Research Article

Tonotopic Morphometry of the Lamina Reticularis of the Guinea Pig Cochlea with Associated Microstructures and Related Mechanical Implications

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ABSTRACT

Morphometry of the lamina reticularis of the guinea pig cochlea was performed using scanning electron microscopy. Seventy-four geometrical parameters of the lamina reticularis, the bundles of stereocilia, and individual stereocilia, in all rows of hair cells and within the individual hair cells, were measured at ten equally spaced locations along the longitudinal direction of the cochlea. Variations of the parameters versus the longitudinal coordinate were statistically analyzed and fitted with polynomials (constant, linear, or quadratic). Our data show that a unique set of geometrical parameters of inner and outer hair cells is typical for every frequency-dependent position at the lamina reticularis. Morphology of the outer hair cell structures varies more than respective parameters of the inner hair cells. Mechanical modeling using the obtained geometrical parameters provides a novel

glance at the mechanical characteristics with respect to the cochlear tonotopy.

Keywords: inner ear, morphometry, SEM, lamina reticularis, stereocilia, guinea pig cochlea

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INTRODUCTION

Cochlear frequency tuning, with high frequencies producing maximal responses closer to the base, is determined primarily by base-to-apex gradients in the mechanical and morphological properties of the cochlear structures (Naidu and Mountain 1998; Raphael and Altschuler 2003; Emadi et al. 2004; Scherer and Gummer 2004). These gradients provide the structural basis for the propagation of the traveling wave, described by Békésy (1960). The lamina reticularis (LR), which forms the upper surface of the organ of Corti, is no exception and incorporates the same structural principle. Stereociliary bundles (SBs) are important structures on the LR. The SBs play a key role in hearing, being the location where transduction of mechanical motion into the receptor potential of an individual hair cell takes place (Hudspeth 1989). Variation of the bundle-specific geometry according to tonotopic position along the LR is of crucial importance for signal processing in the cochlea because it influences

the fluid mechanics in the subtektorial space (Baumgart et al. 2008). Existing morphological studies provide only partial coverage of changes in geometry of structures associated with the subtektorial space relative to their tonotopic position.

Retzius was the first to show that the topology and height of the SBs change along the cochlea (Retzius 1884). This general anatomical principle has been reported for various groups of vertebrates studied to date (Iurato 1967; Lim 1980; Wright 1984; Lim 1986; Tilney and Tilney 1988; Nadol 1988). Furthermore, previous studies have revealed that the lengths of the stereocilia in the same row of the bundle differ not only in the longitudinal direction but also between the hair cell rows at the same tonotopic position (Lim 1980; Hirakawa and Harada 1986). More detailed morphometry of chinchilla's stereocilia (Lim 1986) demonstrated that not only the length of stereocilia but also their diameter is a subject of tonotopic variations.

Further studies (Held 1902; Wright 1984; Strelioff and Flock 1984; Hirakawa and Harada 1986; Furness et al. 1997) focused mainly on comparative morphometry of the tallest stereocilia in the SBs in different species. The length of shorter rows of stereocilia within the bundles, as well as the diameter of stereocilia, has not been investigated in these studies.

A larger set of data on geometry of the guinea pig's stereocilia has been reported by Zetes (1995). Morphometry of stereocilia of the inner hair cells (IHCs) and information on bundle geometry and topology were, however, fragmentary. In the same species, limited information about changes in the SB geometry, topology of the SBs, and the hair cells' cuticular plates (CPs) along the LR has been acquired (Zhou and Pickles 1996). Parameters that define the bundle's V-shape were, however, given without information on the inner structure of the bundles and their tonotopic position.

The radial and longitudinal dimensions of the entire LR and position of the SBs thereon have not been studied systematically. Fragmentary data on this subject have been reported for water buffalos (Tiedemann 1970) and for guinea pigs (Zhou and Pickles 1996), and variations of the distance between the hair cell rows in chinchillas have been studied (Santi et al. 1986).

Precise and reliable modeling of the cochlear mechanics requires a larger set of parametric data. For example, modeling of the fluid flow through the SBs requires information about diameters and lengths of all stereocilia in all bundle rows, as well as about the bundle geometry. The objectives of this study were to provide a consistent set of morphological data about the LR and linked microstructures in guinea pigs, and to investigate the implications on the fluid dynamics in the subtektorial space.

