Anatomical basis of drug delivery to the inner ear

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A B S T R A C T

The isolated anatomical position and blood-labyrinth barrier hampers systemic drug delivery to the mammalian inner ear. Intratympanic placement of drugs and permeation via the round- and oval window are established methods for local pharmaceutical treatment. Mechanisms of drug uptake and pathways for distribution within the inner ear are hard to predict. The complex microanatomy with fluid-filled spaces separated by tight- and leaky barriers compose various compartments that connect via active and passive transport mechanisms. Here we provide a review on the inner ear architecture at light- and electron microscopy level, relevant for drug delivery. Focus is laid on the human inner ear architecture. Some new data add information on the human inner ear fluid spaces generated with high resolution microcomputed tomography at 15 μm resolution. Perilymphatic spaces are connected with the central modiolus by active transport mechanisms of mesothelial cells that provide access to spiral ganglion neurons. Reports on leaky barriers between scala tympani and the so-called cortilymph compartment likely open the best path for hair cell targeting. The complex barrier system of tight junction proteins such as occludins, claudins and tricellulin isolates the endolymphatic space for most drugs. Comparison of relevant differences of barriers, target cells and cell types involved in drug spread between main animal models and humans shall provide some translational aspects for inner ear drug applications.

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Abbreviation List: HCs, hair cells; SGNs, spiral ganglion neurons; SGC, satellite glia cell; ST, scala tympani; SV, scala vestibuli; SM, scala media; IHC, inner hair cell; OHC, outer hair cell; CNS, central nervous system; SGCs, satellite glia cells; CSF, cerebrospinal fluid; CA, cochlear aqueduct; RW, round window; MRI, magnetic resonance imaging; microCT, microcomputed tomography; OSL, osseous spiral lamina; MCs, mesothelial cells; BM, basilar membrane; TCI, tympanic covering layer; EMT, epithelial-to-mesenchymal transition; RWM, round window membrane; BM, Reissner's membrane; TEM, transmission electron microscopy; OW, oval window; Gd, gadolinium; HA, hydroxyapatite

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1. Introduction

1.1. Why inner ear microanatomy favors local drug delivery

Various compounds were identified to rescue neurons and hair cells (HCs) from ototoxic insults and mechanical trauma in animal experiments (Mukherjea et al., 2011; Nguyen et al., 2017). Since endogenous regeneration of mammalian inner ear HCs is very low and limited to the vestibular system, gene therapies were developed to transdiffereniate supporting cells into HCs or utilize stem cells to replace lost receptors (Atkinson et al., 2014; Mittal et al., 2017). Despite this knowledge derived from in vitro and in vivo animal experiments we still lack an effective pharmacological treatment of the human inner ear to combat hearing loss, tinnitus, Meniere’s disease and vestibular deteriorations. Delivery of genes/ vectors or chemical compounds to target cells in the inner ear is still a challenge. Ideal chemical formulation and combination of suitable compounds with an appropriate mode of drug delivery are the current tasks. Knowledge about microanatomy and functional characteristics of cell types in the inner ear is indispensable for a successful pharmacotherapy.

The anatomical location of the human inner ear within the hardest bone isolates the vestibulo-cochlear sensory organ. Additionally low blood flow rates and a limited passage through a blood-labyrinth barrier similar to the blood-brain barrier interfere with systemic application of drugs, so a local administration appears attractive. Local absorption of drugs from the middle ear was first described by Schuknecht using intratympanic streptomycin for treatment of Meniere’s disease (Schuknecht, 1956) and proved suitable also for several other compounds (Berjis et al., 2016; El Kechai et al., 2015; Staecher and Rodgers, 2013; Staecher et al., 2017). Minimally invasive approaches to bypass the tympanic membrane with e.g. cannulas to place drugs close to the inner ear proved safe in routine clinics and for human trials with growth factors (Nakagawa et al., 2014). From the middle ear pharmacologically active compounds need to overcome several barriers to reach HCs, spiral ganglion neurons (SGNs) or other target cells. Fluid filled spaces separated by thinnest membranes, tight cellular barriers and very compact bone results in a compartmentalization of the mammalian inner ear that requires several strategies for drugs to penetrate. Barriers between compartments vary in the degree of permeability which complicates assessment of pharmacokinetic spread and elimination. A profound knowledge of the microanatomy, fluid flow and characteristics of cell types in the inner ear is important to predict routes for small molecules and bigger proteins or genes.

We previously analyzed perilymph-modiolar communication routes in the human cochlea (Kask-Andersen et al., 2006) and the use of nanocarriers to overcome barriers in the cochlea (Gluckert et al., 2015; Pritz et al., 2013b; Ranjan et al., 2012; Roy et al., 2010). Here we provide a review on the inner ear architecture relevant for drug delivery with some additional new data on the human inner ear. Comparison of relevant differences between main animal models and humans shall unravel variations in the mammalian inner ear architecture.

2. Anatomy and targets for drug delivery

2.1. Basic anatomy

A rigid outer wall forms the bony labyrinth that comprises the cochlea, vestibule and semicircular canals. The otic capsule develops early as mesenchymal tissue around the otocyst and is in human adults embedded in the petrous portion of the temporal bone. It is one of the densest bones in the body and retains a considerable fraction of its primordial cartilage as islands of chondral tissue that directly calcifies (Fig. 1a). This results in a meager vascularized bone with low turnover impeding callus formation after fractures. The degree of encapsulation of the otic capsule is very different in mammalian species with a thick encasement in human (Fig. 1a) and cat and only a thin bony jacket in rodents like mice or guinea pigs (Fig. 1b). Especially the bony layer in the apical portion of the guinea pig is very thin and allows penetration of substances into the apex of the cochlea (Mikulec et al., 2009). The perilymphatic fluid space dominates the inner ear, fills the major part of the bony labyrinth in the vestibular system that passes into the scala tympani (ST) of the cochlea. The apical helicotrema interconnects ST with the scala vestibuli (SV) thereby forming the biggest connected fluid space across the inner ear. Within the bony labyrinth a much smaller membranous labyrinth penetrates this canal system and joins the apical poles of the inner ear sensory epithelia to a common compartment. Stereocilia and kinocilia from the vestibular HCs are bathed in this endolymph fluid. Vestibular and cochlear endolymph compartments connect via the small reunion duct. Both fluid systems serve as relay media to transmit vibrations, positional changes and
motion acceleration but seem also to be important for the transport of oxygen, nutrients and waste to compensate for low blood supply of e.g. the cochlear sensory epithelium. The endolymphatic scala media (SM) of the cochlea adds an electrochemical gradient boosting sensitivity of mechanotransduction to movements in the range of atomic size.

