A quantitative comparison of mechanoelectrical transduction in vestibular and auditory hair cells of neonatal mice

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SUMMARY

Vestibular hair cells (VHCs) and cochlear outer hair cells (OHCs) of neonatal mice were stimulated by a fluid jet directed at their stereociliary bundles. Relations between the force exerted by the jet, bundle displacement, and the resulting transducer current were studied. The mean maximum transducer conductance in VHCs (2.6 nS) was about half that of the OHCs (5.5 nS), with the largest recorded values being 4.1 nS and 9.2 nS, respectively. In some OHCs activity of a single, 112 pS transducer channel was observed, allowing an estimate of the maximum number of channels: up to 36 in VHCs and 82 in OHCs, corresponding to about one transducer channel per tip link. The VHC bundles required about 330 nm of tip displacement to activate 90% of the maximum transducer conductance, compared to 150 nm for the OHC bundles. This corresponded to 2 deg of rotation about their pivots for both, due to the greater length of the VHC bundles. The VHC bundles' translational stiffness was one-seventh of that of the OHCs. Conversion to rotational stiffness almost abolished this difference. Rotation of the hair bundle rather than translation determines the gating of the transducer channels, independent of bundle height or origin of the cells.

1. INTRODUCTION

The vertebrate inner ear comprises the auditory and vestibular systems. Both contain hair cells that transduce appropriate stimuli (sound in case of the cochlea, linear acceleration in the sacculus and utricle, rotational velocity in the semicircular canals) into a receptor potential. These cells possess receptive organelles, the stereociliary (or hair) bundles, made up of tens to hundreds of stereocilia in several rows, and a single kinocilium of unknown function which perishes upon maturation in mammalian cochlear hair cells but remains in VHCs. When the bundles are deflected the stereocilia behave as stiff rods that rotate around the points of insertion of their rootlets into the cuticular plate at the cell's apex (Crawford & Fettiplace 1985), and the flow of current through mechano-electrical transducer channels is altered. These channels are probably located near the top of the bundle (e.g. Hudspeth 1982; Denk *et al.* 1995) and may be gated by 'tip links' (Pickles *et al.* 1984; Pickles & Corey 1992) or areas of membrane apposition just below the tip links (Hackney & Furness 1995; Furness *et al.* 1997)

between adjacent stereocilia in neighbouring rows. Mechanisms of vestibular transduction have been most extensively investigated in the sacculus of the bullfrog (e.g. Hudspeth & Corey 1977; Hudspeth 1989). In mammals, the relation between bundle displacement and transducer currents has been studied in cochlear (Kros *et al.* 1993, 1995; Ashmore *et al.* 1993), but not vestibular hair cells.

Here we present a direct comparison, using the same experimental procedures, of the transduction process in vestibular and cochlear hair cells from neonatal mice. Relations between the inferred force applied to the bundle, hair bundle position and transducer currents are substantially different in hair cells from different origins. We attribute these differences to diversity in the structure of the hair bundles. Preliminary reports of parts of this work have been published (Lennan *et al.* 1996; Lennan & Kros 1996).

2. METHODS

(a) Tissue culture

Organotypic cultures were prepared from utricular and saccular maculae or cochleae of one- to two-day-old mouse pups. Culturing procedures were derived from those developed for the organ of Corti by Russell & Richardson

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(1987). Mouse pups were killed by cervical dislocation and surface sterilized by immersion in 80% ethanol for 10 min. The heads were removed and placed in HEPES-buffered (10 mM, pH 7.2) Hanks's balanced salt solution (HBSS, Sigma, UK). Sensory epithelia were dissected out. The otoconial membrane of the vestibular organs was removed prior to explantation onto a collagen-coated glass coverslip. The preparation was fed with 50 μ l medium containing eight parts Eagle's minimum essential medium (MEM) with Earle's salts (Sigma, UK), one part mouse embryo extract and one part horse serum, buffered with 10 mM HEPES, pH 7.2. It was then enclosed in a Maximow depression slide assembly and incubated at 37 °C for a period of 1–4 d.