METHODS

Cochlear preparation for morphometry

Nine cochleae of young adult pigmented male guinea pigs (Charles-River GmbH) weighing 250–300 g were used in this study. Normal hearing of the guinea pigs under xylazine (4 mg/kg) and ketamine (90 mg/kg) anesthesia was confirmed using ABR responses recorded with BERA (ERA-System, Pilot Blankenfelde med. elektr. Geräte GmbH), prior to the removal of the hearing organ. Only animals with normal hearing were used. After sacrificing the animals using CO₂, their cochleae were dissected, and further preparation was performed in cold Hepes–Hanks salt solution with a pH value of 7.2 and an osmolality of 310 mOsm/kg (10 mM HEPES by Merck KGaA, 5.36 mM KCl, 141.7 mM NaCl, 1 mM MgCl₂·6H₂O, 0.5 mM MgSO₄·7H₂O by Sigma-Aldrich Corp. with addition of 1.25 mg/l glucose by Sigma-Aldrich Corp.).

After opening of the bulla and the cochlea in the vicinity of the helicotrema, the cochlear scalae were slowly perfused with 4 % neutrally buffered formaldehyde solution. After the initial perfusion, the specimen was kept in the same solution for 24 h. The subsequent treatment of the specimen and removal of the bony capsule were made in phosphate buffer (PBS solution). The cochlea was divided into half turns, and the bony parts of the modiolus were partially removed. The Reissner's and tectorial membranes were completely removed at this stage. During preparation for the scanning electron microscopy, specimens were dehydrated with an increasing alcohol sequence (30 to 100 % with 10 % steps, 15 min per the concentration step) and dried with liquid CO₂ at the critical point. Thereafter, the specimens were coated with a fine gold layer (15–20 nm, Sputtercoater S150-B, Edwards GmbH). Equal thickness of the layer in different specimens was ensured by the same coating duration (1 min).

This part of the study was performed in accordance with the German Prevention of Cruelty to Animals Act. Permission was obtained from the Dresden Regional Council (24D-9168.11-1-2006-8).

Recording of the compound action potential of the auditory nerve

Six pigmented guinea pigs (250–350 g) from a colony bred at the University of Brighton were anesthetized with the neurolept anesthetics technique (0.06 mg/kg body weight atropine sulfate s.c., 30 mg/kg pentobarbital sodium i.p., 500 µl/kg Hypnorm i.m.). Additional injections of Hypnorm (250 µl/kg) were given every 40 min to maintain a nonreflexive state. Additional doses of pentobarbital (10 mg/kg) were given every 3 h or after elevation of the heart rate. The heart rate was monitored with a pair of skin electrodes placed on

either side of the thorax. Animals were overdosed with pentobarbital at the end of experiments without recovery from the anesthesia. The animals were tracheotomized and artificially respired, and their core temperature was maintained at 38 °C with a heating blanket and a head holder. The right pinna was dissected, and the cochlea was exposed through a lateral opening in the temporal bone to obtain a clear view of the round window. The compound action potential of the auditory nerve (CAP) was recorded with a silver electrode placed near the round window. Silver–silver chloride reference electrodes were inserted into the neck muscles. Signal was amplified $\times 10,000$ and band-pass filtered between 0.25 Hz and 3 kHz using an eight-pole Bessel filter. Resultant noise in the system was 35 μV RMS (85 nV RMS per $\sqrt{\text{Hz}}$) referring to the point of measurements. CAP was observed on an oscilloscope screen, and thresholds of the N1 peak of the CAP were estimated visually using 10-ms tone stimuli at a repetition rate of 10 Hz.

Sound was delivered to the tympanic membrane by a closed acoustic system comprising a Brüel and Kjær 4134 1/2" microphone for delivering tones and a Brüel and Kjær 4133 1/2" microphone for monitoring sound pressure at the tympanum. The measuring microphone was calibrated in situ using a Brüel and Kjær Sound Level Calibrator Type 4230. The microphones were coupled to the ear canal via a conical speculum; the 1.5-mm-diameter opening of which was placed about 1 mm from the tympanum. The closed sound system was calibrated in situ for frequencies between 1 and 50 kHz. Known sound pressure levels were expressed in decibels (sound pressure level) relative to 20 μPa (dB SPL re 20 μPa). Multi-harmonic signals for acoustical calibration and tone sequences for auditory stimulation were synthesized using a Data Translation 3010 board at 200 kHz and delivered to the microphones through a low-pass filter (100-kHz cut-off frequency). Experimental control, data acquisition, and data analysis were performed using a PC with programs written in MATLAB (The MathWorks Inc.). These procedures involving animals were performed in accordance with the UK Home Office regulations.

Scanning electron microscopy investigation

Data on morphometry of the LR, the SBs, and a single stereocilium in all rows of the hair cell were recorded with a raster electron microscope SEM Philips XL30 ESEM. The LR and the SBs were recorded with a magnification of up to 60,000 times and a beam voltage of 30 kV. For reconstruction of true dimensions of stereocilia, every SB was imaged twice with a predefined angular shift and the same magnification (Fig. 1) (see below).