2.2. Target cells - are they equal across mammalian species?

Mammalian animal models may differ in several aspects from primate inner ears. Common ancestors from rodents and primate species separated approximately 75 million years ago (Mouse Genome Sequencing et al., 2002) enough time to evolve changes in orthologue genes or gene activities that may influence efficacy of drugs. Differences in shape and length of the cochlea and semi-circular canals reflect adaptation for frequency spectrum, resolution and sensitivity of motion detection necessary for proper mode of locomotion. Few studies imply functional aspects on a comparative basis of inner ears across mammalian species including man (Makimoto et al., 1980; Nadol, 1988). More knowledge will be necessary to understand why a pharmacological trial fails in human when animal experiments show positive results. Even inbred mouse strains used in otology research show marked differences in hearing performance and susceptibility to damage (Ohlemiller...
et al., 2016; Zheng et al., 1999). However, certain cell types in the inner ear may have specialized more than others during the last 75 million years of evolution as adaptation to specialization and communication skills. In the following section we compare the most important target cells for a pharmaceutical intervention between human and common animal models in inner ear research.

2.2.1. Hair cells
Protection and regeneration of our inner ear receptor cells, the HCs, are one of the main tasks for drug- and gene therapies, since most HCs in the mammalian inner ear do not recover. Only limited regeneration in vestibular end organs was found (Li et al., 2016; Warchol et al., 1993). Apart from a more irregular order of cochlear HC rows in humans compared to other mammalian species (Glueckert et al., 2005b), there do not seem to be different influences to rodents or cats. Specialization into a primary inner hair cell (IHC) and motile outer hair cells (OHCs) is the same and typical for all mammals. Stereocilia of the cochlear mecanotransducers and cell body of OHCs are longer in the apex (Fig. 1c) and shorter in the base. In high sensitivity frequency regions, IHCs show more afferent contacts. Gradients in susceptibility for ototoxic substances and degeneration pattern following acoustic trauma are common across different mammalian species (Lee et al., 2013; Ohlemiller and Gagnon, 2004; Sha et al., 2001).

HC receptor cells seem to be evolutionarily quite conserved in several aspects, so that even toxicity studies from fish lateral line neuromast HCs may predict vulnerability in inner ear HCs (Esterberg et al., 2013; Ou et al., 2012). Marked differences imply their regeneration capacity that distinguish the primal features of fish lateral line HCs. Such data underline the significance of model systems chosen to screen for compounds that may protect our valuable sensory cells. This does not replace drug efficacy studies in mammals including primates.

2.2.2. Spiral ganglion neurons
The majority of bipolar neurons belong to type I SGNs. They transform IHC receptor potentials into action potentials that travel to the ascending neuronal pathway in the central nervous system (CNS). The 3–5% smaller type II neurons provide afferent innervation of OHCs. With ageing, humans loose about 100 SGNs per year (Makary et al., 2011). Decline of innervation may be independent from HC loss (Felder and Schrott-Fischer, 1995; Viana et al., 2015) and affect dynamic range and speech understanding before a profound hearing loss develops. Animal experiments that applied ototoxic drugs to wipe out HCs often resulted in a nearly total loss of neurons following deafness. Toxicity to neurons may be underestimated in humans due to delayed and slow effects. Humans retain many of their neurons decades after complete deafness (Glueckert et al., 2005a). New transgenic animal models selectively ablate HCs without ototoxic drugs changing our picture of a retrograde loss of SGNs following HC loss (Kurioka et al., 2016).

Like sympathetic and sensory ganglia in our body, SGNs are completely enveloped by glia cells (Fig. 2). Unlike CNS neurons there is no direct contact of the neuron with extracellular fluid or matrix. Schwann cells wrap around peripheral and central axons and satellite glia cells (SGCs) sheath the soma (Fig. 2a–d). SGCs have been found to play a variety of roles, including control over the microenvironment by regulating the diffusion of molecules across the cell membrane (Hanani, 2010a, 2010b). Because of this uninterrupted sheath seen in ganglia, a similar role as the blood–brain barrier for larger molecules was suggested (Ten Tusscher et al., 1989). “Naked” portions at nodes of Ranvier are at least covered by nodal microvilli emanating from myelinating Schwann cells and its basement membrane (Fig. 2e). A striking difference of SGNs in human and most other mammals is the lack of myelination of the neuron somata (Fig. 2a–b). Perisomatic myelination of the SGCs may have developed for fastest neurotransmission and timing relevant for most precise sound localization. Humans presumably lost this myelination as an adaptation for speech taking into account a possible delay in action potential propagation (Rattay et al., 2013). Common SGCs form clusters of SGNs in humans (Fig. 2a–b) that can be found to a lesser extent in some apical SGNs in mice. Together with the presence of a gap junction system that couple SGCs, these data suggest that SGCs may play a role in signal processing of functional SGN units (Glueckert et al., 2005a; Liu et al., 2015b). Perisomatic myelination may act as an additional barrier (Fig. 2c–d) not present in human, since transport across several membrane layers may take longer than crossing only two membranes to reach the neuron. Differences in permeability or pharmacokinetics for drugs are not easy to evaluate. There is a mouse model where most SGCs are not myelinated that could be compared with its C57BL/6 background strain to address this issue (Jyothi et al., 2010). Most type II neurons are completely unmyelinated but yet not described to be a primary target for drug delivery.

2.2.3. Other target cells
Other cell types to target are more in the spotlight for gene delivery strategies. Exogenous expression of the transcription factor Atoh1 in sensory epithelium supporting cells (Fig. 1c) is sufficient to induce the trans-differentiation into a HC phenotype and may recover function in the cochlea and vestibule (Baker et al., 2009; Izumikawa et al., 2005). Overexpression of nerve growth factors may promote neural survival or regrowth of peripheral processes of SGN (Fukui et al., 2012; Kawamoto et al., 2003). Mesothelial cells lining perilymphatic spaces are often the main “target” to accomplish endogenous production of nerve growth factors through viral infection or electroporation (Wang et al., 2012). A gene delivery system was developed to rescue hearing in a mouse model of Connexin 26 deletion (Izuka et al., 2015); here cochlear supporting cells as well as lateral wall fibrocytes serve as primary targets. There are no reports to our knowledge about interspecies differences in these cell types regarding pharmacological interventions.

3. Blood supply and blood-labyrinth-barrier
The inner ear blood supply is quite complex and lacks collaterals that make it vulnerable to ischemic effects. A single labyrinthine artery emanating from the anterior inferior cerebellar artery is the only blood vessel into the inner ear. The main cochlear artery spirals up within the modiolus and sends out radial arteries through the SV wall to form a capillary network at the stria vascularis (Fig. 3a). This three cell layered epithelium presents highest density of capillaries to ensure the electro-chemical gradient between perilymph and endolymph in the cochlea. Venules below the spiral prominence (Fig. 3a) gather into bigger veins that travel within the spiral ligament of the ST into the modiolus. These vessels coalesce to the inferior cochlear vein and courses through a small bony channel parallel to the cochlear aqueduct.

