells lining the cochlear outer sulcus have long evaded physiological characterization. A growing body of evidence now suggests that they regulate the solute content of the endolymph and/or the perilymph, and may be essential in safe-guarding the global homeostasis of the cochlea. Immuno-labeling experiments have demonstrated polarized expression of key ion transport proteins, and recent electrophysiological recordings have identified specific membrane conductances. These studies have painted a clearer picture of how this unusual cell type may contribute to the maintenance of sound transduction, and how they may be central to pathological processes associated with various forms of hearing loss.

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1. Introduction

Normal hearing relies on the concerted action of supporting cells, which provide the optimum homeostatic environment for mechano-electrical transduction to occur efficiently. These specialized non-sensory epithelial cells are organized into distinct compartments that support inner hair cells and outer hair cells (Jagger and Forge, 2006). At the lateral extremity of the outer hair cell compartment lie the root cells, which reside in part within the outer sulcus region, and also within the connective tissue forming the spiral ligament (Fig. 1A). As such, they are located strategically at the border of two gap junctional networks, both of which play essential roles in cochlear homeostasis (Hibino and Kurachi, 2006; Kelly et al., 2011; Kikuchi et al., 1995; Nickel et al., 2009). The importance of this junction and the contribution of root cells within the physiology of the outer sulcus are only now becoming clear.

The root cells take their name from characteristic finger-like basolateral projections that extend from their cell bodies and

infiltrate between the mesenchymal fibrocytes of the ligament (Jagger et al., 2010). They display a graded variation of their gross morphological properties along the tonotopic axis of the cochlear spiral, particularly in terms of the number and size of the root processes, and also the functional specializations such as inter-cellular tight junctions. Their physiological membrane properties show a similar position-dependent gradient, suggesting that there are local variations in ion channel expression. In the upper (low frequency encoding) turns of the cochlea the apical membrane of the root cell is exposed to the endolymph within scala media (Marcus and Chiba, 1999). In the lower (high frequency encoding) turns the root cells are completely covered by a layer of cuboidal or columnar Claudius' cells; the root cell membrane is not in direct contact with endolymph (Jagger et al., 2010; Kimura, 1984).

Our appreciation of root cell structure/function has advanced slowly since their first description over one hundred years ago. The improving resolution of available microscopy techniques has provided only incremental increases in our understanding despite numerous studies. Recently the utilization of molecular physiological approaches, such as the use of specific antisera which have localized transport proteins to different parts of the root cell, has added essential detail to the picture. Other studies, including those from this laboratory, have sought to probe the electrophysiological properties of the root cell membrane. This review seeks to draw together these often quite disparate strands of information, in order to better clarify the role root cells may play in normal hearing, and ultimately to speculate on how root cells might be implicated in mechanisms of hearing loss.

Abbreviations: MEBs, membrane-enclosed bodies; Hsp27, heat shock protein-27; AE, anion exchanger; EP, endocochlear potential; ATP, adenosine triphosphate; AQP, aquaporin; CR, canalicular reticulum; AE, anion exchanger; AC, adenyl cyclase; Cx26, connexin26; Cx30, connexin30; EP, endocochlear potential; TRPC, canonical transient receptor potential; AQP, aquaporin; ohc, outer hair cell; cCx26, conditional Cx26; ecgjn, epithelial cell gap junction network; ctgjn, connective tissue gap junction network; mc, marginal cells; Cc, Claudius' cells; rc, root cells; Ps, Promentia spiralis; Sse, Sulcus spiralis externus; CC, Cells of Claudius; sv, Stria vascularis; oC, organ of Corti; os, outer sulcus.

* Corresponding author. Tel.: +44 20 7679 8930; fax: +44 20 7679 8990.

E-mail address: d.jagger@ucl.ac.uk (D.J. Jagger).

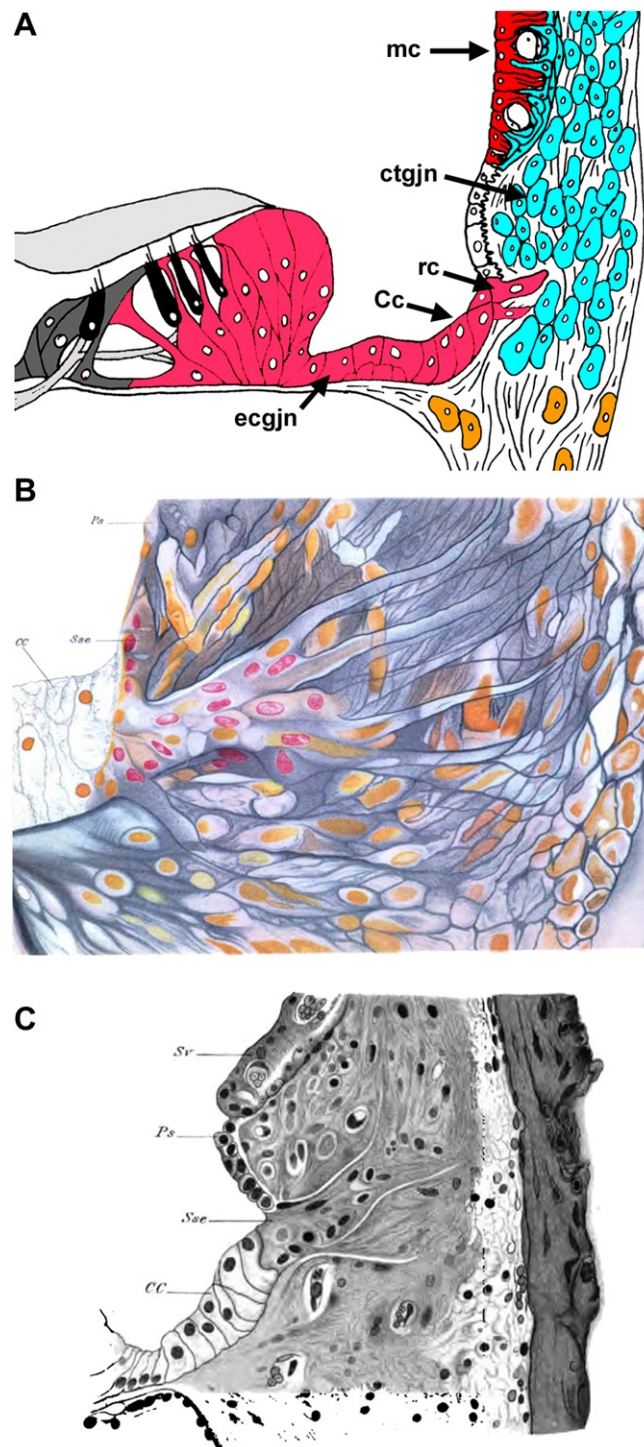


Fig. 1. Root cells reside in the outer sulcus in the basal cochlear turns. (A) Schematic representation of the location of the root cells (rc) underlying Claudius' cells (Cc) at the lateral edge of the epithelial cell gap junction network (ecgjn, pink). The root cell processes extend into the spiral ligament and abut fibrocytes within the connective tissue gap junction network (ctgjn, blue). Intermediate cells within the ctgjn contact the basolateral membrane of marginal cells (mc, red) whose apical membrane faces the endolymphatic duct (scala media). (B–C) Drawings of root cells in light microscopy preparations of the cochlear lateral wall; adapted from Shambaugh (1908). (B) In the lower (high frequency encoding) turns in the guinea pig cochlea the root cells are located beneath the overlying Claudius' cells. Some root cell bodies are located adjacent to those of the Claudius' cells, and others are found deeper within the spiral ligament. The round or ellipsoid nuclei of the root cells are shown in pink. The bundles of root processes, or "pegs", extend into the inferior zone of the spiral ligament amongst the type II fibrocytes and towards the otic bony capsule. (C) In an upper (low frequency coding) turn of the newborn domestic pig cochlea root cells are not evident. Open to the luminal endolymphatic space are apparently small tubule-like structures that extend into the spiral ligament. Abbreviations: Ps, Promentia spiralis; Sse, Sulcus spiralis externus; CC, Cells of Claudius'; Sv, Stria vascularis.