FIG. 1. Estimation of the true geometrical size. Points *F* denote the foci of the observers.

Overall, ten regions from the oval window to the apex of the cochlea were investigated. The regions were equidistant in space, and the region's size was 1–1.5 mm, depending on the length of the entire LR. All measurements were taken at the measurement points (P), which were defined as the center of every region. The points were numbered from P1 to P10 with P1 being located at the base and P10 at the apex of the cochlea.

Data acquisition and analysis

All geometrical measurements (LR, the SBs, and a single stereocilium) were undertaken using "Analysis 3.0" (Soft Imaging System GmbH) (Fig. 2). The measurements included distances between the SBs in different rows of hair cells (Fig. 2b), distances between adjacent bundles in one row of hair cell (Fig. 2f), the widths (Fig. 2d), and the lengths (Fig. 2c) of the CPs. Within a single bundle, the inner angle (Fig. 2h) and the length of legs (Fig. 2g) were estimated, and the number of stereocilia per bundle was counted. For a single stereocilium, the length, the shaft length, and the diameters of its tip (D_{max}) and the coupling place to the CP (D_{min}) were acquired (Fig. 3).

Scanning electron microscopy (SEM) provides only a projected view of the investigated object. To reconstruct the real size of the observed geometrical features, two recordings with the same magnification and known angular difference α were made for every measurement (Fig. 1). The projected lengths l_{p1} and l_{p2} were measured on both images. The true length l_w was calculated using the following formula:

$$l_w = \left| \frac{\sqrt{l_{p1}^2 + l_{p2}^2 - 2l_{p1}l_{p2}\cos\alpha}}{\sin\alpha} \right|. \quad (1)$$

The angle α used was 10 ° in most cases. Some specimen alignments required a larger angle of 15 ° to 25 °. All reconstructed true sizes of the LR and the SBs were saved in a database and mapped tonotopically.

FIG. 2. Measurements on the lamina reticularis: (a) the width of LR, (b_1 – b_3) distances between the stereocilia bundles, (c_{OH1} – c_{OH3}) lengths of the cuticular plates, (d_{OH1} – d_{OH3}) widths of the cuticular plates, (e_{OH1} – e_{OH3}) distances between bundle tips, (f_{OH1} – f_{OH3}) widths of gaps between bundles, (g_{OH1} – g_{OH3}) side length of the OHC bundles, (h_{OH1} – h_{OH3}) inner angle of the OHC.

Tonotopic mapping of the lamina reticularis

Preparation of specimens for SEM (including dehydration) causes shrinkage of the organ of Corti. Hence, estimates of the tonotopic mapping of frequencies along the LR were made taking into account this shrinkage, which was assumed to be homogeneous. To reproduce the correct tonotopy in this case, the position of every measuring point must be anchored to the location of the corresponding

frequency related to the total length of the LR. This allows extrapolating existing frequency mapping data to a shrunken specimen. In our study, this procedure was utilized to ensure the correct tonotopy using the frequency map of the guinea pig basilar membrane by Tsuji and Liberman (Tsuji and Liberman 1997).

Statistical analysis

The means and the standard errors of the mean (SE) were calculated for all parameters measured. Polynomial regression analysis was used to determine the significance criterion of the parameters with $p < 0.05$. All statistical tests were performed using IBM SPSS 16 (IBM Corporation, Armonk, NY, USA).

Since the frequency dependence of the measured parameters was heterogeneous, we proposed pooling parameters which show similar tendencies into three different groups. A type of approximation that provided the best fit was assigned to each of these groups. The criterion for the best fit was the significance of the respective approximation calculated in a t test.

Group 1 consists of parameters which show no significant variation along the LR. These parameters were approximated by constants (zero-degree polynomials):

$$f(x) = a_0, \quad (2)$$

where x is the location in the longitudinal direction from the base to the apex and $f(x)$ is the approximated parameter value. Parameters of the second group show a significant linear change. For these parameters, linear approximations (first-degree polynomials) were chosen:

$$f(x) = b_1x + b_0. \quad (3)$$

In the third group, parameters with a strong nonlinear variation over the length of the LR were combined. The parameters of this group were fitted with quadratic functions (second-degree polynomials):

$$f(x) = c_2x^2 + c_1x + c_0. \quad (4)$$

FIG. 3. Measurements of a stereocilium.

RESULTS

All acquired tonotopic measurements were summarized in a three-dimensional model of the LR (Fig. 4). In this model, mean values were used. A complete set of high-precision data used for the spatial reconstruction and the parameters of approximation curves are given in Supplemental Tables.