Maintenance of inner ear fluid homeostasis may have favored the formation of a barrier function between the vascular system and the inner ear fluid. Similar to the blood–brain-barrier, endothelial cells lack fenestrations and seal their lumen with vast tight- and adherens junctions (Fig. 3e–d). Pericytes as well as perivascular resident macrophage-like melanocytes are in intimate contact with endothelial cells and add multiple basement membrane layers (Fig. 1e) to tightly regulate exchanges from the blood to
interstitial fluid. This is especially pronounced within the stria vascularis (Zhang et al., 2012) and reviewed recently (Shi, 2016). Strial basement membranes were found to be negatively charged (Suzuki and Kaga, 1996) that may establish a charge selective barrier similar to the glomerular basement membrane in the kidney that restricts the transmembrane flux of anionic proteins. Especially highly positive charged substances might obstruct the strial ultrafilter of blood-labyrinth-barrier (BLB) capillaries.

More and more data deliver direct evidence on impaired function of the BLB in Menière disease. Transport of chelated gadolinium (Gd) is compromised in Menière’s patients likely due to endolymphatic hydrops (Shi et al., 2014). Menière disease was recently associated with a deteriorated BLB evaluating MRI post-contrast measurements of signal intensities (Pakdaman et al., 2016). Concurrently specific ultrastructural changes of capillaries constituting the BLB were identified in Menière patients’ utricles taken out during surgery (Ishiyama et al., 2017). Hence, systemic pharmacotherapy should not aim to disrupt this tightly regulated homeostasis function, also because its regulation is largely unknown and severe inner ear disorders like Menière disease are associated with.

4. Fluid spaces, fluid flow and barriers

4.1. Perilymphatic spaces and modiolus—we are connected!

Perilymph fills the major space of the bony labyrinth (Fig. 4a–b) and merges with the extracellular space of the central modiolus housing cochlear neurons (Fig. 4c) (Rask-Andersen et al., 2006). By its ionic composition perilymph reveals as a typical extracellular fluid, the source is still under debate. It may be produced as a blood ultrafiltrate, originated from the cerebrospinal fluid (CSF) and
transported via the cochlear aqueduct (CA) (Fig. 4a) or is a mixture of both. Although interconnected, composition of this fluid varies between ST and SV (Juhn et al., 2001; Wangemann, 2006) due to active homeostatic mechanisms that vary along the tonotopical axis.

The very dense endosteal bony layer of the otic capsule encompasses this fluid space interrupted by the round- and oval window as well as the cochlear- and vestibular aqueduct (Fig. 4a). The CA is a small bony canal that originates at the floor of the ST close to the round window (RW) and establishes a communication between the perilymphatic and the subarachnoid space of the posterior cranial cavity. CA is approximately 1 cm in length (range from 2.4 to 14.6 mm) the narrowest diameter (isthmus) varies between 0.06–0.3 mm (Bachor et al., 1997; Guo et al., 2016). In guinea pig the CA is only 2 mm long (Shinomori et al., 2001) but not narrower than in humans (Ghiz et al., 2001). Flow may not be restricted to one direction. Communication between perilymph and CSF was described (Kaupp and Giebel, 1980) but may have been favored by large volumes introduced into perilymph of such studies (Ghiz et al., 2001). A main function of the CA may also be a pressure release route from the cochlea (Carlberg et al., 1982). Complete bony obstruction of the CA in human was reported (Rask-Andersen et al., 1977).

A parallel accessory canal with a diameter of 0.13–0.27 mm in human (Guo et al., 2016) guides the inferior cochlear vein to drain blood from the cochlea to join the internal jugular vein (Fig. 4a).

Marker studies suggest that inner ear fluids are relatively unstirred and solely dependent on diffusion rates (Salt et al., 2015). Even in prolonged local application of drugs, concentration gradients remain in the fluids (Salt and Ma, 2001). Another source for CSF fluid may occur via the inner ear canal (inner acoustic meatus) and the vestibulocochlear nerve. This nerve is surrounded by a perineurium that progresses into the dura of the brain. At its distal ending the VIIIth nerve penetrates the bone in the fundus region of the human inner ear via fenestrations of various sizes that guide central axons (Fig. 5a–b). In other mammals such as guinea pigs this opening is more prominent (Fig. 1b) without bony restrictions towards the modiolus. CSF fluid may pass into the modiolus and volume fluctuations may open the reverse path. Big pressure changes into the inner ear must be avoided otherwise it may be very likely that drugs introduced into the inner ear may show up in the brain and even to the contralateral ear (Stover et al., 2000).

Dimensions of fluid spaces vary considerably across species (Thorne et al., 1999) and also within a single species as so in human (Avci et al., 2014; Erixon et al., 2009). We evaluated the fluid spaces in 24 human inner ears acquired with a microcomputed tomography (microCT) at 15 μm voxel resolution and manual segmentation. We distinguished vestibular and cochlear portions in the human inner ear (see Table 1). Values are in the range of previously published papers (Buckingham and Valvassori, 2001) (total 192.5 mm³; endolymph, 34.0 mm³; perilymph, 158.5 mm³).

ST length ranges from 28 to 40 mm in humans (Wright et al., 1987) and are around 17 mm in guinea pigs and 4.3 mm in mice (Nadol, 1988). As fluid spaces of the human cochlea are also considerably larger than those of animal models a single site applications of a drug may act only at a limited region. Insertion of catheters to provide a more even spread implies the high risk of damage while elution of drugs from a cochlear implant may add few additional risk of trauma. Enhanced substance spread could also be performed with superparamagnetic nanocarries in an external magnetic field before they release their payload (Barnes et al., 2007; Ramaswamy et al., 2015).

SGNs reside in a bony canal of the modiolus termed Rosenthal’s canal (named after the neuroanatomist Friedrich-Christian Rosenthal 1780–1846). Bony columns guide the peripheral axons of the bipolar neurons as fascicles towards the osseous spiral lamina (OSL) where nerve fibers fan out to innervate the sensory epithelium (Figs. 4c, 5a–b). The ST portion of the bony modiolar wall is surprisingly porous with plenty of fenestrations forming a trabecular meshwork with broad communication canals into the central modiolus (Fig. 5a). Likewise, the delicate osseous spiral lamina reveals also plenty of bony fenestrations. SV face presents a different view with a smooth bony surface poor in fenestrations. Prominent holes guide the spiral modiolar arterioles to the apical portion of SV.
wall and further to stria vascularis. Trabecular bone close to the OSL provides bony opening into the modiolus, but to a lesser extent than in the ST (Fig. 5a).

There are suggestions for direct communication routes from perilymph to the modiolus in humans (Kucuk et al., 1991; Rask-Andersen et al., 2006; Shepherd and Colreavy, 2004). In animals these may also exist as reported also in cat (Shepherd and Colreavy, 2004). Results from the distribution of nanocarriers such as liposomes and polymerosome particles suggest even intramodiolar routes from ST basal turn into the modiolus towards ST & SV in more apical turns (Buckiova et al., 2012). A radial communication between ST and SV via the spiral ligament has also been demonstrated in animals and humans (Salt et al., 1991a, 1991b; Zou et al., 2005) suggesting cells facing perilymphatic spaces allow fluid flow into the central modiolus across all turns.