2. The structural and functional properties of outer sulcus root cells – cyto-architecture and membrane physiology

2.1. Morphological studies of root cell specialization

2.1.1. Early light microscopy

The unusual cyto-architecture of the root cell was noted by the 19th century anatomists, including Claudius, Deiters, Böettcher, Katz, and Retzius, and its morphological distinction from other cell types led to unresolved conjecture and debate. For more than one hundred years the precise nature of their physiological role has been proposed to range from that of a simple anatomical anchor for the sensory epithelium, contractile elements responsible for setting the tension of the basilar membrane, glandular fluid pumps essential for the formation of endolymph, to regulators of cochlear fluid ion homeostasis.

The first rigorous study of root cell morphology was carried out by Shambaugh (1908). Guinea pig and domestic pig cochlear sections from various planes were exposed to Mallory reticulum stain and analyzed using light microscopy. The resulting drawings clearly demonstrated the extensive cytoplasmic continuity of the root cells and the other cell types in the outer sulcus, particularly in terms of their cytoplasmic homogeneity, and how the basolateral root processes extend deep into the inferior region of the spiral ligament and between cells of the spiral prominence (Fig. 1B). Shambaugh emphasized the intimate physical association between the root processes and the rich capillary network in the ligament, suggesting that the root cells play an active role in auditory function that involves an exchange between the blood and cochlear fluids. Sections cut perpendicular to the axis of the root processes revealed small tubule-like structures that possessed a lumen positioned between the epithelial clumps, and which opened into the endolymphatic space (Fig. 1C). These tubular structures were easily missed in sections cut parallel to the long axis of the root processes. Shambaugh concluded that the root cell clumps formed a “secreting or glandular epithelium”, that in partnership with stria vascularis, is responsible for the formation of the constituents of endolymph.

The observations of Shambaugh, including the possibility of secreting tubules, were largely confirmed by studies of the outer sulcus of the rhesus monkey (Lawrence, 1956). Contrary to the theory of endolymph secretion, the observation of cell debris within the outer sulcus (Saxen, 1951) suggested that the root cells may perform phagocytic functions, removing debris from the cochlear duct and depositing it into the capillary network. Similarly, root cells were observed to accumulate substances injected into the sub-arachnoid space (Altmann and Waltner, 1950), supporting a role in the re-absorption of low molecular weight species.

2.1.2. Electron microscopy

Essential details of root cell ultra-structure were provided by the first comprehensive study of the outer sulcus using electron microscopy (Duvall, 1969). In the guinea pig, small groups of parallel root processes were seen to form bundles or “pegs” which extended into the surrounding spiral ligament. The root cell processes were separated from the adjacent fibrocytes and capillaries by a basal lamina or basement membrane. Individual root processes could be branched, but there were no anastomoses. Within single processes there were oval nuclei with prominent nucleoli, non-polarized Golgi apparatus, small mitochondria, numerous free ribosomes and extensive rough endoplasmic reticulum. Importantly, root cells did not possess tight junctions in the lower two turns where they do not border the endolymphatic space. Instead, tight junctions were prominent at the apices of the overlying Claudius’ cells, whose apical membranes contact the

endolymph. Tight junctions were evident between root cells in the upper turns where they border the endolymph, and the apical membrane also possessed numerous microvilli suggesting micro-pinocytotic activity. This was supported by the observation of numerous micro-vesicles in the region close to the plasma membrane. In addition, Duvall described unusual and large “membrane-enclosed bodies” (MEBs) within root cells (and within other cells in the region), which contained fine ground substance or debris. Duvall concluded these bodies may represent a macro-transport chain that may allow transfer of material, including cell debris and electrolytes, between different cell types in the outer sulcus and into the capillary network.

Subsequent electron microscopy studies have further extended the description of root cell ultra-structure (Galic and Giebel, 1989; Kimura, 1984; Kucuk and Abe, 1990; Spicer and Schulte, 1997). Within a single region of the outer sulcus distinct “light” and “dark” root cells have been identified (Galic and Giebel, 1989). These apparent cell sub-types were endowed with differing amounts of organelles and intracellular vacuoles, suggesting a divergence in their relative rates of protein synthesis. “Giant” and unusually long mitochondria have been observed within the root cells, oriented in the plane parallel to the axis of the root processes (Galic and Giebel, 1989; Kimura, 1984). Root processes have also been shown to contain variable amounts of tubulo-cisternal endoplasmic reticulum, or “canalicular reticulum” (CR), and in processes more richly endowed with CR there are more numerous mitochondria (Spicer and Schulte, 1997). The functional role of this membranous network remains to be elucidated, but it may be relevant that comparable structures are also features of cells in ion transporting epithelia, particularly in strial marginal cells (Forge, 1982).

A systematic analysis of root cell morphology along the length of the cochlear partition of gerbils quantified the progressive changes in the size of their cell bodies and their root processes (Spicer and Schulte, 1996). As predicted by earlier studies, in the basal (high frequency encoding) turns the cell bodies are smaller but the root bundles have a larger cross-sectional area. Comparable tonotopic variations of the root cells’ morphology are also evident in the guinea pig (Jagger et al., 2010), in the mouse (Fig. 2), and in the rat (Fig. 3). The tonotopic variations in the morphology of root cells coincide with local variations in the fine structural features of the neighboring type II fibrocytes. In the higher frequency encoding regions there are more numerous type IIb fibrocytes surrounding the root process bundles. This sub-class of fibrocyte is identified by its unusually high numbers of mitochondria, which extend into the numerous projections from the cell body (Spicer and Schulte, 1996). In the lower frequency encoding regions there are relatively more numerous type IIa and type IIc fibrocytes, which lack the cytoplasmic projections and have fewer and smaller mitochondria. Gap junctions are plaque-like aggregations of intercellular channels that allow cytoplasmic communication between adjacent cells. Electron microscopy studies have identified these specializations between adjacent Claudius’ cells, between Claudius’ cells and root cells, and between aligned root cell processes (Kikuchi et al., 1995). Importantly, gap junctions do not span the basement membrane between the root cell processes and the enveloping spiral ligament fibrocytes and/or capillaries.