FIG. 4. Simplified tonotopic scheme of the lamina reticularis with the bundles of stereocilia. Created with SolidWorks (Dassault Systèmes SolidWorks Corp.). **A** View at an angle of 45 ° to the plane the lamina reticularis. **B** Top view.

Morphometry of the lamina reticularis

In the longitudinal direction, borders of the LR were defined as the distance between the outer edges of the CPs of the first and the last IHCs. In the radial direction, the LR was defined to range from the inner edge of the CPs of IHCs to the outer edge of the third row of outer hair cells (OHC3) (Fig. 2a). Three zones were defined on the surface of the LR, namely, surface of the pillar cells (PC zone), inner hair cell zone (IHC zone), and outer hair cell zone (OHC zone). The PC zone is located between the other two zones and forms a cover of the inner tunnel.

The width of the LR was defined as the distance between IHC and OHC3 bundles (Fig. 2a) plus half-CP lengths of IHC and OHC3 (Fig. 2c_{IHC, OHC3}). The width of the IHC zone was defined to be equal to the CP length of IHC (Fig. 2c_{IHC}). The width of the PC zone was taken equal to the distance between IHC and OHC1 bundles (Fig. 2b₁). The width of the OHC zone was defined as the sum of the distances between OHC1 and OHC2 bundles (Fig. 2b₂) and between OHC2 and OHC3 bundles (Fig. 2b₃) plus half-CP lengths of OHC1 and OHC3 (Fig. 2c_{OHC1, OHC3}). This definition implies that the zones of the LR are overlapping and sum of their widths is not equal to the width of the LR.

The measured length of the entire LR was 16.28 ± 2.23 mm ($N=9$). This corresponds well to invasive and noninvasive measurements of the basilar membrane (Thorne et al. 1999; Poznyakovskiy et al. 2011). LR

width varied nonlinearly from 23.17 µm at the base to 42.28 µm at the apex, with a maximum width of 42.59 µm at P8. The total area of the LR, derived by the integration of a polynomial fit of the LR width as a function of its length, was 0.5568 mm². Width of all three zones showed a similar increase over the length of LR, although a peak width was absent in the IHC zone. The values rose from 2.47 to 4.12 µm in the IHC zone, from 9.53 to 15.89 µm in the PC zone (with a maximum of 17.67 µm at P7), and from 11.01 to 22.20 µm in the OHC zone, which had a peak value of 22.54 µm at P8 (Fig. 4). The total area of the IHC zone was 0.0503 mm², the PC zone was 0.2590 mm², and the OHC zone was 0.2368 mm².

All three LR zones encompass the full length of the organ of Corti. In guinea pigs, the width of all three zones grows from the basal end toward P8 and decreases again slightly in the apical part of LR. It appears that this decrease is not solely related to mechanical adjustments but also to the limited space near the helicotrema, where the radius of the LR curvature decreases and constrains LR geometry.

Topology of the stereociliary bundles

An example of the topology of the SBs for all four rows of hair cells at each measurement point is given in Fig. 4. Adjacent IHCs are located very close to each other, with their cell membranes being almost in direct contact. There is a small distance between the

SBs of adjacent IHCs, but these SBs are located close enough to be connected by the side links in most cases (Hackney et al. 1988). For this reason, we did not include the distance between adjacent IHCs as a measurement parameter.

For OHCs within a single row, the distance measured between the bundle apices (tip distance; Fig. 2 $_{e_{OHI}, e_{OHI}, e_{OH3}}$) remained constant along the LR, with values of approximately 5.8 μm . Conversely, distances between the bundle bases of adjacent OHCs within a single row (gap distances, Fig. 2 $_{f_{OHI}, f_{OH2}, f_{OH3}}$) increased from the base to the apex of the cochlea (Fig. 5A). The gap distance for OHC1 (Fig. 2 $_{f_{OHI}}$) increased slightly from 0.56 to 0.94 μm by a factor of approximately $\times 1.5$, compared to $\times 3.1$ (from 0.83 to 2.38 μm .) and $\times 3.9$ (from 0.90 to 3.41) for the OHC2 and OHC3 gap distances, respectively (Fig. 2 $_{f_{OH2}, f_{OH3}}$). These differences became statistically significant, however, only between P4 and P10 regions (Fig. 5A).

Radial distances between the OHC rows (OHC1 to OHC2, OHC2 to OHC3; Fig. 2 $_{b_2, b_3}$) showed a steeper growth in the basal, high frequency region of the LR (Fig. 5B). For the OHC2 to OHC3 distance, a minor decrease near the apex was observed. The OHC1 to OHC2 distance increased in the apical direction from 4.34 to 8.98 μm and the OHC2 to OHC3 distance increased from 3.36 to 7.52 μm , with a local maximum of 8.52 μm around P9. Distance between the IHC and OHC1 stereocilia (Fig. 2 $_{b_1}$) correlated with variation of the width of the PC zone and increased from 9.53 to 15.89 μm with a peak of 17.94 at P9 (Fig. 5B).