4.2. Mesothelial cell layer-more than a pavement!

Perilymphatic spaces are delineated by mesothelial cells (MCs) (Fig. 5c–i) that derive from mesodermal tissue (Mutsaers, 2004). They commonly form a very thin monolayer and rest on a basement membrane supported by a complicated collagen meshwork (Rask-Andersen et al., 2006) (Fig. 5d and e). Although perilymphatic fluid is the primary media for local pharmacotherapies and MCs are the first cellular barrier, only little is known about this cell type in the inner ear. MCs line most internal organs and cavities such as the peritoneal, pleural and pericardial. It is the first line of defense against invading microorganisms by initiating inflammatory and immune responses and provide a slippery non adhesive surface (Mutsaers et al., 2016). They form tight junctions to avoid paracellular flow (Fig. 5d) or appear more loosely arranged between
bony columns (Fig. 5e) and underneath the basilar membrane (BM) where they may form thick layers called tympanic covering layer (TCL) (Fig. 5i). The TCL layer is more prominent apically where they form a thick meshwork of cells (Fig. 1c) while in higher frequency regions this layer becomes thinner with flat MCs (Cabezudo, 1978; Liu et al., 2015a) (Fig. 7c). Acellular debris can frequently be found between TCL cells (Fig. 5i). MCs secrete phosphatidylcholine, the major constituent of lamella bodies and pulmonary surfactant (Mutsaers, 2002). For the inner ear they may provide a frictionless, lubricant surface for smooth fluid movements. They are able to transport fluid and cells thereby “cleaning” their luminal compartment through pinocytotic vesicles, intracellular cavities or stomata. Stomata are cavities at the junctions of MCs, 3–12 μm in diameter that allow for rapid removal of fluid and cells (Ohtani et al., 2001) and provide a direct access to underlying space in the modiolus (Fig. 5c). In pathological or severe damage conditions MCs develop adhesion, may convert into fibrocytes and even trigger fibrosis (Jia et al., 2016). MCs have the ability to convert their phenotype comparable to changes seen in the epithelial-mesenchymal transition (EMT), hence they express vimentin and desmin together with cytokeratins characteristic for epithelial cells. They are important for wound healing as MC proliferation was reported as reaction of ruptures of the round window membrane (RWM) (Sone, 1998). Fibrosis following cochlear implant insertion trauma is likely also be related to this EMT and may influence transcellular transport mechanisms of MCs.

Especially the region between the bony columns in the basal turn seems to provide a direct access into the modiolus (Fig. 5a, b and f). Various melanocytes populate the perilymphatic surface area. These dendritic cells with its typical brown endogenous pigment play a key role in innate immune responses. Little is known about the physiology of these melanocytes. In the stria vasularis the pigmented cells share characteristics of both macrophage and melanocyte phenotypes and control the integrity of the intrastrial fluid–blood barrier by affecting the expression of tight- and adherens-junction proteins (Tapia et al., 2014). Their location directly underneath the MC layer (Fig. 5f–h) suggests similar functions or a combination of immune response and tight junctional permeability control.

TCL cells show an incomplete junctional seal underneath the basilar membrane. Thicker layers in the apical turns (Liu et al., 2015a) present like a labyrinth in the labyrinth (Figs. 1c and 5i). A more or less dense meshwork of spindle shaped cells appears to be highly interwoven into each other. Tight junctions alternate with free passage canals to the BM. Different textures of the TCL in different turns suggested the function as a dapping layer (Angelborg and Engstrom, 1974) to support mechanical movements of the BM. Their high phagocytic activity is more relevant for drug studies. We often found dense agglomerations of nanocarriers tagged with fluorochromes in the TCL (Glueckert et al., 2015). In human material acellular substances often fill the intercellular space (Fig. 5i). Like with other MCs a regulatory function on the border between compartments may be an explanation. These cells form a micro environment underneath the BM and may regulate transport between fluid spaces of the sensory epithelium and the ST. Other functions may involve repair or maintenance of the BM.

Taken together the ability of fast transport mechanisms of the MCs cell layer and a highly fenestrated bone especially at the ST suggests that the passage of perilymph may occur through the modiolus into the inner acoustic meatus. Mammalian mesothelium is considered essentially similar regardless of species or anatomical site (Mutsaers, 2004), so tissue from big body cavities could serve as models to analyze drug uptake/permeation mechanisms of MCs. Their enormous flexibility in phenotypical appearance from epithelial to mesenchymal characteristics and phagocytic activity may attribute MCs as main candidates for drug clearance and metabolic degradation of chemical compounds. Lack of blood vessels in the scalae and the BLB argues for a vital role of this cell type in clearance of drugs from perilymph. Elimination of drugs that are locally applied to perilymph is one of the major factors influencing both the local concentration achieved and spread towards the apex. A better understanding of MC physiology may be valuable to prolong drug activity and enhance spread within the inner ear.

4.3. Endolymphatic compartment-splendid isolation

Endolymph composition is unique in mammals with concentrations high in potassium and low in calcium, similar to intracellular fluid. The endolymphatic compartment is much smaller than the surrounding perilymphatic space and comprises only 17.3% of total labyrinth fluid in human, the SM is only 3.3% of the lymphatic volume in human according to our own data (Table 1). Via the vestibular aqueduct this compartment is connected to the endolymphatic sac (Fig. 4a). Functions imply regulation of the volume and pressure of endolymph, immune response of the inner ear, and the elimination of endolymphatic waste products by phagocytosis (Couloigner et al., 2004; Rask-Andersen and Stahle, 1980; Rask-Andersen et al., 1991; Salt, 2001).

Most compounds soluble in perilymphatic fluid should behave the same in the endolymph. Endolymph shows even lower protein concentrations (Wangemann et al., 1995) that may enhance effectiveness of a drug. Exchange of substances between perilymph and endolymph is strictly limited to transcellular routes (Juhn et al., 2001). However, the transport of horseradish peroxidase from perilymph to endolymph was reported previously (Saijo and Kimura, 1984). Uninterrupted strands of tight junctions seal uncontrolled paracellular loss. Loss of the EP driving force results in deafness. Various claudin proteins form a complicated expression pattern with variable barrier functions across different areas. Many cell borders show co-expression of different claudin proteins whereas in the basal cells and adjacent fibrocytes of the stria vasularis claudin 11 is present to insulate the cochlear battery (Fig. 6c) (Kitajiri et al., 2004b; Liu et al., 2017). Deletion of claudin 11 leads to a loss of the EP and deafness but not to a decreased K⁺ concentration in the endolymph (Gow et al., 2004; Kitajiri et al., 2004a). The EP seems to be generated in the basal cell area whereas maintenance of high K⁺ levels are attributed to marginal cells of the stria vasularis (Wangemann et al., 1995).