2.1.3. Immuno-detection of expressed proteins

Light microscopy highlighted the ultrastructure of these unusual cells, and electron microscopy then refined the subcellular description which enabled the formulation of novel hypotheses of how the root cells contribute to normal hearing. However, our understanding of their physiology, particularly in ion transport processes, has been accelerated by the use of antisera raised against specific structural and functional proteins. Outlined below are some of the key studies which have enabled these advances.

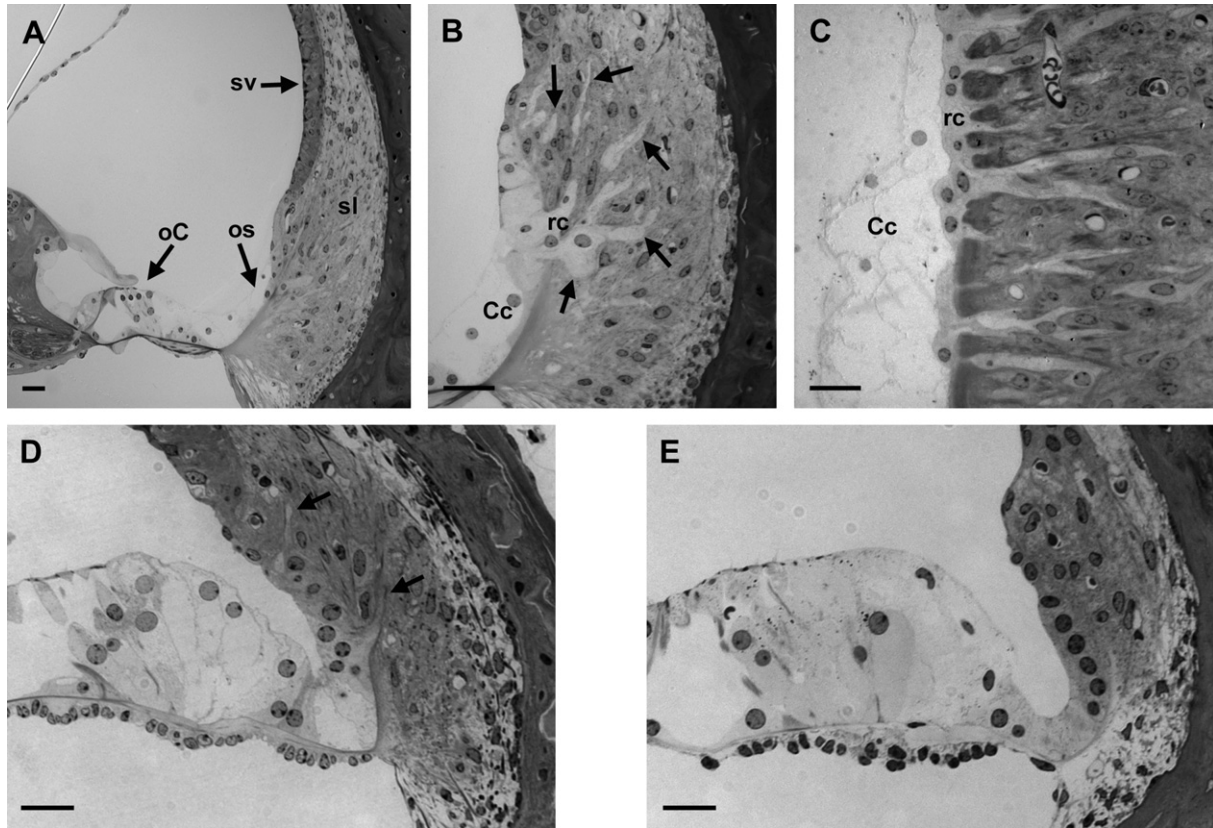


Fig. 2. Light micrographs of the outer sulcus in the adult mouse cochlea. Cochleae were fixed using glutaraldehyde, and sections were cut at 1 μm thickness and stained using toluidine blue. (A) Radial section through the mid-basal turn region, located approximately 20% along the cochlear duct. The hair cells are located within the organ of Corti (oC), surrounded by non-sensory supporting cells forming the epithelial gap junction network. This network extends to the outer sulcus (os) where it meets the cochlear lateral wall, consisting of the spiral ligament (sl) and stria vascularis (sv). (B) Detail of the outer sulcus region in the mid-basal turn (~20% distance), showing Claudius' cells (Cc) overlying the root cells (rc). Some root processes (arrows) are apparent, extending among the fibrocytes of the spiral ligament. (C) A section of the lower basal turn spiral ligament (~10% distance), cut parallel to the plane of the basilar membrane. The root cell body layer is discontinuous; the root processes are often bifurcated and lie in close proximity to blood vessels. (D) In the lower apical turn region (~80% distance) the inferior portion of the spiral ligament is narrower, and the root processes are fewer in number and shorter (arrows). The root processes extend from cells within the endolymph-facing epithelial layer. (E) In the upper apical turn region (~95% distance) there are few if any detectable root processes extending from cells within the endolymph-facing epithelial layer. Scale bars 20 μm .

2.1.3.1. Components of the cytoskeleton and basement membrane. Root cell bodies and their processes are immuno-labeled using an antibody raised against acetylated α -tubulin, with dense bundles of microtubules apparent running through the length of the root process (Jagger et al., 2010). Although the root processes do not appear to contain unusually large amounts of F-actin, they are strongly immuno-labeled using an antibody raised against Heat shock protein-27 (Hsp27) which is known to act to regulate actin polymerization (Leonova et al., 2002). The basement membrane of the root cells delineating the root process from the surrounding spiral ligament tissues is rich in multiple subtypes of type IV collagen (Cosgrove et al., 1996), and this basement membrane is not detectable in a mouse line which is deficient in the COL4A3 subtype (Cosgrove et al., 1998), a model of Alport Syndrome which features sensorineural hearing impairment.

2.1.3.2. Oxidative, ion transporting & 2nd messenger generating enzymes. The outer sulcus region appears to be an area of very high metabolic energy consumption, an observation that points to high transport activities. Early histochemical studies showed that the region of the spiral ligament housing the root cell processes is particularly active for the metabolic enzymes succinate-dehydrogenase and cytochrome oxidase (Vosteen, 1960). Na, K-ATPase, an anti-porter of cytoplasmic Na^+ in exchange for extracellular K^+ , has been shown to be extensively expressed in cochlear

tissues and to be essential for normal cochlear function. Histochemical studies revealed unusually high ATPase activity in the spiral ligament (Iinuma, 1967), which increased from lower to upper turns. Antibodies that do not distinguish between the various subunits of the enzyme label cells in the outer sulcus region of the gerbil at the light microscopic level, and have been localized to the root cell process membrane using immuno-electron microscopy (Nakazawa et al., 1995). In this study the root cell apical membrane was devoid of labeling. Subsequently, immuno-histochemical detection showed that the root cells of the rat cochlea express the $\alpha 1$ and $\beta 1$ subunits, but not $\alpha 2$ -3 or $\beta 2$ (Peters et al., 2001).