Topological arrangement of the SBs on the LR differs between IHCs and OHCs. The IHC bundles stand closely side by side, with bundle edges often contacting each other, as has been described previously (Hackney et al. 1988). Altogether, this forms a fence-like structure without gaps between the IHC bundles. This arrangement does not fully block fluid motion in the radial direction because fluid can still move through the remaining gap between the IHC stereocilia and the tectorial membrane.

The OHC rows, on the contrary, have gaps between the SBs. The gap width grows with an increasing rate toward the apical end (Fig. 5A). This growth is associated with decreased number of stereocilia per bundle toward the apex, nonmonotonic variation in the inner angle of the bundle (Fig. 5C), and growing diameter of the stereocilia. At the same time, distance between the adjacent tips of V-shaped bundles stays constant along the whole LR.

Morphometry of the stereociliary bundles

Since the SBs of adjacent IHCs are in contact with each other, the width of an IHC bundle can be considered equal to the width of the CP in corre-

sponding IHC (Fig. 2 $_{d_{IH}}$). OHC1 had roughly constant leg lengths (Fig. 2 $_{g_{OHI}}$) along the LR. This length ranged from 3.5 to 4 μm . The same parameter for OHC2 and OHC3 (Fig. 2 $_{g_{OH2}, g_{OH3}}$) stayed equal to approximately 3.5 μm until P7 for OHC2 and P5 for OHC3. These values decreased, however, to 1.85 and 1.63 μm , respectively, at P10.

The inner angles of the SBs (Fig. 2 $_{h_{OHI}, h_{OHI}, h_{OH3}}$) were 123.50° for OHC1, 121.20° for OHC2, and 108.58° for OHC3 (Fig. 5C) in the most basal, hook region of the cochlea. All three OHC inner angles decreased to a minimum toward the middle of the cochlea. The minimal values of these parameters were 77.29°, 74.29°, and 76.26° for OHC1, OHC2, and OHC3, respectively, observed at P5. They, however, increased again thereafter, reaching 110.0° for OHC1, 101.3° for OHC2, and 134.0° for OHC3 at the cochlear apex.

The height of the IHC bundles, defined as the length of the tallest stereocilia, changed linearly along the LR from 1.88 μm in the hook region to 3.60 μm at the apical end (Fig. 5D). The OHC bundles had roughly equal height of a slightly less than 1 μm at the base of the cochlea, which increased toward the apex. The greatest increase in the height was observed in OHC3, followed by OHC2 and OHC1, respectively. Maximum values of the SB height were 2.39 μm for OHC1, 3.66 μm for OHC2, and 4.24 μm for OHC3.

Morphometry of stereocilia

Guinea pig SBs consist of three rows of stereocilia of different lengths. Morphometry of a single stereocilium can be described by measurements of its length, diameter, and shaft parameters (Fig. 3). The tallest stereocilia within the SB consisted of nearly cylindrical bodies with conical shafts. The length of the conical shaft was about 25 % of the total length of the tallest stereocilium and changed proportionally along the LR. It was difficult to estimate the shaft length for the middle and short stereocilia due to their conical shape; this shape made measurement of the shaft length for these types of stereocilia imprecise, and consequently, greater variation was observed for this parameter. However, the mean shaft length was also close to 25 % from the total length of a stereocilium.

For both OHCs and IHCs, length of the tallest stereocilia determined the resulting height of the SBs; the lengths of the middle and short rows of stereocilia of the OHC SBs changed less along the cochlea. Lengths of the middle rows of the OHC stereocilia changed linearly from the base to the apex: from 0.71 to 1.33 μm (OHC1), from 0.64 to 1.52 μm (OHC2), and from 0.90 to 1.75 μm (OHC3). Length of the shortest stereocilia was approximately 0.5 μm for all

FIG. 5. Mean values $\pm$ SE and approximations for the LR and bundle parameters (cf. Fig. 2, Supplemental Table 2): **A** widths of the gaps between adjacent bundles in the same row in μm (cf. Fig. 2f); **B** distances between the SB rows in micrometers (cf. Fig. 2e); **C** SB inner angles in degrees (cf. Fig. 2h); **D** SB heights in micrometers (cf.

Fig. 4). Here and in the following figures, the x-axis also shows measurement points (*lower*) and corresponding tonotopic frequencies according to Tsuji and Lieberman (1997) (*upper*).

three OHC rows at P1. Longitudinally, the length increased toward the apex; to $0.74 \mu\text{m}$ (OHC1), to $0.76 \mu\text{m}$ (OHC2), and to $1.26 \mu\text{m}$ (OHC3). For the IHCs, the lengths of the middle and shortest rows were constant along the cochlea; at approximately 1.2 and $0.77 \mu\text{m}$, respectively.