Although very thin, the 2-cell layered Reissner’s membrane (RM) provides a potent barrier for ions and chemical compounds (Fig. 6a–b). Selective transport for sodium was found that contributes to maintaining the low Na⁺ concentration in endolymph fluid (Yamazaki et al., 2011a) similar to semicircular canal duct epithelial cells (Yamazaki et al., 2011b). Trans-epithelial exchange of Na⁺, K⁺, Cl⁻ and Ca²⁺ via the RM was proposed (Lang et al., 2007) and water transport mediated by aquaporin channels calculated (Eckhard et al., 2014). However for bigger molecules RM and epithelium of the vestibular portion of the endolymphatic compartment seems to be rather impermeable. A water shunt region in the cochlear apex was proposed that may explain the experimentally determined phenomenon of endolymphatic longitudinal flow towards the cochlear apex (Eckhard et al., 2014; Hirt et al., 2010).

4.4. Reticular lamina & cortilymph: shear stress proof tight junctions and the third lymph

Tricellulins are important especially in the reticular lamina (Fig. 7a) that seals the SM against the organ of Corti. Vibrations and shear stress sets special requirements for these mosaic pattern
Mesothelial cells in the perilymphatic compartment and possible fluid pathways from the scala tympani into the modiolus in human: a: Scanning electron micrograph (SEM) from a macerated human temporal bone showing the bony structure of the modiolar wall in the basal turn. All soft tissue was chemically removed. Between bony columns guiding nerve fiber fascicles from Rosenthal's canal (RC) to the osseous spiral lamina (OSL) pathways without relevant hindrance lead into the central modiolus (yellow arrows indicate possible fluid routes). The scala vestibuli (SV) bony surface is rather smooth with few prominent holes that act as outlets (black arrows) for radial arterioles running to the stria vascularis. FU; fundus region, osl; osseous spiral lamina (most part removed), RC; Rosenthal's canal, SM; spiral modiolar vein, ST; scala tympani. b: Hematoxilin-Eosin stained section through the basal turn of a human cochlea between two bony columns. Yellow arrow marks fluid pathways into the modiolus. FU; fundus region of the inner ear canal, RC; Rosenthal's canal, SM; scala media, ST; scala tympani, SV; scala vestibuli. c: Scanning electron micrograph of the scala tympani modiolar surface at the cell border of two mesothelial cells. Pinocytotic vesicle or stomata are frequently seen at the cell borders. d: Transmission electron micrograph of a mesothelial cell (MC) at the scala tympani (ST) aspect of the osseous spiral lamina. MCs rest on a thin collagen network and connect with tight junctions (arrow). MPA; myelinated peripheral axon. e: SEM of the region shown in 5b of a surgical specimen: Between bony columns thin layers of MCs (white arrows) leave big pores open (yellow arrow indicate fluid pathways from scala tympani into the modiolus), RC; Rosenthal's canal. f: Hematoxilin Eosin stained section; higher magnified view of Fig. 5b that corresponds to location of region to Se-. The thin porous layers of MCs are visible in sections. Various brown colored cells with numerous processes represent melanocytes. g: TEM overview of melanocytes (ME) between mesothelial cell layers (MC) at the modiolar...
Fig. 6. Tight junctional seals in the human cochlea: a: The endolymphatic compartment is sealed by Reissner’s membrane (TEM image), a delicate two cell sheet layer with cochlear duct epithelium facing the scala media (SM) and a mesothelial (MC) layer facing the scala vestibuli (SV). b: Immunostaining for the tight junction protein occludin depicts the uninterrupted seal by both cell types in Reissner’s membrane. scala media; SM, scala vestibuli; SV. c: Claudin 11 and Na⁺-K⁺-ATPase immunostaining identifies this important tight junction protein in strial basal cells and adjacent layers of type I fibrocytes of the spiral ligament (SL). Marginal strial cells are highly positive for Na⁺-K⁺-ATPase to maintain high K⁺ levels in the scala media (SM). BV: blood vessel.

Table 1
Temporal Bone (TB) dimensions of fluid spaces from 24 human individuals evaluated by manual segmentation of 15 μm voxel resolution microCT data sets. R and L represent left and right inner ears. vestib. EL vestib. PL refers to endolymphatic and perilymphatic spaces in the vestibular system, Fluid spaces in the scala media endolymph (S. media EL), scala tympani perilymph (S. tymp. PL), scala vestibuli perilymph (S. vest. PL) and total perilymphatic (PL total) and endolymphatic fluid (EL total). Fluid total; total inner ear fluids.

| TB   | vestib. EL | vestib. PL | S. media EL | S. tymp. PL | S. vest. PL | PL total | EL total | Fluid total |
|------|------------|------------|--------------|-------------|-------------|----------|----------|-------------|
| 1R   | 18.68      | 77.74      | 6.43         | 24.27       | 26.43       | 128.44   | 25.11    | 153.55      |
| 2L   | 19.45      | 82.60      | 6.18         | 30.21       | 20.51       | 133.31   | 25.63    | 158.95      |
| 3R   | 20.39      | 78.68      | 5.32         | 28.64       | 15.54       | 122.85   | 25.71    | 148.56      |
| 4L   | 25.38      | 80.50      | 5.11         | 27.60       | 14.07       | 122.16   | 30.49    | 152.65      |
| 5L   | 25.15      | 69.69      | 4.10         | 32.70       | 24.38       | 126.77   | 29.25    | 156.02      |
| 6L   | 25.87      | 86.02      | 5.35         | 32.18       | 25.56       | 143.76   | 31.22    | 174.98      |
| 7R   | 25.13      | 91.99      | 5.72         | 27.61       | 26.83       | 146.42   | 30.86    | 177.28      |
| 8R   | 28.41      | 96.23      | 5.33         | 30.69       | 22.48       | 149.69   | 33.74    | 183.43      |
| 9L   | 26.32      | 78.26      | 5.15         | 32.49       | 28.13       | 138.90   | 31.48    | 170.38      |
| 10R  | 26.42      | 92.17      | 5.08         | 28.85       | 31.60       | 152.62   | 31.50    | 184.12      |
| 11R  | 26.51      | 80.42      | 4.76         | 29.29       | 23.66       | 133.37   | 31.26    | 164.63      |
| 12L  | 24.53      | 98.72      | 7.14         | 37.19       | 25.44       | 161.35   | 31.67    | 193.02      |
| 13R  | 30.85      | 105.68     | 5.34         | 30.19       | 23.07       | 158.94   | 36.19    | 195.13      |
| 14R  | 19.22      | 75.82      | 5.12         | 27.47       | 21.43       | 124.71   | 24.34    | 149.05      |
| 15L  | 28.02      | 100.18     | 5.08         | 26.75       | 33.26       | 160.18   | 33.09    | 193.27      |
| 16R  | 32.57      | 94.65      | 6.20         | 22.46       | 22.04       | 139.15   | 38.76    | 177.91      |
| 17R  | 30.98      | 101.43     | 6.06         | 34.97       | 28.74       | 165.14   | 37.03    | 202.17      |
| 18R  | 26.81      | 100.33     | 5.93         | 42.71       | 27.82       | 170.86   | 32.74    | 203.60      |
| 19R  | 23.66      | 83.06      | 5.79         | 27.80       | 22.52       | 135.38   | 29.44    | 166.82      |
| 20L  | 12.57      | 82.02      | 5.37         | 26.78       | 20.80       | 139.59   | 17.94    | 157.53      |
| 21L  | 14.77      | 80.84      | 7.38         | 27.42       | 16.51       | 124.77   | 22.15    | 146.92      |
| 22L  | 30.97      | 125.32     | 7.56         | 42.12       | 24.48       | 191.92   | 38.53    | 230.44      |
| 23L  | 25.50      | 102.43     | 4.74         | 29.23       | 15.83       | 147.50   | 30.24    | 177.74      |
| 24L  | 16.93      | 80.44      | 5.77         | 24.17       | 18.41       | 129.02   | 22.70    | 151.71      |
| Mean | 24.38      | 90.13      | 5.67         | 30.17       | 23.31       | 143.62   | 30.04    | 176.66      |
| SD   | 5.26       | 12.41      | 0.84         | 5.03        | 4.97        | 17.66    | 5.25     | 21.51       |