For the experiments, the collagen-coated coverslip with the adhering cultures was transferred to a chamber with a volume of 1.1 ml. This chamber was perfused with a peristaltic pump at a flow rate of 7 ml h⁻¹. The composition of the extracellular solution was 137 mM NaCl, 0.7 mM NaH₂PO₄, 5.8 mM KCl, 1.3 mM CaCl₂, 0.9 mM MgCl₂, 5.6 mM D-glucose, 10 mM HEPES-NaOH, and its pH was 7.5. Amino acids and vitamins for Eagle's MEM were added from concentrates (Gibco, UK).

Cultures were observed and photographed using a Zeiss ACM (Germany) top-focusing microscope equipped with Nomarski differential interference contrast (DIC) optics ($\times 40$ water immersion objective and $\times 16$ eyepieces). For the scanning electron microscopy (SEM), some explants were prepared as described by Forge & Richardson (1993) and observed with a Leica S420 (Germany) scanning electron microscope.

(b) Electrophysiological recording and mechanical stimulation

Cells were voltage-clamped in the configuration whole-cell patch clamp using an Axopatch 200A amplifier (Axon Instruments, USA). Patch pipettes drawn from soda glass capillaries (Clark Electromedical Instruments, UK) were coated with ski wax (Astra-Gruppen, Norway) to reduce electrode capacitance. Their resistance in the extracellular solution was 2–3 M Ω . Patch pipettes were filled with 148 mM CsCl, 0.1 mM EGTA-NaOH, 3.5 mM MgCl₂, 2.5 mM Na₂ATP, 5 mM HEPES-NaOH; pH 7.3. In some OHCs the composition was 135 mM CsCl, 5 mM EGTA-NaOH, 0.1 mM CaCl₂, 3.5 mM MgCl₂, 2.5 mM Na₂ATP, 5 mM HEPES-NaOH; pH 7.3. Experiments were performed at room temperature (22–25 °C).

The membrane capacitance of vestibular hair cells used in this study was 4.9 ± 0.9 pF ($n = 7$, mean $\pm$ s.d.), which was not significantly different (two-tailed *t*-test) to that of the OHCs: 5.2 ± 0.4 pF ($n = 11$). In this paper, means are given $\pm$ s.d., and $p < 0.05$ in a two-tailed *t*-test is taken as the criterion for statistical significance. No correction was made for the voltage drop across the residual series resistance after compensation, which was at most 3 mV. The linear leak conductance at the holding potential of -84 mV (adjusted for a -4 mV liquid junction potential measured between pipette and bath solutions) was typically 0.5–1.0 nS, including the resting transducer conductance.

To expose the basolateral surface of the cells, a small tear was made in the epithelium using a suction pipette (tip diameter 5 μ m) filled with the normal extracellular solution. The cells were cleaned with the same instrument before gigaohm seals were applied to the basolateral surface. Hair bundles were mechanically stimulated by a fluid jet driven by a piezoelectric disc (Kros *et al.* 1992). The fluid jet was brought to the hair cell through a pipette and applied along the bundle's axis of bilateral symmetry, either from the side of the kinocilium (still present in neonatal cochlear hair cells)

or opposite, from the side of the shorter stereocilia. The angle of the pipette to the horizontal was 20 deg, and its tip (diameter 7–11 μ m) was placed 10–15 μ m from the hair bundle. Mechanical stimuli were force steps of 50 ms duration.