Diameters of a single stereocilium were measured (Fig. 3) at the widest point (near the top; D_{max}) and at the narrowest point (the point of coupling to the CP, bottom of the rootlet; D_{min}). D_{min} measured around $0.1 \mu\text{m}$ in all rows for both types of hair cell. D_{max} for the IHC stereocilia did not vary longitudinally and stayed constant at approximately $0.37 \mu\text{m}$ for the tallest stereocilia, $\sim 0.34 \mu\text{m}$ for the middle stereocilia, and $\sim 0.17 \mu\text{m}$ for the shortest stereocilia. For the tallest stereocilia of the OHCs, the diameter increased linearly from the base to the apex, from 0.19 to $0.28 \mu\text{m}$ (OHC1), from 0.19 to $0.33 \mu\text{m}$ (OHC2), and from 0.20 to $0.35 \mu\text{m}$ (OHC3). D_{max} of the middle stereocilia in the OHCs increased from 0.18 to $0.20 \mu\text{m}$ (OHC1), from 0.16 to $0.19 \mu\text{m}$ (OHC2),

and from 0.17 to $0.26 \mu\text{m}$ (OHC3). The shortest stereocilia had constant D_{max} diameters of about $0.15 \mu\text{m}$ in all OHC bundles.

Dimensions of the cuticular plate

Dimensions of the CP of the hair cells were measured in the radial (length) and longitudinal (width) directions relative to the LR. The CP of an IHC is oval-shaped and was approximately $6.97 \mu\text{m}$ in the longitudinal direction. The length varied between 2.27 and $3.92 \mu\text{m}$. The CPs of the OHCs exhibited large variation in size along the cochlea and also between the OHC rows at one measurement location. The OHC CPs are V-shaped in the basal region of the cochlea, triangular in the medial region and round in the apical region. The widths of CPs (Fig. 2d) were approximately constant with values around $5.5 \mu\text{m}$. The lengths (Fig. 2c) changed from 3.16 to $5.33 \mu\text{m}$ (OHC1), from 3.28 to $6.06 \mu\text{m}$ (OHC2), and from 3.48 to $6.06 \mu\text{m}$ (OHC3).

Number of stereocilia within the bundle

The number of stereocilia changed along the cochlea only in the OHCs. For the IHC, the value was constant with approximately 58 stereocilia per cell along the entire LR. OHC1 and OHC2 had approximately 106 stereocilia per cell at the basal end of the cochlea. A maximum value of approximately 117 and 116 stereocilia per cell, respectively, was reached at P3. The number decreased again thereafter; the drop rate was greater for OHC2 than for OHC1. Near the apex, the number of stereocilia per cell was 72 for OHC1 and 33 for OHC2. The same pattern of the change in the number of stereocilia but with greater decrease toward the apex was observed for OHC3, which showed an increase from 98 stereocilia per cell at P1 to 110 at P3 and a subsequent decrease to 27 at P10.

DISCUSSION

Mechanics of stereocilia plays one of the key roles in the mechano-electrical transduction process of hearing (Howard et al. 1988; Hackney and Furness 1995; Gillespie and Walker 2001; Raphael and Altschuler 2003; Fettiplace and Hackney 2006). Direct observation of mechanical responses of SBs in vivo and in situ is difficult due to limited accuracy and resolution of measurement devices in liquid environments (Langer et al. 2001; Fridberger et al. 2006). To gain further insight into mechanical behavior of stereocilia, analytical and numerical models can be used. For this purpose, knowledge of geometrical and material properties of a single stereocilium and their bundles is essential.

Previous studies have shown that the inner structure of stereocilia does not depend on their tonotopic position within the cochlea (Engström et al. 1962; Tilney and Tilney 1988). This may imply that their representative material properties (e.g., Young's modulus) are largely constant throughout the cochlea. Mechanical fluid properties are also approximately constant along the LR. Therefore, geometrical changes of the stereocilia and their arrangement into bundles could be the dominant factor which determines the variations in the mechanical properties of the bundles and fluid mechanics along the LR.