wall (human). ST; scala tympani. h: Higher magnified view of melanocytes seen in 5g: Melanocytes (ME) never directly face the scala tympani (ST) and are always shielded by mesothelial cells (MC). i: Tympanic covering layer (TCL) at the scala tympani (ST) side of the basilar membrane (BM). TCL cells form a compartment with aecular substances (colored yellow) fragmentally sealed by tight junctions (arrows). There is a direct connection towards the ST (yellow arrow). The organ of Corti face reveals infoldings that enlarges the surface of the basal pole of Deiters cells (DC) and is structurally similar to the basement membrane (colored green).
Fig. 7. The human organ of Corti-pathways to hair cells: a: Human reticular lamina (apical turn) provides a tight and robust seal between scala media (SM) and the fluid space in the organ of Corti filling the tunnel of Corti (TC) and Nuel’s space (NS) that enable outer hair cells (OHC) to change their length. DC: Deiters cells, HC: Hensen cells, IHC inner hair cell, IP; inner pillar, OP; outer pillar. b: Mechanical stress lead to a special tight-junction with adherens junction characteristics between outer hair cells and Deiters phalangeal processes (DP). SM; scala media. c: Human organ of Corti basal turn: A connection between scala tympani (ST) and cortilymph (Tunnel of Corti (TC) and Nuel’s space (NS)) was proposed (yellow arrow) present as a slit between Deiters (DC) and Hensen cells (HC). BC; Boettcher cells, BM; basilar membrane, IP; inner pillar, OHC; outer hair cells, OP; outer pillar, SM; scala media. d–e: TEM image of Deiters-Hensen-cell slit in the high frequency region (7d) and low frequency region (7e). The densification of the basal pole attachment to the basilar membrane is much more prominent in the apical turn. Intercellular gaps (asterisks) are frequently found between Deiters (DC) and Hensen (HC) cells, the multi layered basement membrane is not interrupted at the slit region (yellow arrow). f–g: Habenula perforata in a human middle turn: Unmyelinated nerve fibers fascicles as well as epithelial cells of the organ of Corti produce a basal lamina (colored in green in Fig. 7g) but possibly leave spaces between also under in vivo conditions. There is very likely a communication between scala tympani (ST) through the tympanic covering layer (TCL). BM; basilar membrane, IHC; inner hair cell, IP; inner pillar cell, S; putative afferent synapses likely enlarged through postmortem toxic effect of glutamate, SM; scala media, TC; tunnel of Corti.
shaped cell borders. Oscillation induced forces set highest demands on junctions between Deiters cells and motile OHCs (Fig. 7b). This cell-cell contact is unique in a way that the OHCs–Deiters cells connection form a novel hybrid tight junction with adherens junction organization (Nunes et al., 2006). Loss of tricellulin leads to HC degeneration and non-syndromic deafness (DFNB49) in human but does not impair EP or endolymphatic ion composition (Kamitani et al., 2015; Riazuddin et al., 2006).

Nuel’s space surrounds the lateral surfaces of OHCs and Deiters’ phalangeal processes. Together with the fluid filling the tunnel of Corti this compartment is termed cortilymph, the third extracellular fluid space (Fig. 7a, c). Composition is similar to perilymph regarding K⁺ concentration at rest but changes with sound evoked stimulation as extracellular K⁺ accumulates by HC activation (Johnstone et al., 1989). K⁺ is then recycled back into the stria vascularis by a connexin gap junction network.

A leaky reticular lamina would intermingle endolymph with fluid spaces of the sensory organ and in turn lead to a loss of HCs. Hence, any damage of the sensory epithelium surface, especially in combination with acoustic stimulation must be avoided for any safe drug application.

4.5. Basilar membrane-leaky to cortilymph?

A communication between ST and cortilymph was suggested to occur at the base of the Hensen-Deiters’ junction (Beagley, 1965). TEM imaging and tracer studies suggest this slit as a patent pathway between ST and the organ of Corti even able to generate hydrops of the sensory epithelium (Nomura et al., 2016). We recently analyzed the human BM and confirm thinnest portions situated underneath the OHCs/Deiters cells (Liu et al., 2015a). Further a discontinuity in the basement membrane between the Hensen cells was found in the apical region in cat (Cabezudo, 1978). These gaps provide structural evidence of the free communication between the intercellular spaces in the organ of Corti and the ST through the BM, as previously reported by several authors (Altmann and Walther, 1950; Duvall, 1972; Ilberg and Vosteen, 1969; Masuda et al., 1971; Tonndorf et al., 1962). At this level in the pars pectinata the upper layer of BM filaments was very thin and a discontinuity between the bundles was observed. This pathway appears extremely attractive to target sensory HCs via the perilymphatic compartment. Whether this Hensen-Deiters slit is the only path from ST through the BM to cortilymph is not clear. The slit is located next to prominent septa of the BM that extend (5–6 μm in height and diameter) into the organ of Corti (Santi and Johnson, 2013). These acellular protrusions likely serve as an anchoring support for Deiters cells to mechanically support or uncouple Hensen’s cells base. In chemically fixed specimens it seems to be a site where adherent junctions are less developed and so para-cellular gaps appear (Figs. 1c and 7c, d and e). The protrusions are most prominent in the apex where additional smaller protrusion support Deiters cells (Fig. 7c–e). To what degree such intercellular gaps occurs as a fixation artefact not present at physiological conditions or act as patent pathways into cortilymph needs to be further elucidated. We always found several basement membrane layers at this slit region in human that may act as a filter for certain high molecular weight compounds (Fig. 7e).

A natural port through the BM is the habenula perforata where nerve fibers pass into the sensory epithelium. These oval to round holes (1–3 μm in diameter; lurato, 1967), underneath the IHC are occupied by unmyelinated nerve fibers and few specialized glia cells that are surrounded by a basement membrane (Fig. 7f–g). Adjacent supporting cells add another basement membrane but leave a small cleft open as seen in TEM images (Fig. 7g) that may even be smaller under in-vivo conditions.

