

Supporting Information

Materials and Methods

Preparation. Briefly, preparations were made from the first three turns of the mature guinea-pig cochlea. Animals (pigmented) weighed 250-500 g, had a positive Preyer's reflex, and were killed by rapid cervical dislocation under CO₂ anesthesia. Immediately after removal of the bulla (\approx 1 min postmortem), the sample was placed in ice-cooled Hanks' balanced salt solution (Sigma; supplemented with 4.1 mM NaHCO₃ and 10 mM Hepes buffer, adjusted with glucose to 320 mOsm for the first cochlear turn and 300 mOsm for the other turns; pH 7.35). The solution warmed up to room temperature (20-22°C, controlled) within the preparation time (\approx 15 min). The preparation was fixed to a custom-made support within the experimental chamber by using plasticine; the fluid volume was 27 ml.

Further requirements for a viable preparation were: (i) orderly arrangement of cells, (ii) cylindrically shaped OHCs, and (iii) RL aligned along its entire length with the focal plane of the microscope. OHCs began to swell, as detected optically with a total magnification of 400, at \approx 60 min postmortem in the first cochlear turn, and \approx 90 min postmortem in the other two turns. Swelling meant that conditions *i* and *ii* were no longer satisfied; the experiment was then terminated. Condition *iii* was always satisfied.

Intracochlear Electrical Stimulation. Electrical stimulation of the cochlea with a multitone signal was by two platinum electrodes (diameter: 0.3 mm) placed in scala vestibuli and a gold electrode in scala tympani (width: 1.1 mm), as illustrated in Fig. 1A. The gold electrode also served as a mirror for intracochlear illumination, via a light source guided with a glass fiber (diameter: 1 mm). The electric field strength per spectral point between scalae vestibuli and tympani was typically 37 V/m at low frequencies, decreasing to \approx 24 V/m at high frequencies. We estimate (*Estimation of Maximum Transmembrane Potential Change*) a maximum transmembrane potential change of

0.6 mV for the shortest (30 μm) and 1.7 mV for the longest (90 μm) OHCs. Using two electrodes in scala vestibuli enabled the extracellular electric field to be larger in the direction parallel to the principal axis of the OHC than in the orthogonal direction along the cochlea, by a factor of 9. Because of the small radial distance between OHCs compared with the distance between the electrodes, the stimulus can be assumed to be equal for all three rows of OHCs. That the electrically induced motion of the sample was not caused by either electrophoretic fluid drag or surface charges was checked by measuring the velocity of: (i) a Kleenex tissue fiber (8- μm diameter placed across the width of the cochlea in the absence of the organ of Corti) or (ii) a cotton fiber (40- μm diameter held 3 mm from one end in extracellular solution). Amplitudes were, respectively, at least 20 dB below that for the cochlear structures or less than the background noise level. Pharmacological experiments led to the same conclusion (see *Results* in text).

Velocity Measurement. The LDV was composed of a laser system (OFV-302, wavelength 633 nm, power 1 mW), equipped with a demodulator (OFV-3000, bandwidth 100 kHz), both from Polytec (Waldbronn, Germany). The laser beam was focused on the object by coupling it into the optical path of an upright microscope (Axioskop 2FS, Zeiss) via a beam splitter (AHF Analysentechnik, Tübingen, Germany), which was highly reflective only > 590 nm, but transparent for shorter wavelengths. The microscope objective was a water-immersion objective with magnification $\times 40$, numerical aperture 0.8, and working distance 3.61 mm (Zeiss Achromat). The laser spot had approximately a Gaussian profile, with full-width at $1/e^2$ of maximum power of 0.63 μm (quantified with a knife-edge method). The velocity spectrum was corrected for the measured transfer function of the LDV. Phase is positive for motion toward the microscope objective, which corresponds to motion toward scala vestibuli. The noise floor, expressed as displacement but measured as velocity, decreased from 100 pm at 480 Hz to 1 pm at 67 kHz (effective averaging time: 25 s).

The best assurance of negligible contribution of reflected light from outside the focal plane was that the standard deviation of the vibration phase of different points at

approximately the same anatomical location was less than $\approx 5^\circ$. A systematic error was unlikely because the relative interferometric reflection phase of an unwanted contribution, from an optically rough surface, is equally distributed over 2π and would induce an error with standard deviation related to the amplitude of the unwanted contribution (1).