Currently, it is not yet possible to measure the structures of LR in as fine detail as it is stereocilia in the entire cochlea in vivo. The only method that provides sufficient resolution is electron microscopy; however, this technique is limited by the dehydration of tissue during preparation, which causes tissue shrinkage in all dimensions. Edge et al. (1998) demonstrated that complete dehydration of the basilar membrane and the organ of Corti using 90–

100 % alcohol leads to shrinkage of approximately 50 %. Conversely, fixation using formaldehyde causes no statistically significant changes (Edge et al. 1998). Our own investigations comparing in vivo LSM of tonotopic areas, where the size of structures allows for live microscopy (low-frequency, apical end) with fixed and dehydrated specimens, showed a similar shrinkage factor (approximately 50 %) for the LR (Yarin et al. 2006), single stereocilia, and stereociliary bundles (Yarin et al. 2007). The similarity between these shrinkage factors could mean that the LR shrinkage is homogenous in the entire organ of Corti and that the shape of its structures is largely preserved. This means that in order to create a scale model of the subreticular space on the basis of our data, all line measurements must be corrected by a factor of 2.

Fluid dynamics around the stereociliary bundles

It has been assumed that the V-shaped arrangement of the OHC stereocilia allows a directed fluid flow from the scala media toward the inner sulcus, with the gaps between the bundles acting like nozzles (Pau and Pau 2006). Performing dimensional analysis of the fluid mechanics with the bundle geometry presented in this study demonstrates, however, that a jet is not formed between the V-shaped SBs under normal physiological conditions. A plane laminar directed jet toward the inner sulcus would be formed only if the translational Reynolds number was significantly larger than one (White 1991), because this dimensionless number relates the convection to the viscous dissipation. Here, our estimate of the translational Reynolds number is based on the tip-to-tip distance between the SBs in the longitudinal direction, the mean fluid velocity, and on properties of the endolymph, which is similar to water. The tip-to-tip distance is chosen as a relevant length because the jet will spread over approximately this length until it meets a neighboring jet. Furthermore, the momentum flux is also estimated analytically using the average velocity based on the tip-to-tip distance and not on the width of the gaps between the SBs. With the values of this study, the translational Reynolds number is approximately

$$Re = \frac{\rho \cdot e}{\eta} u \cong 0.01 \cdot u [\text{mm/s}], \quad (5)$$

with the density ρ (1,000 kg/m³; estimated from water), the dynamic viscosity η (0.7 mPa s; Dahl and Kleinfeldt 1970), the tip-to-tip distance e (Fig. 2e) and the velocity u . Therefore, a jet is formed only if the velocity exceeds 100 mm/s. The basilar membrane velocities are in the order of about 10 mm/s at 100 dB SPL (Ruggero et al. 1997). The fluid velocity can be

locally accelerated by squeezing effects by approximately $\times 10$ (Pau and Pau 2006). This is still insufficient to produce a fluid jet because the essential convective effect starts to influence the flow field for values of the translational Reynolds number close to one. Furthermore, it is worth noting that the pressure loss is changing nonlinearly only with values of the translational Reynolds number larger than 1 when additional vortices produce increased energy losses, as has been observed by Frommer and Steele (1979) in macroscopic experiments.

Instead of creating fluid jets, variable gaps between the OHC SBs could play a role in fine regulation of fluid motion in the subtektorial space, especially in areas of the maximum hearing sensitivity. If fluid has to pass through the gap between the SBs, then the pressure needed to keep the flow rate constant increases with decreasing the gap width, which is a critical parameter. If the V-shaped SB is simplified to a cylinder with the axis aligned along the normal direction to the RL, then the fluid behavior between the SB rows can be analyzed by considering pressure loss over a row of cylinders, as presented by Yeom et al. (2009) for a wide range of parameters. Additional pressure loss for the fluid motion in the radial direction is caused by the Poiseuille flow because the LR and tectorial membrane are regarded as fixed walls. The relevant length, over which the pressure decays are being calculated, is the average distance between rows OHC1 to OHC2 and OHC2 to OHC3. The ratios of two drag coefficients for radial and longitudinal fluid movement calculated for different SB rows are plotted in Fig. 6. This ratio provides information about how much the resistance for the Poiseuille flow in the subtektorial space increases if hair bundles are introduced. A value higher than one,

which is observed for all locations along the cochlea, implies that it is easier for the fluid in the subtektorial space to move in the longitudinal direction rather than in the radial direction. The pressure drop increases for all locations by approximately $\times 7$ for the OHC1. However, for the OHC2 and OHC3 rows, it decreases toward the apex and the base of the cochlea reaching an absolute maximum for frequencies corresponding to maximum hearing sensitivity (minimal CAP thresholds at Fig. 6) in guinea pigs. The exact reason for the increase in the radial drag at this location is not known, but we suggest that it serves as a protective mechanism for the IHC stereocilia against excessive mechanical impact induced by enhanced fluid motion in the subtektorial space. The longitudinal flow of fluid in the subtektorial space can also facilitate fluid mechanical coupling in addition to the longitudinal elastic coupling along the tectorial membrane and, hence, contribute to enhancing the cochlear sensitivity (Russell et al. 2007). It should be noted that the auditory thresholds reported for guinea pigs differ depending on technique and strain of animals used (e.g., see Wever et al. 1963; Heffner et al. 1971; Harrison et al. 1984; Liberman and Gao 1995). What is important for the current study is that most of the authors report minimum auditory thresholds within the range of 8 to 30 kHz which corresponds to our findings.