The stiffness of the hair bundles was estimated as follows (figure 1*d*). The motion of the hair bundles appears to be predominantly determined by viscous fluid forces under our experimental conditions (see comments following Kros *et al.* 1993). Although few analytical equations exist for viscous forces on rigid bodies, they have been worked out for spheres and spheroids (Happel & Brenner 1965), as an extension of Stokes's law:

$$F = 6\pi\mu R_{\text{eff}}V, \quad (1)$$

where F is the force, μ is the viscosity of the extracellular solution (taken as 10^{-3} Pa s at room temperature), R_{eff} is the effective radius of the spheroid (equal to the radius, R , in case of a sphere), and V is the linear undisturbed driving velocity of the fluid. This calculation does not take possible effects of the proximity of the apical surface of the hair cell into account. For fluid motion perpendicular to the axis of symmetry of a prolate spheroid:

$$R_{\text{eff}} = \frac{8a}{3} \cdot \left\{ \frac{1}{\left[\frac{\phi}{\phi^2 - 1} + \frac{2\phi^2 - 3}{(\phi^2 - 1)^{3/2}} \cdot \ln(\phi + \sqrt{\phi^2 - 1}) \right]} \right\}, \quad (2)$$

where $\phi = c/a$ (the elongated semiaxis, c , divided by the radius of the largest circular cross-section, a). The fluid velocity was estimated as 8.6×10^{-4} m s⁻¹ per Volt driver voltage to the jet, by driving one end of a glass fibre of known stiffness and a radius of 3.5 μ m with a jet pipette of 4.5 μ m radius, and treating the fibre as a prolate spheroid with a 3.5 μ m radius, a , and a 4.5 μ m elongated semiaxis, c (equations (1) and (2)). The OHC bundles were then modelled as prolate spheroids with a 2.2 μ m radius a (half the mean bundle height of the eight cells tested, all of which came from the apical coil of the cochlea, where the stereocilia are tallest) and a 2.5 μ m elongated semiaxis, c (half the bundle width). For VHC bundles, a was taken to be 1.5 μ m (half the bundle width) and c 4.8 μ m (half the mean bundle height, five cells). So for the OHCs, the elongated semiaxis is parallel to the cells' apical surface, for the VHCs it is perpendicular (figure 1*d*). The height of each bundle was measured as that of the tallest stereocilia; the kinocilium was not taken into account. According to equation (2), R_{eff} was 2.32 μ m for the OHCs and 2.69 μ m for the VHCs. From equation (1), the forces exerted by the fluid jet on the bundles were calculated. These forces centre halfway down the bundle, and, analogous to Kros *et al.* (1992), the equivalent force that would have to be applied by a fibre attached to the top of the bundle to exert an equal torque about the pivot on the cell's surface would be one half of that provided by the jet. Forces on the bundle are given in this paper as the equivalent forces at the tips of the hair bundles, and were 1.89×10^{-11} N per Volt driver voltage for OHCs, and 2.19×10^{-11} N per Volt for VHCs. This analysis, though still quite approximate, is more appropriate for a viscously driven system than that described in Kros *et al.* (1992), where the force exerted by the jet on the bundle was simply assumed to be proportional to the area of the bundle facing the jet. Using the original analysis, identical qualitative conclusions about differences in stiffness between VHCs and OHCs to those reported here would have been reached, but absolute stiffness estimates of the hair bundles would have been 30% smaller for the VHCs and 34% smaller for the OHCs. To correct for any static inflow or outflow from the stimulus pipette, the resting current was

recorded before approaching the cell with the pipette tip. The driver voltage for the fluid jet was low-pass filtered at 0.5 kHz (f_{-3dB} , 8 pole Bessel).

(c) Optical measurement of bundle displacements

The experimental set-up used for the measurement of bundle movements has been developed previously by Kros *et al.* (1993, 1995). An image of the top of the bundle, illuminated by a bright laser spot, was projected onto a pair of photodiodes through the Nomarski optics of the microscope. The calibration procedure has been described before (Kros *et al.* 1993, 1995). Briefly, calibration was performed by scanning the photodiodes and the laser illumination in concert across the bundle over a range of 800 nm. The scan gave photovoltage as a near-linear function of bundle position. The slope of this relation was used for conversion of photovoltages generated during experiments into bundle displacements. The quoted bundle displacements were likely to be underestimated by between about 16% for a 4 μ m tall OHC bundle, and 8% for a 10 μ m VHC bundle, due to the limits imposed by the optical sectioning ability of the interferometer (G. W. T. Lennan, G. S. G. Géléoc & C. J. Kros, in preparation). No correction has been applied for this source of error. After most experiments, controls were performed, obtaining an optical signal from the edge of the cuticular plate to ensure that only the bundle, and not the whole cell, was displaced by the jet. No movement could be detected at the level of the cuticular plate in case of the VHCs; in the OHCs it was about 10% (maximum 22%) of the movement at the tip of the bundle. Values quoted for bundle movements include this potential overestimate. Signals from the photodiode pair, as well as the membrane currents and the voltage across the piezoelectric disc driving the fluid jet, were low-pass filtered at 2.5 kHz (f_{-3dB} , 8 pole Bessel) and digitized at 5 kHz before storage on hard disk. Data acquisition and analysis were performed using ASYST software (Keithley Instruments Inc., USA). Curve fitting was done with the programme FigP (Biosoft, UK), using linear regression or a Levenberg–Marquardt nonlinear least-squares algorithm.