Anion exchanger (AE) proteins are also noticeably concentrated to the outer sulcus, suggesting the cells here play important roles in local pH-regulation and possibly in the balancing of endolymphatic pH. The AE2 $\text{Cl}^-/\text{HCO}_3^-$ exchanger is expressed by root cells, particularly within their processes invading the spiral ligament (Stankovic et al., 1997). In addition, there is punctate cytoplasmic immuno-labeling for a vacuolar H^+ -ATPase (Stankovic et al., 1997), raising the possibility of proton secretion by the root cells. An anion transporter protein extensively expressed in the outer sulcus region is linked to Pendred Syndrome, a clinical condition which features congenital deafness. Pendrin (SLC26A4) has been suggested to play roles in the trans-membrane transport of iodine, sulfate, chloride, and bicarbonate in a number of tissues throughout the body. RNA in

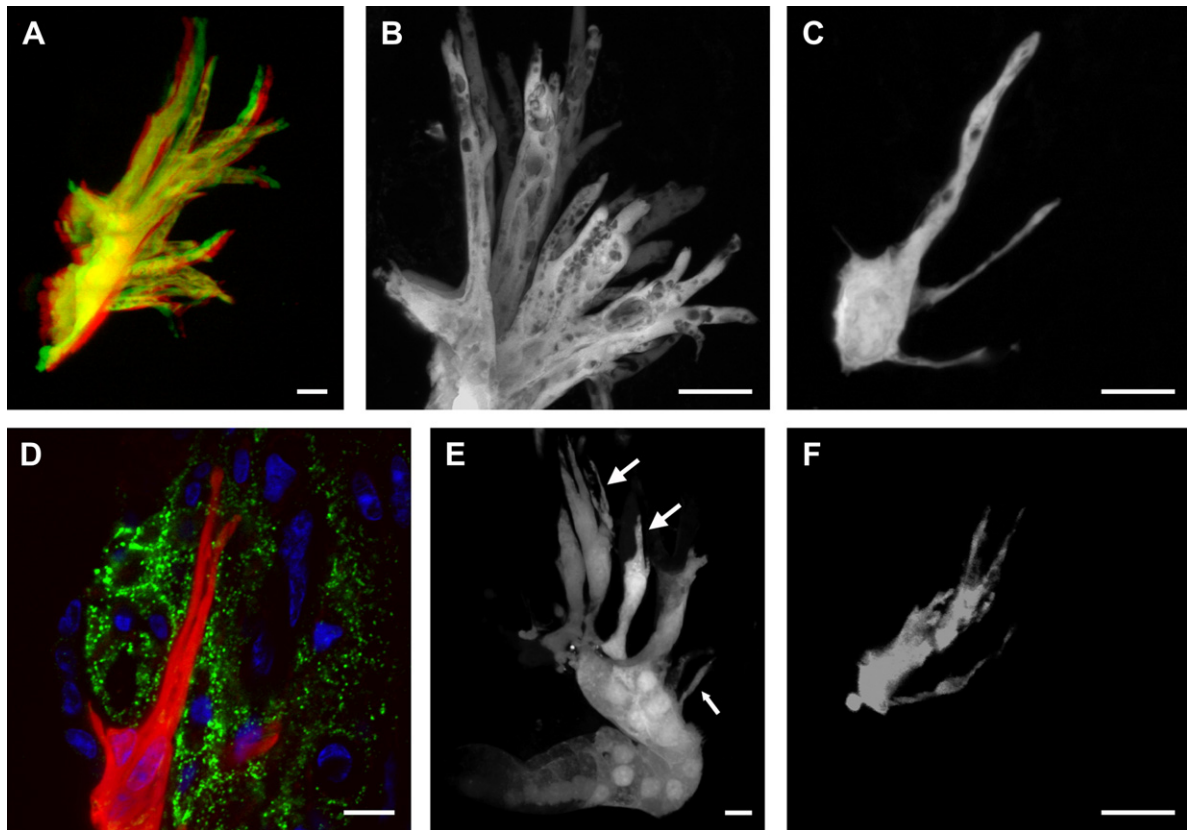


Fig. 3. Investigations of root cell structure and function in slice preparations of the cochlear lateral wall. (A–B) Gross structure of root cells in turn 2 of the lateral wall of the guinea pig, delineated by neurobiotin injected during 10-min whole-cell patch clamp recordings in 200 μm thick slices. Following paraformaldehyde fixation injected neurobiotin was detected using fluorescent streptavidin, and imaged on a confocal microscope (see Jagger et al., 2010). (A) 3-D projection of root processes extending into the spiral ligament (best viewed using red/green or red/blue stereo-glasses), revealing extensive intercellular transfer of neurobiotin via gap junctional coupling. (B) Detail of numerous root processes for several coupled root cells extending into the region behind the spiral prominence. Unbranched and branched root processes are evident. The distribution of neurobiotin is not even throughout the root processes, highlighting the presence of membrane-enclosed bodies (MEBs). (C–F) Gross structure of outer sulcus cells in the rat cochlea revealed by neurobiotin fills. (C) Following a whole-cell recording in the presence of the gap junction uncoupling agent 1-octanol, neurobiotin is restricted within a single root cell. The cell has one large-diameter process containing MEBs extending towards the superior region of the spiral ligament, and two fine processes extending more inferiorly. (D) Detail of the relationship between neurobiotin-coupled root processes (red) and the surrounding fibrocytes in the spiral prominence region. Nuclei are labeled with DAPI (blue). (E) In the lower apical turn, neurobiotin distribution delineates small root processes (small arrow) on the basolateral surface of the cells within the epithelial layer forming the outer sulcus. Four distinct dye-coupled cells are also apparent superior to this layer, each with larger root processes extending into the spiral ligament (large arrows). (F) In a 1-octanol uncoupled cell in the lower apical turn there is a single fine root process and a larger branched process. Scale bars 10 μm .

situ hybridization localized *Pds* gene expression to the outer sulcus of the neonatal mouse (Everett et al., 1999), and the protein was subsequently localized specifically to the root cells of adult mice, including to their root processes (Royaux et al., 2003). Semi-quantitative immuno-electron microscopy has suggested that expression of pendrin is relatively higher in the endolymph-facing apical membrane than in the root processes (Yoshino et al., 2006), and so may play more prevalent roles in the upper cochlear turns.

Adenylyl cyclase (AC) is a membrane-bound enzyme which is responsible for the synthesis of the 2nd messenger cAMP, a cytoplasmic mediator for activated G-protein-coupled neurotransmitter and hormone receptors. Root cells, including their processes, are highly immuno-reactive for the enzyme subtypes ACI and ACVIII (Drescher et al., 2000). However, they are not reactive for subtypes ACII–IV or ACVII.

2.1.3.3. Gap junction proteins. There are numerous gap junction plaques at the intercellular junctions between root cells, and between root cells and overlying Claudius' cells (see Section 2.1.2). The gap junction channel subunits Connexin26 (Cx26) and/or Cx30 have been immuno-localized to the outer sulcus region in a number of studies (Ahmad et al., 2003; Forge et al., 2003; Jagger et al., 2010; Kelsell et al., 1997; Kikuchi et al., 1995; Liu and Zhao, 2008; Sun

et al., 2005). Both Cx26 and Cx30 have been detected in the root cells (Liu and Zhao, 2008), raising the possibility of heteromeric Cx26/Cx30 gap junction channel assembly in these cells (Forge et al., 2003; Sun et al., 2005), though extensive intercellular transfer of the large anionic dye Lucifer yellow (see Section 2.2.1) points to a predominant expression of homomeric Cx26 channels (Jagger et al., 2010).