Tonotopic specialization of the stereociliary bundles

V-shaped SBs of the OHCs could be fully characterized by its inner angle, the number of stereocilia within the bundle, and by the diameter of stereocilia. One may hypothesize that the V-shape is a specialization for making the bundle more rigid and, hence, for providing sufficient elastic coupling between the organ of Corti and the tectorial membrane. On the other hand, the number of stereocilia determines the number of ionic mechano-electrical transduction channels in the OHCs. V-shaping allows for packing more stereocilia into a single bundle than would be possible in a straight bundle. Statistically significant reverse frequency-adjusted correlation between the number of stereocilia per cell and the bundle angle (Fig. 7) confirms this suggestion. From this point of view, the bundle's V-shape might fulfill both above mentioned requirements in the presence of additional geometrical constraints, i.e., limited cell width and required gap distance between the SBs.

SB height is set by the length of the tallest stereocilia, and variations in the bundle height and other changes in the bundle geometry occur in the longitudinal, as well as in the radial, direction along

FIG. 6. Correlation between the neural hearing threshold in guinea pigs and the ratio between drag coefficients (pressure losses) of the radial and longitudinal fluid movement over rows of the OHC stereocilia. The blue line denotes CAP hearing threshold ($N=6$, error bars = σ) (right scale).

FIG. 7. Tonotopic variations of the inner angle of stereociliary bundles and the number of stereocilia per bundle in: **A** OHC1; **B** OHC2; **C** OHC3. The *numbers in the lower corner* denote the partial correlation coefficient between the angle and the number of stereocilia adjusted for frequency and its significance, respectively.

the LR. This implies that each tonotopic place along the cochlea has a respective set of the OHC bundles with a unique combination of geometrical parameters specific for a given frequency (Fig. 2). Moreover, since the tips of the tallest OHC stereocilia are imbedded into the tectorial membrane, their length uniquely specifies the height of the subreticular space in the OHC region at each tonotopic place.

Tonotopic specialization of stereocilia

Significant variations in the diameters of a single stereocilium in bundles along the LR were only observed in OHC stereocilia, and, among these, only in the tall and middle rows of stereocilia. All diameters stay approximately constant at the coupling point to the CP. These structural features of the OHC stereocilia are most likely to be explained with the supposed enhancement of both axial bending stiffness and the mechanically more important bending stiffness of the stereocilia. This could be very important for the resistance of stereocilia bundles against applied mechanical forces and pressure loads due to the fluid flow. In high-frequency regions of the LR, where these forces are the largest due to greater pressure loss over rows of OHC (Fig. 6), the bending tension on stereocilia rootlets might be reduced by the smaller length of the bundle compared to the low-frequency region.

Tonotopic variations of the zones of lamina reticularis and their structures

The OHC zone of the LR shows greater tonotopic variations in most of the parameters than the IHC zone. The only exceptions are the depths and widths of the CP and the shaft length of the tallest stereocilia, for which variations are similar in IHCs and OHCs. On the other hand, within the OHC zone, the same parameters show the similar character of variation between rows, except for the gap distance. In general, the OHC zone shows little constancy for most of the parameters which could

be associated with the specific function of OHCs in the frequency-dependent amplification.

Tonotopic variations are smaller within the IHC zone, and the most significant change was observed for the length of the tallest stereocilia. This change could reflect variations in the volume of the subreticular space and adjustment of the IHC bundle height for optimization of the fluid flow between the tectorial membrane and the SBs (for review see Lukashkin et al. 2010).

Summary

Morphometry of the LR and SBs changes along the cochlea from the oval window to the apex in longitudinal and radial directions. The LR zones show significant differences in changes of the geometrical parameters. The OHC zone shows tonotopic variation for a greater number of parameters than the IHC zones. This could be due to the frequency-dependent amplification function of the OHCs. The unique alignment of the SBs of the OHCs and IHCs associated with each frequency-dependent position along the LR creates the complex geometry of the subreticular space, with large longitudinal and radial differences influencing fluid flow.

Consideration of fluid dynamics with the parameters obtained in this study suggests that the fluid flow around the OHCs occurs mainly in the longitudinal direction, along the rows of the SBs, rather than in the radial direction. Fluid pressure drop in the radial direction varies tonotopically along the cochlea and reaches its maximum in the area of the most sensitive hearing thresholds of guinea pigs.

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