3. RESULTS

(a) Morphology of the vestibular cultures

Vestibular and cochlear cultures were observed by DIC optics and SEM to assess the condition of the hair cells. The morphology of neonatal cochlear, but not vestibular, cultures has been illustrated before (e.g. Russell & Richardson 1987; Furness *et al.* 1989). Each vestibular culture contained about 300 VHCs, with their stereociliary bundles separated by 6–12 μ m (figure 1*a*). Only preparations with well-maintained hair bundles were used for the mechano-electrical transduction studies (figure 1*b*). Bundle dimensions seemed quite uniform under the light microscope, being about 3 μ m wide where they insert into the cuticular plate, and 8–12 μ m tall (excluding the much taller kinocilium). Under SEM, the hair bundles generally appeared well preserved, with their stereocilia arranged in rows increasing in height towards the kinocilium. The arrangement of the stereocilia on a VHC after 3 d *in vitro* is shown in figure 1*c*. SEM revealed a number of hair cells with extremely short

bundles and very slender stereocilia: presumably these bundles were immature, and they were not evident under the light microscope. There were no obvious differences between utricular and saccular hair bundles at this stage, which is why results from both epithelia were pooled.

VHC bundles contained 38 ± 7 ($n = 15$) stereocilia, with 31 ± 6 apposition sites between neighbouring stereocilia in different rows, which is where the tip links are situated. By comparison, neonatal OHCs from the mid-apical coil ($n = 6$) had 129 ± 6 stereocilia in four rows with 97 ± 4 potential tip link sites. The mean stereociliary diameter of VHCs (232 ± 59 nm, $n = 15$) was larger than that of OHCs (145 ± 12 nm, $n = 6$, $p < 0.001$). Nevertheless, the spacing of adjacent stereociliary rootlets between rows was similar for the VHCs (260 ± 37 nm, $n = 13$) and OHCs (253 ± 10 nm, $n = 6$), and appeared, as for mature guinea-pig OHCs (Furness *et al.* 1997), not to vary much between different rows.

(b) Transducer currents as a function of bundle displacement

When hair bundles were stimulated with force steps of 50 ms duration, transducer currents were observed in seven VHCs and eight OHCs. In all cells, with the exception of two of the VHCs, bundle movements were recorded simultaneously. Families of bundle movement records and matching transducer currents are shown for a VHC in figure 2*a*, and for an OHC in figure 2*c*. Excitatory bundle movements (in the direction of the kinocilium) elicited inward transducer currents, inhibitory movements (in the opposite direction) shut off the small fraction of the current flowing at rest. Bundles of both types of hair cell responded with a rapid initial movement with a time-course similar to the force step, followed by a slower further relaxation to a steady position.