Stimulus Signal. Maximum displacement amplitudes (at low frequencies) were of the order of 1 nm, so that a linear relationship between stimulus and response could be safely assumed, which was also verified experimentally (second and third harmonics were >30 dB below the fundamental). Therefore, a multitone signal could be safely used as the command voltage. It contained 81 frequency components between 480 Hz and 67 kHz, with equal amplitude but random phase, uniformly distributed on the interval $[0, 2\pi]$. Frequency spacing was almost logarithmic with a ratio of ≈ 1.07 between adjacent frequencies. To reduce harmonic distortion products in the measured velocity, care was taken that no frequency was within 1% of the first four harmonics of a lower frequency.

Estimation of Maximum Transmembrane Potential Change. The maximum transmembrane voltage change U_{tm} was estimated from the extracellular voltage gradient along the OHC by assuming the cell to be a voltage divider with a ratio of 1:1 between its apical and basal halves. The maximum electric field gradient between the stimulus electrodes in scalae vestibuli and tympani was 37 V/m per frequency point. Therefore, for an OHC of length L_{OHC} (in units of μm), we have $U_{tm} = 18.5 L_{OHC}$, in units of μV . The amplitude values given in the text are relative to the voltage difference between the stimulus electrodes in scalae vestibuli and tympani. However, because U_{tm} is estimated to be ≈ 1 mV (the range is from 0.6 mV for a 30- μm cell to 1.7 mV for a 90- μm cell), the 1-mV reference potential can be understood to be approximately equal to the voltage drop across the OHC basolateral wall.

The influence of the electrical impedance of the RL on the current path was neglected in this calculation because (i) the organ was small compared with the cross-sectional area through which the current passed (1 cm^2), and (ii) current could flow around the RL.

Moreover, we had no means of monitoring the extracellular potential within the organ of Corti up to the high experimental frequencies. However, the true voltage gradient in the chamber can only be smaller than our estimate, so that the estimate provides an upper bound.

Results

In-Phase Motion of TM and IHC Above CF. Examination of the high-frequency phase responses in the second and third turns (Fig. 2 *B* and *D*) indicates that in-phase motion of TM and IHC is not a salient feature of the first turn; the phase difference asymptotes to 360° (i.e., 0° modulo 360°) at high frequencies in all turns. Indeed at 24 kHz, the CF for the first-turn recording site, the IHC phase relative to the TM phase was $298 \pm 47^\circ$ ($n = 23$) and $271 \pm 44^\circ$ ($n = 16$) for the second and third turns, respectively. The asymptotic phase difference of 360° derives from the additional 180° phase roll-off of the TM relative to its underlying IHC, which in turn presents a total phase roll-off of only 180° (Fig. 2 *B* and *D*). Expressed in terms of a filter function, the additional asymptotic phase roll-off of 180° for the TM implicates a second-order, apparently nonresonant, low-pass filter. For the second and third cochlear turns, the 90° frequency for this filter was located, on average, at 19.4 kHz ($n = 38$). The most parsimonious explanation for this high-frequency in-phase motion of IHC and overlying TM is that as frequency increases the impedances of the RL and TM tend to that of the fluid, so that the system acts as a highly coupled composite structure moving in unison. [Both the real and imaginary components of the RL impedance are known to decrease with increasing frequency (2)]. Moreover, it is expected that the pulsating mode will also vanish for excessive slip at the TM and RL, as found originally by Lloyd and Redwood (3) for the case of a narrow fluid layer between elastic plates. This interpretation concurs, of course, with the customary notion that TM and RL cannot move independently at high frequencies because of viscous coupling between their surfaces (4-6).

Discussion

Size of the Displacement at Low Frequencies. The vibration amplitudes of the OHC RL at low frequencies, typically 1-4 nm/mV with the larger values at the more apical positions, are consistent with values predicted from the following three parameters: (i) the small signal gain of the electromotility of isolated OHCs [4 nm/mV for a 30- μ m long cell from the first cochlear turn (7-9)], (ii) the axial mechanical impedance of isolated OHCs [0.017 N/m for a 30- μ m long cell from the first cochlear turn (7)], and (iii) the stiffness of the organ of Corti [0.112 N/m for the first cochlear turn (2)]. The values in parentheses yield an amplitude of 0.5 nm/mV for the first cochlear turn, if it is assumed that only the OHC at the measurement point is responsible for the motion. Clearly, the motion is correspondingly larger if a group of OHCs contribute to motion at a point. Moreover, these amplitudes are of the same order as those observed on the *in vivo* BM, as calibrated by the OHC ac receptor potential. In other words, if at neural threshold the *in vivo* iso-output tuning curves of the OHC ac receptor potential [40 μ V (10, 11)] and BM displacement [0.35 nm (5)] approximately superimpose (12), then assuming linearity at low SPL (10, 11, 13), a 1-mV transmembrane potential change is associated with a BM displacement of 9 nm.