Drugs that are able to enter to intercellular clefts may be able to enter cortilymph, although gap junctions present in the organ of Corti narrow these gaps. Connexin proteins form hemi-channels as well as cell-cell communication gap junctions vital for cochlear function. Gap junctions are present in organ of Corti supporting cells, glia cells, fibrocytes, and other cell types. Fast K⁺ recycling into the stria vascularis is accomplished via these intercellular communication routes. Few studies focus on permeation of cytoplasmic molecules through connexin channels, so it is unknown how gap junctions contribute to drug distribution in the inner ear. Limiting factors are molecule size and charge. More care should be taken on drugs not to interfere with gap junction action. Several small compounds have already been found to inhibit connexin function. One example represents the amino acid taurine that is able to directly and reversibly inhibit homomeric and heteromeric channels that contain Cx26 (Locke et al., 2011).

“Leakiness” of the BM may largely depend on molecular formulation of drugs. Kinetics of this passage may also vary between the locations of application. A thicker and narrower BM in the basal turn could influence penetration speed compared to a wider and thinner BM in the apical region. The thicker TCL layer in the apical region full of MCs may compensate for that higher speed of penetration or act as a cleaning filter for compounds close to the basilar membrane.

5. Application sites

5.1. Round window and oval window- gateways to the inner ear

The two natural fenestrations of the perilymphatic space open towards the middle ear and are occupied by a thin membrane and the smallest bone of our body. These openings act in concert as vibrations entering the inner ear through the stapes footplate at the oval window cause vibrations with opposite phase to the membrane of the RW (RWM). This allows the fluid in the inner ear to propagate and sets the BM in motion (Fig. 8a).

The RWM is a continuation of the mucous membrane lining the middle ear followed by a connective tissue layer containing a collagen- and elastic fiber matrix populated with fibroblasts, blood- and lymph vessels and comprises even some nerve fibers (Rask-Andersen et al., 1999, 2004). MCs cover the perilymphatic aspect in the ST (Fig. 8b–c). While the connective tissue layer is thinnest in rodents resulting in a RWM thickness of 10–30 μm in guinea pigs (Tanaka and Motoymura, 1981), 12 μm in rats (Nordang et al., 2003) and below 10 μm in mice (Fig. 8b) it is dominating feline (20–40 μm in cat) and primate RWM, thickening it to about 70 μm in human (Figs. 8c) and 40–60 μm in rhesus monkey (Goycoolea and Lundman, 1997; Sahni et al., 1987). Thickening of the fibrous layer with age suggests decreasing compliance (Goycoolea and Lundman, 1997). Size and shape of the human RW was evaluated recently (Atturo et al., 2014). Ultrastructure of the inner and outer cell layers suggests a participation in absorption and secretion with age suggests decreasing compliance (Goycoolea and Lundman, 1997).
inflammation permeability increases but established pathological changes make this membrane even less permeable to protect inner ear function (Cureoglu et al., 2005; Goycoolea et al., 1980; Ikeda and Morizono, 1988; Kawauchi et al., 1989; Lim et al., 1990; Schachern et al., 1987). Induction of an inflammatory reaction to enhance drug permeation through the RWM would only be beneficial for a short period of time, but may be an option for single shot applications. Uptake of substances and particles from the middle ear cavity is seemingly decided by the mucosal epithelial cells and mainly a selective and active process. Cell lines or primary culture from the human or murine middle and inner ear epithelial cells may provide good models to predict uptake and transport in vitro (Lim and Moon, 2011; Mulay et al., 2016).

Fig. 8. Entry ports from the middle ear in mice and men: a: Semithin section of the mouse round window membrane area in the basal turn. SM; scala media, ST; scala tympani, SV; scala vestibuli. b: Higher magnified view of the round window membrane. ST; scala tympani. c: Human round window membrane set to the same scale as mouse corresponding structure in 8b: The connective tissue layer is considerably enlarged in human. d: Section through a human temporal bone visualizing the position of the oval window with the stapes (stap) footplate facing the vestibular perilymphatic space (PL). Macula sacculi (M.sac.) is very close to the oval window. M.stap.; musculus stapedius. ST; scala tympani, tymp.; tympanic membrane, SV, scala vestibuli. e: Higher magnified view of the stapes footplate seen in 8d: The bone is very thin an covered by connective tissue, blood vessels and middle ear epithelium. PL; perilymphatic space. f: Volume rendering of a human stapes depicts the tenuousness of the footplate. g–h: Micro CT images of human stapes footplates. Green line in 8g corresponds to the plane seen in 8h. i: Color coded graph of two stapes footplates from one human individual depicts high variations of shape and the thickness map. The upper stapes is seen in Fig. 8f–h.

The RW niche, a funnel-shaped depression in the otic capsule and favors retention of substances at the RWM especially when solid gel-like matrices are placed. Clearance through the middle ear Eustachian tube by the mucosa and ciliated epithelium can be delayed thereby. Interestingly, the rhesus monkey was shown to exhibit a secretory immunodefense-like structure residing at the RWM rim at the niche (Engmer et al., 2008). A
previous study demonstrated in 33% of human temporal bones an obstructed RW by either a pseudomembrane or a fibrous or fat plug (Alzamil and Linthicum, 2000). In these cases the oval window (OW) could compensate as an entry port.

The OW is nearly fully occupied by the stapes footplate, only a narrow annular ligament enables the smallest bone of our body to transmit vibrations into the inner ear (Fig. 8d–e). Permeability of substances via the OW has long been underestimated. Trans-tympanic gentamicin treatment for Meniere’s disease gave hints for the clinical significance of this pathway to control vertigo since vestibular sensory structures are relatively selectively ablated. Transport of Gd tracer through the OW in vivo was demonstrated in rats with MRI imaging (Zou et al., 2012). This pathway appears to be even more effective than the RW pathway for cochlear uptake in humans. Animal studies calculated Gd permeation of the combined substances via the OW has long been underestimated. These shortcuts rely on active mechanisms of MCs. MicroCT imaging can provide valuable data for stapes measurements (Elkhouri et al., 2006; Hagr et al., 2004; Sim et al., 2013). Two human stapes from the same individual presented here (Fig. 8f–i) depicts how thin the footplate bone appears around its center. Undermined by various canals and lacunae (Fig. 8g–h) mineral portion of hydroxylapatite (HA) content measures 926.6 mg HA/ccm and 965.6 mg HA/ccm that is in the range of bigger lamellar bones. Footplate thickness ranges from 30 to 500 μm in the presented human (Fig. 8i). Considerable variability in thickness is present even within the same subject (Fig. 8i).

Stapes footplate application of gentamicin proofed effective to deliver a sufficient dose for the vestibular system (King et al., 2017). Since penetration of substances into perilymph through the bone of the otic capsule in guinea pigs was presented (Mikulec et al., 2009), it is likely that the thin bone of the footplate is permeable for several drugs.