2.1.3.4. Voltage-gated and ligand-gated ion channels. Clues towards the physiological role of the root cells may be taken from studies of ion channel protein subunits expressed in the apical and basolateral membranes. These functional proteins mediate the flow of ionic current through the membrane, and so determine the resting membrane potential of the cell, and regulate the asymmetric distribution of key ions during sound transduction. In a recent study from our labs (Jagger et al., 2010) we identified significant expression of Kir4.1 in root cells of the adult guinea pig. This member of the weakly rectifying K^+ channel family is known to play crucial roles in the redistribution of K^+ in the brain and in the retina (Nagelhus et al., 2004; Olsen and Sontheimer, 2008), and likely dominates the membrane properties of various glial cell types. In the cochlea, this subunit is most conspicuous in the membrane of strial intermediate cells (Ando and Takeuchi, 1999),

where it contributes to the generation of the highly positive endocochlear potential (EP) (Hibino and Kurachi, 2006; Marcus et al., 2002). Kir4.1 is also expressed in supporting cells of the organ of Corti (Eckhard et al., 2012; Hibino et al., 1997; Taylor et al., 2012), where its role seems less certain. Using immunofluorescence we detected Kir4.1 within root cells at all locations along the cochlear partition, and specifically to the root process membrane using immuno-electron microscopy (Jagger et al., 2010). More recently, Kir4.1 immunofluorescence has been observed in the outer sulcus of rat and human cochleae (Eckhard et al., 2012), and we have confirmed Kir4.1 expression in root processes of mice and rats (unpublished). Another K⁺-selective channel, TASK-1, has been localized to outer sulcus cells contacting the endolymph during the postnatal development of the rat cochlea (Kanjhan et al., 2004). Expression of this member of the two-pore domain K⁺ channel family was more prevalent at postnatal day 13 (P13) than it was in adult tissue (P105), and so its physiological role may be limited to trans-epithelial K⁺ distribution before hearing fully matures. The K⁺ concentration of endolymph is known to reach adult-like levels as early as P7 (Bosher and Warren, 1971), in advance of the maturation of EP, and TASK-1 may act as a regulated shunt pathway for K⁺ from the endolymph to the perilymph during the developmental period.

Immuno-detection studies have pointed to several non-selective cation entry pathways in cells of the outer sulcus. Ionotropic P2X₂ receptors, which are activated by purinergic agonists such as the neurotransmitter adenosine triphosphate (ATP), have been detected throughout the epithelial cell network of the sensory epithelium which is exposed to the endolymph, including the outer sulcus cells in the upper turns (Housley et al., 1999; Jarlebark et al., 2000). P2X₂ expression appears restricted to the apical pole of the outer sulcus cells (Jarlebark et al., 2000), suggesting that receptor activation would occur exclusively via ATP in the endolymph. Interestingly, the spatial pattern of the expression of P2X₂ receptors is matched by that of NTPDase2, an ectonucleotidase that preferentially hydrolyses nucleoside triphosphates such as ATP (Vlajkovic et al., 2002). The co-expression of these proteins points to a system wherein endolymphatic ATP can transiently increase the cation permeability of the apical membrane of outer sulcus cells, before the activation is terminated by the action of NTPDase2. Another potential route for cation entry has been raised by the detection of canonical transient receptor potential (TRPC) channels in outer sulcus cells. Immunofluorescence for the TRPC3 subunit has been localized to the cell body and root processes in the lower turn of the adult mouse cochlea (Phan et al., 2010; Tadros et al., 2010). TRPC3 opening is associated with IP₃-receptor binding and phospholipase-C activation, and the resulting Ca²⁺-entry may act to refill depleted Ca²⁺-stores (Raybould et al., 2007), or possibly modulate intercellular gap junctional coupling (Tadros et al., 2010).

2.1.3.5. Aquaporins. Aquaporins (AQPs) are channel-like proteins responsible for the trans-membrane flux of water molecules, and are often co-expressed with ion channels to ensure balanced osmotic gradients (Nagelhus et al., 2004). Several AQP subunits have been immunolocalized within the outer sulcus region, revealing possible expression gradients along the cochlear partition, and possibly inter-species variations. In neonatal rats, AQP5 has been identified as the subunit expressed by all outer sulcus cells in their endolymph-facing membrane (Hirt et al., 2010). With advancing development, as the cells in the lower turns become covered by Claudius' cells, AQP5 expression is expressed only by endolymph-facing cells in the upper turns. Hirt et al., 2010 also reported that AQP4 is expressed within the basolateral (root process) membrane but not the apical membrane, revealing differential localization of two AQP subtypes within the same cell.

This suggests that individual AQPs are trafficked to opposite poles of the cell for specific functions. Recently, AQP4 immunofluorescence has been co-localized with Kir4.1 immunofluorescence in the outer sulcus of the rat and human cochlea (Eckhard et al., 2012), suggesting co-operative functions between these proteins as seen elsewhere (Nagelhus et al., 2004).

2.2. Functional studies of membrane conductance

How epithelia regulate the ion content of fluids that surround them can often be best determined by the use of in vitro electrophysiological techniques, which allow the real-time monitoring of ionic currents flowing across the cell membrane. These recordings can separate and identify currents based on their sensitivity to specific pharmacological agents. Novel whole-mount preparations have allowed access to the apical endolymph-facing membrane of cells in the outer sulcus of the apical cochlear turns. Recently developed slice preparations of the cochlear lateral wall have also allowed access to the root cells in the lower turns, which are normally more difficult to access being hidden beneath the overlying mass of supporting cells.

2.2.1. Gap junctional coupling

Numerous studies have revealed gap junction plaques and extensive connexin expression between the cells in the outer sulcus (see Section 2.1.3.4). Consistent with this observation, whole-cell patch clamp recordings from root cells in lateral wall slice preparations of the lower and upper turns of the guinea pig cochlea revealed a large voltage-independent conductance that was attenuated by the gap junction uncoupler 1-octanol (Jagger et al., 2010). Both the cationic tracer neurobiotin (molecular weight 287; charge +1) and the anionic tracer Lucifer yellow (443; -2) transferred from the recorded cell to many others, characteristic of gap junctional coupling via homomeric Cx26 channels. Dye transfer was prevented by pre-incubation with 1-octanol. These observations have been replicated in slice preparations from the adult rat cochlea (Jagger, unpublished). Dye injections have also revealed novel detail of the fine structure of the root processes (Fig. 3A–F), including distribution of the membrane-enclosed bodies (MEBs; Fig. 3B–C) observed previously by Duvall (1969).