Estimation of IHC Stereocilia Deflection for Counterphasic Motion of the TM and RL. We chose the coordinate axes, x and z , respectively, parallel and orthogonal to the plate surface, with the z -origin midway between the plates (Fig. 3B). The TM and RL are located, respectively, at $z = \delta$, and $z = -\delta$, where 2δ is the depth of the subtektorial space.

We assume linearity because the measured displacements were of the order of a nanometer, and these are much smaller than the effective thickness of the plates and their separation, which in turn are of the order of micrometers.

We neglect plate inertia because point impedance measurements on the organ of Corti suggest that it is negligible in the frequency range (<3 kHz) where counterphasic motion was observed (2).

We assume that the plates are isotropic and homogeneous, with equal flexural rigidity, D , and are without tension. The value of D for the RL in the IHC region can be readily derived from the impedance measurements of Scherer and Gummer (2); it is 0.04 nN m and 0.1 nN m for the second and third cochlear turns, respectively. Also, it will be shown that the wavenumber for fluid motion is inversely proportional to $D^{1/6}$, so that small differences in the true D values will have negligible effect on the assessment of fluid motion.

For the fluid we assume: (i) incompressible and viscous flow, (ii) zero radial velocity at both plates, (iii) the transverse velocities at the plates are equal and opposite, and (iv) wave propagation only in the positive x -direction; that is, in the direction of the inner sulcus. This latter assumption appears justified because the mechanical impedance in the opposite direction, laterally along the narrow subtektorial space, is expected to be larger than in the medial direction, which opens up into the relatively large inner sulcus.

For small amplitude displacements in an incompressible fluid, the velocity components v_x and v_z are defined by gradients of two scalar field potentials, ϕ and ψ (14):

$$v_x = -\frac{\partial \phi}{\partial x} - \frac{\partial \psi}{\partial z} \quad [2]$$

$$v_z = -\frac{\partial \phi}{\partial z} + \frac{\partial \psi}{\partial x}, \quad [3]$$

where ϕ satisfies Laplace's equation:

$$\nabla^2 \phi = 0, \quad [4]$$

ψ satisfies the heat equation:

$$\frac{\partial \psi}{\partial t} = \frac{\mu}{\rho} \nabla^2 \psi, \quad [5]$$

and fluid pressure, p , is given as:

$$p = \rho \frac{\partial \phi}{\partial t}. \quad [6]$$

The coupling of the elastic motion of the TM plate into fluid motion is defined by the boundary condition:

$$D \frac{\partial^4 \eta}{\partial x^4} = p - 2\mu \frac{\partial v_z}{\partial z}, \quad [7]$$

where $\eta(x, \delta, t)$ is the (transverse) displacement of the TM.

For transverse velocity at the TM given as $v_z(x, \delta, t) = V \exp(j(kx - \omega t))$, where V is the velocity amplitude and k is the (complex-valued) wavenumber for traveling-wave propagation in the x -direction, solution by separation of variables gives:

$$v_x(x, z, t) = V \frac{(\cosh kz \cosh l\delta - \cosh k\delta \cosh lz)}{(\sinh k\delta \cosh l\delta - \frac{k}{l} \cosh k\delta \sinh l\delta)} \exp(j(kx - \omega t + \pi/2)) \quad [8]$$

$$v_z(x, z, t) = V \frac{(\sinh kz \cosh l\delta - \frac{k}{l} \cosh k\delta \sinh lz)}{(\sinh k\delta \cosh l\delta - \frac{k}{l} \cosh k\delta \sinh l\delta)} \exp(j(kx - \omega t)), \quad [9]$$

where

$$l^2 = k^2 - \frac{j\omega}{\mu/\rho}, \quad [\mathbf{10}]$$

and k , which is derived from the boundary condition Eq. 7, is related to ω according to the transcendental equation:

$$Dk^5 \left(\tanh k\delta - \frac{k}{l} \tanh l\delta \right) = \rho\omega^2. \quad [\mathbf{11}]$$

Experimentally, we found that $|k\delta| \ll 1$. Then, according to Eq. 10, an upper bound for $|l\delta|$, is 0.27 in the first turn (evaluated at 3 kHz and $\delta = 2 \mu\text{m}$), and 0.28 in the third turn (evaluated at 0.8 kHz and $\delta = 4 \mu\text{m}$). Therefore, to a first-order approximation, we can approximate the hyperbolic functions in Eqs. 8 and 9 by the first two terms of their Taylor series expansion. Then, the velocities simplify to:

$$v_x(x, z, t) = \frac{3}{2} \frac{V}{k\delta} (1 - (z/\delta)^2) \exp(j(kx - \omega t + \pi/2)) \quad [\mathbf{12}]$$

$$v_z(x, z, t) = \frac{3}{2} \frac{Vz}{\delta} \left(1 - \frac{1}{3} (z/\delta)^2\right) \exp(j(kx - \omega t)). \quad [\mathbf{13}]$$

Notice that the depth dependence of the radial velocity is parabolic, whereas that of the transverse velocity is cubic. Most importantly, because we found experimentally that $|k\delta| \ll 1$, Eq. 12 means that the radial fluid velocity is amplified relative to transverse TM (and RL) velocity throughout most of the subtectorial space. (Clearly, radial velocity is zero near the TM and RL, because of the nonslip boundary condition.)