6. Conclusion

Local drug delivery via the RWM is a safe and effective way to target cells in the cochlea. SGNs are accessible via ST as well as HCs very likely via the cortilymph compartment that seems to be leaky towards the ST. The OW offers another path that may be more effective for certain substances than the RW application (King et al., 2013). Still we lack knowledge about the exact path of compounds through the oval window layer. Studies including the fibrous annular ligament of the OW or the thin bone of the stapes footplate (Tanaka and Motomura, 1981) (Fig. 8e). Middle ear face of the footplate is populated by mucosa cells resting on a connective tissue layer containing various blood vessels (Fig. 8e). The perilymphatic aspect is covered by MCs. MicroCT imaging can provide valuable data for stapes measurements (Elkhouri et al., 2006; Hagr et al., 2004; Sim et al., 2013). Two human stapes from the same individual presented here (Fig. 8f–i) depicts how thin the footplate bone appears around its center. Undermined by various canals and lacunae (Fig. 8g–h) mineral portion of hydroxylapatite (HA) content measures 926.6 mg HA/ccm and 965.6 mg HA/ccm that is in the range of bigger lamellar bones. Footplate thickness ranges from 30 to 500 μm in the presented human (Fig. 8i). Considerable variability in thickness is present even within the same subject (Fig. 8i).

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about drug efficacy along the tonotopical axis. This should help to address clinical questions for dosage and requirements for drug stability in perilymph.

Targeting the endolympathic compartment implies risks to damage a barrier that results in degeneration of HCs. Only drugs or nanocarriers that are able to leave these barriers intact will succeed for a safe application. Drug formulation will always be the key for a successful pharmacotherapy. Combinations of drugs and genes with various nanocarriers may be extremely useful especially for the inner ear (Pritz et al., 2013a). They do not only cross solubility barriers but may in future enable cell specific targeting. Considering the active and passive mechanisms of cellular transport, fluid pathways and dimensions in the inner ear, a site selective pharmacotherapy seems feasible in the near future.

7. Material and methods

7.1. Ethics

Human bodies were donated to the Division of Clinical and Functional Anatomy of the Innsbruck Medical University by people who had given their informed consent prior to death for the use of their bodies for scientific and educational purposes (McHanwell et al., 2008; Riederer et al., 2012). All specimens were anonymized. Post mortem delays until fixation ranged from 6 to 12 h. Surgical human materials from Sweden was approved by the local ethics committee (no. 99398, 22/9 1999, cont. 2003, Dnr. 2013/190), and subjects gave informed consent. The study adhered to the rules of the Declaration of Helsinki. There was no evidence for any malformation in any human temporal bones. Guinea pig and cat images were taken from archival material used in previous studies (Rattay et al., 2013). Guinea pig were from a study performed in pigmented 250–350 g guinea pigs of both gender from Elm Hill Breeding Labs, Chelmsford, MA in a previous project (Glueckert et al., 2008). Animals were housed in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, with free access to food and water throughout the duration of the experiment. Veterinary care and animal husbandry was provided by the Unit for Laboratory Animal Medicine at the University of Michigan, and all protocols were approved by the University Committee for the Use and Care of Animals at the University of Michigan. Experiments were performed in accordance with the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23, revised 1978). A concerted effort was made to minimize both the number of animals used in this previous study, and the suffering of subjects involved in the study. Re-evaluation from archival celloidin and plastic embedded sections emenate from previous research projects and were published by Spoedlin and others (Spoedlin and Schrott, 1989; Spoedlin and Schrott, 1990).

7.2. MicroCT imaging of temporal bone specimens

24 temporal bones from body donors were excised and fixed in Karnovsky’s formaldehyde-glutaraldehyde solution for several weeks. To ensure rapid fixative penetration, oval and round windows were penetrated with a needle and the fixative gently perfused with a Pasteur pipette. Specimens were post-fixed in 2% osmium tetroxide (OsO4) for 2 days. After thorough washes in PBS the excess bone was removed in most of these specimens with a drill to meet maximum specimen size for the microCT scanner. Specimens were decalcified in EDTA pH 7.2–7.4 for 6–8 weeks at 37 °C in a Milestone® HISTOS 5 microwave tissue processor and thoroughly washed in PBS for 5 days. Subsequently, they were transferred to 50% and 70% ethanol 3 × 2 hours each, rotated on an
overhead shaker (Heidolph® Reax) for 2 days and mounted in plastic sample holders again in 70% ethanol. This procedure ensured that air bubbles present in PBS get removed. Scans from the decalcified specimens were acquired using an XRadia MicroXCT-400 at 45 kVp and 109 µA with an isotropic voxel size of 15 µm.

All scans from human inner ears were exported in DICOM format. Scans were imported to Amira® 6.2 and nerves and structures of the membranous labyrinth were manually segmented switching between the three orthogonal planes using the Segmentation editor. Segmented structures such as the membranous labyrinth, perilymphatic compartments of the whole inner ear, vestibular end organs, vestibulocochlear nerve were visualized using volume and surface renderings, endolymphatic duct as well as the cochlear aqueduct were traced.

7.3. MicroCT imaging of stapes specimens

Stapes specimens were vertically mounted in small plastic containers in 1.5% low melt agarose. Quantitative micro-computed tomography was performed using a Scanco μCT35 System (SCANCO Medical AG, Brüttisellen, Switzerland) in order to assess mineralization of the stapes. Image acquisition was performed at 70 kV source voltage and 114 µA intensity with an angular increment of 0.18° between projections and an integration time of 1.6s per projection. Voxel resolution of reconstructed slices was 10 µm (isotropic). Mineral concentrations were measured using the hydroxyapatite-phantom based densitometry calibration of the scanner.

For measurement of the mineral concentrations, image volumes were imported into the 3d software package Amira 6.4 (FEI Visualization Sciences Group, Mérignac Cédex, France). First, average mineral density was calculated for the whole stapes specimen. Subsequently, the stapes image volume was cropped to the basal plate using the VolumeEdit tool, and average mineral density for the thin parts of the basal plate was calculated. For the cropped basal plate volume, a polygon surface model was created and local surface thickness was measured. Surface thickness values were plotted on the polygon model using a false color lookup table in order to show the thinnest areas of the basal plate.

7.4. Transmission & scanning electron microscopy

For detailed protocols the reader is referred to Glueckert et al., 2005a,b (Glueckert et al., 2005a, 2005b). Transmission Electron Microscopy was done at the Institute of Zoology and Center of Molecular Bioscience Innsbruck, University of Innsbruck, Technikerstr. 25, A-6020 Innsbruck, Austria with a Zeiss Libra 120.

7.5. Immunostaining

All procedures, antibodies, immunostainings and imaging strategies are described in Liu et al. (2017).

Conflicts of interest

All authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Contributions

R.G. wrote the manuscript, made the figures and participated in scanning- and transmission electron microscopy work; L.J. Ch. did segmentation work and fluid space measurements; H.R.-A. participated in writing the manuscript, did all immunostaining work and participated in scanning-as well transmission electron microscopy work; W.L. did immunostaining work; S.H. did all microCT imaging and stapes measurements; A.S.-F. participated in writing the manuscript and participated in scanning- and transmission electron microscopy work; all authors provided input and proofreading of the manuscript.

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