2.2.2. Non-selective cation conductances

Experiments employing the vibrating current density probe technique demonstrated a cation-permeable conductance in the apical (endolymph-facing) membrane of root cells of the third cochlear turn in the gerbil (Marcus and Chiba, 1999). The conductance had little voltage-dependence and was dependent on Na⁺ and K⁺, but not Ca²⁺ or Cl⁻, and was sensitive to millimolar concentrations of Gd³⁺, lidocaine and amiloride, suggesting it is mediated by non-selective cation channels. Patch clamp recordings have identified a channel with a single channel conductance around 30 pS, with comparable pharmacological sensitivity (Chiba and Marcus, 2000; Marcus et al., 2002). Vibrating current density probe recordings have also demonstrated a suramin-sensitive purinergic trans-epithelial conductance in the outer sulcus of the upper turn of the gerbil cochlea (Lee et al., 2001). The agonist sensitivity of this receptor-mediated cation entry was consistent with the activation of ionotropic P2X₂ receptors, agreeing with the immunolocalization of this subunit to the outer sulcus (see Section 2.1.3.5).

2.2.3. K⁺-selective membrane conductance

Distinct K⁺-selective conductances have been identified in the apical membrane of root cells in the upper turns of the gerbil cochlea (Chiba and Marcus, 2000). Channels with a conductance of

around 270 pS that were dependent on the Ca^{2+} concentration, and were blocked by charybdotoxin, suggest the presence of BK-like channels. Additionally, a channel was identified with a conductance of only around 7 pS, though its pharmacology was not investigated. Whole-cell patch clamp recordings from outer sulcus cells in the upper turns of the gerbil cochlea identified a K^+ -selective conductance in the basolateral membrane (Chiba and Marcus, 2001). This conductance displayed little voltage-dependence, and was blocked by extracellular Ba^{2+} , but not by charybdotoxin, suggesting it was distinct from the BK conductance in the apical membrane.

Whole-cell recordings from octanol-uncoupled root cells in the second turn of the guinea pig cochlea revealed weakly rectifying K^+ currents, which were blocked completely by 1 mM Ba^{2+} (Jagger et al., 2010). Currents in the fourth (low frequency coding) turn were of much smaller amplitude though, and the current density (normalized to the membrane surface area) was only around a third of that in the second turn. There are similarities between the basolateral conductance observed in the outer sulcus of gerbils and guinea pigs, and the weak voltage-dependence and sensitivity to Ba^{2+} point to the Kir4 family of weakly rectifying K^+ channels. The immunolocalization of Kir4.1 to the root cell processes in a number of species (see Section 2.1.3.5) suggests that this channel sub-type contributes to ion homeostasis in the outer sulcus, particularly in the lower cochlear turns.

3. What are the likely functions of root cells?

It is clear from data accumulated over more than 150 years that the root cells are likely to play numerous roles within the normal homeostatic function of the outer sulcus region, roles which are more complex than simply anchoring the sensory epithelium to the spiral ligament. There are structural and functional specializations of both the apical membrane, and the basolateral extensions provided by the root processes or “pegs”. In all species examined thus far there are graded variations in the anatomical and polarized functional properties of the root cells which depend on their position along the cochlear partition (Figs. 2 and 3). This variation suggests quite distinct mechanisms proceed in the lower frequency coding turns, compared to those in the high frequency coding regions. A common thread appears to be a central role for root cells in cochlear fluid homeostasis.

3.1. Cation absorption from the endolymph

In the outer sulcus of the upper cochlear turns the epithelial cells are poorly endowed with slender root processes, resulting in them being more commonly termed “outer sulcus cells” (Chiba and Marcus, 2000; Lee et al., 2001; Marcus and Chiba, 1999). It appears that the main function of the outer sulcus cells here is focused within their apical membrane which directly contacts the endolymph. The common consensus appears to be that the cells absorb Na^+ and K^+ from the endolymph via the apically located non-selective cation channels (Kim and Marcus, 2011). This would help to maintain the relatively low endolymphatic $[\text{Na}^+]$, optimizing the K^+ -mediated mechano-electrical transduction currents passing through hair cells. Elevations of ATP levels in the endolymph has a suppressive effect on EP (Munoz et al., 1995), most likely via activation of P2X_2 ionotropic receptors (Housley et al., 1999; Jarlebark et al., 2000; Munoz et al., 1999). The flow of Na^+ and K^+ into the outer sulcus, and the concomitant decreased epithelial resistance of this region may be sufficient to explain the suppression of EP and hearing sensitivity. Absorbed Na^+ could be effluxed to the spiral ligament perilymph via the Na, K-ATPase, and

K^+ may be lost to the perilymph through basolateral membrane channels.

The specific cation absorption mechanisms identified within the outer sulcus may provide candidates for the leakage pathways necessary to support the stria-derived standing current driving hair cell transduction (Zidanic and Brownell, 1990). Under this mechanism the standing (or “silent”) current is continually recycled back to the cochlear lateral wall, and is diverted by acoustic stimulation to pass through the hair cell transduction channels. Mapping of current density profiles measured by microelectrode recordings suggested that in the absence of stimulation the current is returned to the lateral wall through leak pathways passing via scala vestibuli and scala tympani, and it also passes through hair cell transduction channels, ~20% of which are open at rest. Zidanic and Brownell (1990) suggested a parallel route though, in which endolymph constituents are exchanged to the perilymph through the outer sulcus. The passive cation permeability of the outer sulcus cell apical membrane (Kim and Marcus, 2011) could provide this route under normal circumstances, and be augmented by the purinergic current during periods of prolonged acoustic stimulation (Housley et al., 2002).

3.2. K^+ transport between gap junctional compartments?

In the high frequency coding regions of the cochlea the epithelial cells of the outer sulcus do not contact the endolymph, and so they appear poorly placed to alter the endolymphatic ion content. Their basolateral membrane surface area is amplified by the presence of the numerous root processes (Figs. 2 and 3). The root processes are usually long and sometimes bifurcated, and bundles of these cytoplasmic extensions penetrate deep behind the spiral prominence and into the inferior reaches of the spiral ligament. In these regions there are numerous specialized type IIb fibrocytes which express Na, K-ATPase, and which are “situated to lower K^+ in the fluid bathing root bundles to a level effecting its efflux from the sulcus cells” (Spicer and Schulte, 1996). This efflux would be enabled by the expression of Kir4.1 channels in the root cell process membrane (Eckhard et al., 2012; Jagger et al., 2010). A comparable mechanism may exist within stria vascularis, where the Na, K-ATPase expression in the basolateral membrane of marginal cells may act to lower the $[\text{K}^+]$ of the intra-strial fluid, to encourage efflux of K^+ through Kir4.1 channels in the intermediate cell membrane (Hibino and Kurachi, 2006). It seems possible, therefore, that the basolateral membrane of the root cell acts as a continuous K^+ sink, for a source located beyond their apical membrane.