Likewise, approximating the tanh functions in Eq. 11 with the first two Taylor series terms, we obtain:

$$k = j^{1/6} \left(\frac{3\mu\omega}{D\delta^3} \right)^{1/6} . \quad [14]$$

The term $j^{1/6}$ has six complex-valued roots, but only two yield a wave traveling in the positive x -direction, whose amplitude does not increase with x ; namely, $j^{1/6} = 0.97 + j0.26$, and $j^{1/6} = 0.26 + j0.97$. However, the second of these roots yields a wave that decays to $1/e$ within ≈ 1.5 cell diameters and is therefore not a viable solution. This completes the derivation of Eq. 1. Notice that because k is complex-valued, radial fluid velocity does not lead transverse TM velocity by 90° , as it does for inertial fluid flow, but rather by 75° .

To obtain the deflection of the tip of the IHC stereocilia, we applied the analysis of Billone and Raynor (15), who derived the viscous drag on a stereocilium for Couette fluid motion in the subtektorial space. In their analysis, the velocity field is derived from the relative radial motion between the TM and RL. In our analysis, the velocity field is generated by the counterphasic motion of the TM and RL. As presented above (Eqs. 12 and 13), counterphasic motion results in a radial component that is much larger than the transverse component. Thus, the present analysis differs from ref. 15 only in the form of the velocity field: here, the velocity field is a parabolic function of vertical position in the subtektorial space (Eq. 12). Assuming as in ref. 15 that the stereocilium acts as an ideal, frictionless and massless, clamped cylindrical beam of uniform cross section and modulus of elasticity, one can readily show that the displacement of the tip of the IHC stereocilium is given by:

$$s_{tip}(t) = \xi_m |s_r| \exp(j(-\omega t + \pi + \phi_r)) , \quad [15]$$

where ξ_m is the amplitude of the radial fluid displacement, and $|s_r|$ and ϕ_r denote, respectively, the amplitude and phase of the function:

$$s_r = \frac{L^2}{\delta^2} \left\{ \left(2 \frac{\delta}{L} - 1 \right) - \frac{2}{\theta^2 L^2} \left[\frac{\theta \delta (\sin \theta L + \sinh \theta L) - \sin \theta L \sinh \theta L}{1 + \cos \theta L \cosh \theta L} \right] \right\}, \quad [16]$$

where the argument of the trigonometric and hyperbolic functions is

$$\theta L = j^{1/4} q L \quad [17]$$

with

$$q = \left(\frac{8\pi c \mu \omega}{D_S} \right)^{1/4}, \quad [18]$$

where L and D_S are, respectively, the length and flexural rigidity of a stereocilium; c is a constant depending on the ratio of stereocilium radius to distance between the centers of adjacent stereocilia, which for the IHC stereocilia is $c \approx 4$ (15). For calculations, we used $D_S = 9 \times 10^{-21} \text{ Nm}^2$ (derived from ref. 16).

To relate the phase of tip displacement to radial fluid displacement, integrate Eq. 12 with respect to time; for a given kx , one obtains a phase of $(-\omega t + \pi - \pi/6)$, where $\pi/6$ is the phase of k . For the transverse fluid displacement, the phase is $(-\omega t + \pi/2)$. Then, the phase of the tip displacement is $\phi_r + \pi/12$ relative to radial fluid displacement, or $\phi_r + \pi/2$ relative TM displacement.

Notice that at low frequencies, $s_r \approx -j \frac{(qL)^4}{8} \frac{L^2}{\delta^2} \left(2 \frac{\delta}{L} - 1 \right)$, meaning that ϕ_r tends to $-\pi/2$ and $|s_r|$ decreases with a slope of 6 dB/oct. This asymptotic low-frequency phase value means that the tip displacement is in-phase with TM displacement at low frequencies. As frequency is increased, ϕ_r tends to zero, the stereocilia becoming “softer” with the

stereocilia just following the radial fluid motion, so that the phase of stereocilia displacement leads TM displacement by $\pi/2$.

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