A popular hypothesis is that this source of K^+ is located within the epithelial cell gap junction network (Ahmad et al., 2003; Forge et al., 2003; Gale and Jagger, 2010; Hibino and Kurachi, 2006; Kikuchi et al., 1995; Spicer and Schulte, 1996), formed from the syncytium of supporting cells in the organ of Corti and extending through the Claudius' cells and root cells (Jagger and Forge, 2006; Jagger et al., 2010; Taylor et al., 2012). Following mechano-electrical transduction by outer hair cells (ohcs), Deiters' cells are proposed to take up K^+ from the fluid bathing the hair cell basolateral membrane, which may be mediated by the co-operative action of transporters and K^+ channels (Eckhard et al., 2012; Hibino and Kurachi, 2006). Following its lateral translocation to the root cells and its subsequent uptake by type IIb fibrocytes, the K^+ may be transferred via another trans-cellular route to the strial intermediate cells within the connective tissue gap junction network (Forge et al., 2003; Hibino and Kurachi, 2006; Kikuchi et al., 1995; Liu and Zhao, 2008), which is based on the intercellular expression of Cx26 and Cx30. Type II fibrocytes within the inferior region of the spiral ligament are dye-coupled via type I fibrocytes to strial basal cells and intermediate cells (Kelly et al., 2011), providing a viable K^+ -recirculation pathway. The importance of the K^+ -buffering

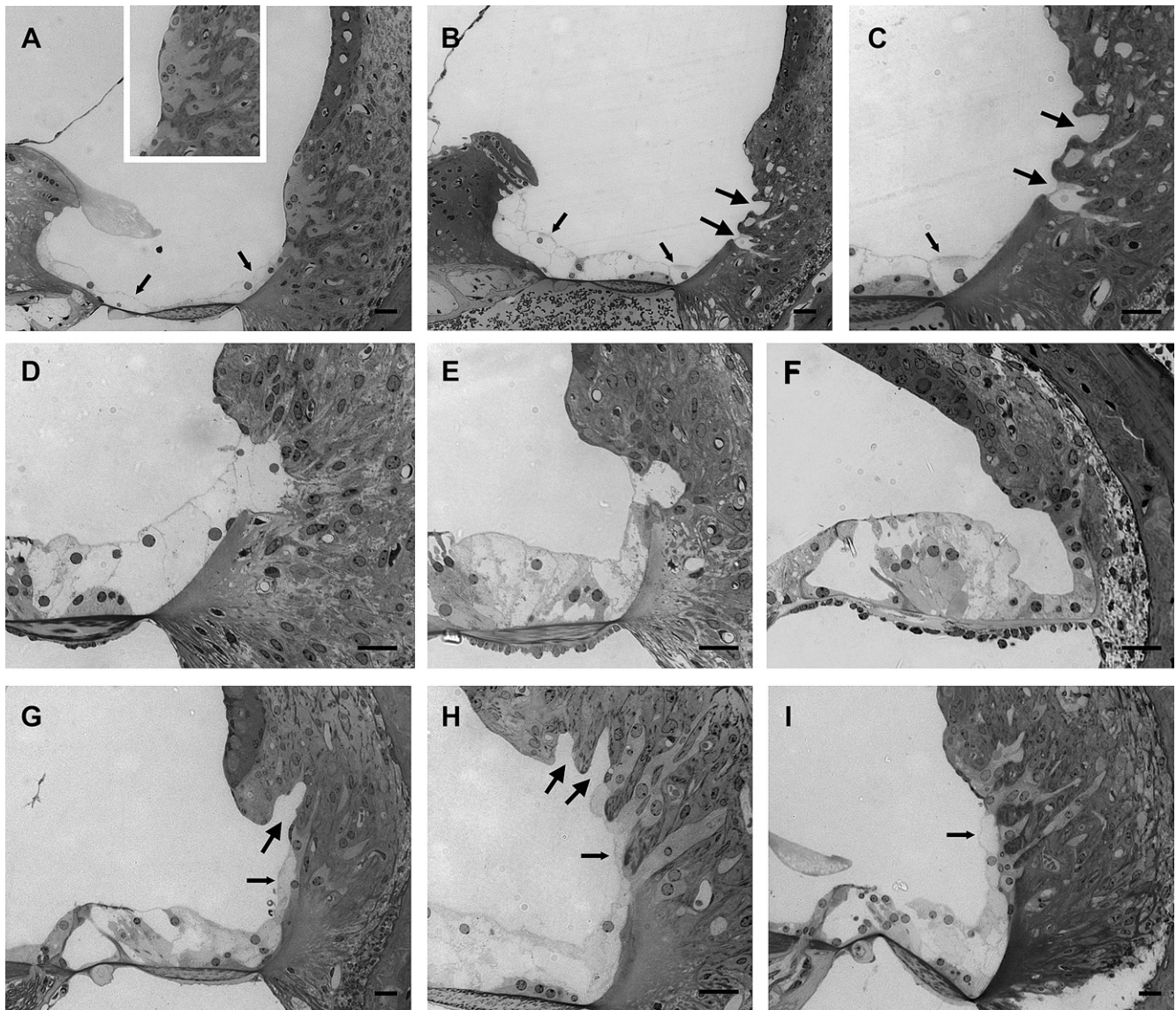


Fig. 4. Root cell degeneration in mouse models of age-related and inherited hearing loss. Cochleae were fixed using glutaraldehyde, and sections were cut at 1 μm thickness and stained using toluidine blue. (A–C) Cochleae of conditional connexin 26 (cCx26) null mice. (A) In the lower basal turn region of a 30 day-old cCx26 null mouse the organ of Corti has degenerated, leaving a flattened epithelium stretching between the inner sulcus and the outer sulcus (small arrows). Root cells are evident in the inferior portion of the spiral ligament (detailed within the *inset*). (B) In the lower basal turn region of a 60 day-old cCx26 null mouse the flat epithelium appears shortened (small arrows), and large indentations are evident in the root cell region (large arrows). (C) Detail of the affected region. (D–F) Cochleae of connexin 30 null mice. (D) In the basal turn of a 30 day-old mouse the Claudius' cells extend towards the spiral prominence, and appear to be resident within a large indentation within the spiral ligament. Few root cells are evident. (E) In the mid-turn region of a 30 day-old mouse the Claudius' cells have also extended into an indentation. (F) The outer sulcus in the apical turn of a 67 day-old mouse appears largely normal, with small root processes still evident within the spiral ligament. (G–I) Cochleae of 24 month-old CBA/Ca mice. (G) In the upper basal turn the organ of Corti appears largely normal, with evidence of surviving inner and outer hair cells. Within the spiral ligament there is a large indentation in the spiral prominence region (large arrow), with an apparent loss of Claudius' cells and their underlying root cells. Where the Claudius' cells are still present (small arrow) root cells are also apparent. (H) Detail of a comparable region in a littermate. Two large indentations are evident, but some root cells remain. (I) In the lower basal turn the Claudius' cells extend towards the spiral prominence epithelial cells, and numerous root cells are present throughout the inferior region of the spiral ligament. Scale bars 20 μm .

mechanism in the lower turns is perhaps best demonstrated by the significantly greater K^+ load which is likely to be directed from ohc to the epithelial cell gap junction network. In the guinea pig cochlea, the K^+ -derived input conductance of ohc in turn 2 is more than ten-fold higher than that of ohc in turn 4 (Mammano and Ashmore, 1996).

4. Do root cells play roles in inherited and acquired cochlear dysfunction?

Although the exact contribution of root cell function to normal hearing remains open to debate, it is evident that they lie at

a potentially crucial operational locus between sensitive tissues, and so they may represent a previously unappreciated site of cochlear pathology. The root cells express a number of functional membrane proteins previously implicated in clinical cases of inherited hearing loss, and animal models have revealed potentially pathological changes to the root cell region during conditions of stress and/or ageing.

Root cells at all points along the cochlear partition are coupled to adjacent root cells, and to the overlying Claudius' cells in the lower turns, and so form the lateral limits of the epithelial cell gap junctional network (ecgjn, Fig. 1A). Thus, it seems likely that their

normal function would be deleteriously affected by inherited mutations in the *Gjb2* and/or *Gjb6* genes, which code for Cx26 and Cx30. Connexin gene mutations are a prominent cause of clinical non-syndromic hearing loss (Bitner-Glindzicz, 2002; Kelsell et al., 1997; Nickel et al., 2009), and also syndromic hearing loss with skin disease (Kelsell et al., 2001). Connexin deafness is often associated with hair cell loss which may result from disruption of normal homeostatic functions of the supporting cells. In the *Cx26^{OtogCre}* mouse model in which there is a targeted ablation of Cx26 specifically from cochlear supporting cells (Cohen-Salmon et al., 2002), the hair cells and their supporting cells are observed to die soon after the onset of hearing, though the status of root cells in this animal have not been reported. Similarly, in a tamoxifen-inducible conditional Cx26 (*cCx26*) null mouse there is rapid loss of hair cells in the basal turn and degeneration of the organ of Corti (Sun et al., 2009). In ongoing work from our labs using this model, we have observed a progressive loss of root cells in the basal turn spiral ligament (Fig. 4A–C). In *Cx30* null mice we have also observed peculiarities in the outer sulcus, where root cells in the basal and mid turns are sparse by P30, and are apparently replaced by Claudius' cells expanding into the void left behind (Fig. 4D, E). The outer sulcus in the apical turn appears largely unaffected in this model (Fig. 4F). Whether the root cells are directly affected by the loss of connexin proteins in these models, or are lost following connexin-associated dysfunction of Claudius' cells remains to be determined.

A mouse model in which Kir4.1 has been ablated also suffers from a profound hearing loss, in part caused by a failure to generate the endocochlear potential (Marcus et al., 2002), but also suffers a catastrophic degeneration of the sensory epithelium soon after the normal age of hearing onset (Rozenfurt et al., 2003). Mis-sense and nonsense mutations of *KCNJ10* which codes for Kir4.1 result in SeSAME (Seizures, Sensorineural deafness, Ataxia, Mental retardation, and Electrolyte imbalance) syndrome (Scholl et al., 2009). Although the profound hearing impairment may be largely attributable to loss of endocochlear potential, deletion of Kir4.1 from the supporting cells may affect the handling of K^+ in these patients. Together, these studies suggest that perturbations of K^+ homeostasis are likely to result from certain gene mutations, and indeed may be the ultimate cause of sensory cell death and profound deafness. Similarly, genetic ablation of pendrin results in the hearing loss associated with Pendred Syndrome (Royaux et al., 2003), suggesting that abnormal root cell-associated anion transport is sufficient to cause hearing loss.

There is evidence from animal models that the outer sulcus may be involved with the cochlea's response to pathological stress. Root cells of mice and guinea pigs can be immunostained for NF κ B P65, a transcription factor necessary for the production of inflammatory cytokines, and also I κ B α , an inhibitory protein that regulates the actions of NF κ B (Adams, 2002). This suggests that root cells are able to produce inflammatory cytokines, but are also likely to express receptors for these autocrine and paracrine signaling molecules. This finding suggests that corticosteroids, which control the activation state of NF κ B and are used clinically to treat various forms of hearing loss, may have a site of action within the outer sulcus.

Further evidence supporting a role for root cells in the response to stress comes from the observation that noise exposure leads to an increase in their expression of P2Y₁₄, a G-protein coupled purinergic receptor associated with the mobilization of intracellular Ca^{2+} stores (O'Keefe et al., 2010). Noise exposure results in significant rises in endolymphatic [ATP] (Munoz et al., 2001), which may influence outer sulcus physiology via activation of both P2X and P2Y receptors (Housley et al., 2002). Although the release of ATP into the endolymph during noise exposure may enable a protective effect, via its P2X₂-dependent suppression of EP and

the resulting reduced activation of hair cells (Munoz et al., 1999, 1995), there is evidence suggesting prolonged effects of ATP may be damaging to cochlear function (Thorne et al., 2002). Perhaps of relevance to our understanding of the causes of presbycusis is the observation that the ATP-mediated suppression of EP during noise exposure is lost in aged mice (Telang et al., 2010). This suggests that the protective shunt located at least in part within the outer sulcus, may be less effective in old age and so rendering us more susceptible to noise-induced injury.

Following application of gentamicin to the round window, a procedure that causes hair cell death and subsequent degeneration of the sensory epithelium, the aminoglycoside is found to be concentrated within the root cells (Imamura and Adams, 2003). This suggests that root cells are less vulnerable than hair cells to this ototoxic drug, and that they can survive even after loss of the sensory epithelium. Further, this may suggest that root cells play a role in sequestration of ototoxic drugs from the cochlear fluids.

Root cell numbers have been observed to decrease in hearing-impaired ageing CBA/CaJ mice (Ohlemiller et al., 2010), a loss which occurs in the upper basal turn specifically, and an effect that is more pronounced in females. We have also observed this phenomenon in our colony of ageing CBA/Ca mice (Fig. 4G–I), and suggest that the root cells are lost following retraction of the Claudius' cells which normally cover the root cells in this region. This pathology appears similar to that in *cCx26* mice (Fig. 4A–C) where Claudius' cells retract from the outer sulcus. However, the significance of root cell loss in ageing mice to our understanding of human presbycusis is currently unclear, as the effect may be strain specific (Ohlemiller et al., 2010).

5. Conclusions

The cells of the outer sulcus appear to form a crucial component of the non-sensory epithelial syncytium that supports normal function of the sensory hair cells, ensuring auditory sensitivity and frequency selectivity, whilst also preserving the longevity of our hearing. In comparison to the supporting cells of the organ of Corti, relatively little is known about the physiology of the outer sulcus, particularly of the root cell processes which are difficult to access within the spiral ligament. Based on the body of accumulated evidence, the primary roles of epithelial cells in the outer sulcus appear to be centered on fluid homeostasis, and these roles are likely to vary depending on their location along the cochlear duct. Certain key questions remain, however. There has been little functional evidence in support of the presence of Shambaugh's "secretory tubules", and any contribution to the secretion of fluid into scala media has been largely discounted. Future studies should focus on how root cells contribute to the management of fluid composition during sound coding, and also on their involvement in the progression of certain forms of hearing loss. The root cells in the lower cochlear turns appear particularly sensitive to changes in their local environment, which may be related to the status of the Claudius' cells that shield them from the high K^+ endolymph. A better understanding of the interplay between these cell types appears crucial. It would be fascinating to further dissect the physiology of the outer sulcus under normal and diseased conditions, and hopefully reveal the contribution of the mysterious root cell to cochlear homeostasis.

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