The relationship between transducer current and bundle displacement was measured just after the completion of the rapid initial phase, and is plotted as the current–displacement transfer functions of figure 2*b, d*. The shapes of the current–displacement relationships were sigmoidal for both cells, and were best fitted with second-order Boltzmann curves, pointing to at least two closed states and one open state of the transducer channels:

$$C_1 \xrightleftharpoons[\beta_1]{\alpha_1} C_2 \xrightleftharpoons[\beta_2]{\alpha_2} 0. \quad (3)$$

Then, the probability, p_o , of the channel being open is given by:

$$p_o = \frac{1}{1 + K_2(1 + K_1)}, \quad (4)$$

where

$$K_{1,2} = \frac{\beta_{1,2}}{\alpha_{1,2}} = \exp(\alpha_{1,2}(x_{1,2} - x)). \quad (5)$$

Figure 1. Views of organotypic vestibular cultures at different magnifications. (a) Utriculus from a two-day-old mouse after two days in culture, observed with DIC optics. Scale bar, 100 μm . (b) Surface of the utricular epithelium after 3 d in culture, as seen with the experimental microscope. Hair bundles are orientated with their kinocilia towards the striola, which runs diagonally across the picture from top left to bottom right, where a couple of otoconia can be seen. DIC optics. Scale bar, 25 μm . (c) High-magnification SEM showing the apical surface of a saccular hair cell after three days *in vitro*. The kinocilium has become detached from the stereocilia. Scale bar, 1 μm . (d) The model used to estimate the fluid forces on the hair bundles. VHC and OHC bundles were modelled as prolate spheroids of different orientation (see text). The elongated semiaxis, c , is 4.8 μm for the VHC and 2.5 μm for the OHC; the radius, a , of the largest circular cross-section (dotted line) is 1.5 μm for the VHC and 2.2 μm for the OHC. The line segments on which the bundles rest represent the apical surfaces of the hair cells. Fluid flow is perpendicular to the plane of the page.

$K_{1,2}$ are the equilibrium constants of the two transitions; $x_{1,2}$ are the displacements at which the set points of transition between states are found; $a_{1,2}$ are the displacement sensitivities of the transitions (Crawford *et al.* 1989, 1991).

It can be seen that the VHC has a smaller maximum transducer current than the OHC, and that larger movements of the tip of the hair bundle are required to elicit a given fraction of the maximum current. These differences in current size and operating range between the two cell types were consistently present, as demonstrated by the averaged transfer functions of five VHCs and eight OHCs shown in figure 3a, and the parameters of table 1 (transfer functions of another VHC and OHC that contributed to the average are shown in Lennan *et al.* (1996) and Kros *et al.* (1995), respectively). Currents have been converted into conductances, dividing them by an electrical driving

force of 87 mV, using a reversal potential of +3 mV previously found under similar ionic conditions (Kros *et al.* 1992). This allows for more direct comparison with data from other species where different holding potentials have been used.

The various transformations of the averaged transfer functions in figure 3b, 3c and 3d demonstrate the main reasons for the differences in transduction properties of VHCs and OHCs. Figure 3b presents the transfer functions normalized with respect to the maximum conductance. VHCs are clearly less responsive to displacement than OHCs. In figure 3c displacements are converted to rotations about the pivot at the apical surface of the cell, according to:

$$\alpha = \arcsin \frac{d}{h} \quad (6)$$

Figure 2. Mechano-electrical transduction in a VHC (*a, b*) and an OHC (*c, d*). (*a, c*) Driver voltages to the fluid jet, bundle displacements, and transducer currents are shown (single traces). Positive driver voltages and displacements are for excitatory stimuli, which elicit negative, inward transducer currents. Zero current is taken as the holding current in the absence of a force stimulus. Note the difference in the scale of the driver-voltage records. The VHC responds to smaller driver voltages (and hence fluid velocities) with larger displacements but smaller currents. $V_{\text{hold}} -84$ mV. Bundle height 10 μm (VHC), 4.5 μm (OHC). (*b, d*) Transducer currents as a function of bundle displacement (both measured around $t = 4$ ms). Note the different scales of the ordinates in *b* and *d*, and that the currents have been inverted. Here, zero current is set as the holding current when the force stimulus closes the transducer channels. Curves fitted to equations (4) and (5). VHC: $a_1 = 0.054 \text{ nm}^{-1}$, $a_2 = 0.0072 \text{ nm}^{-1}$, $x_1 = 43.2 \text{ nm}$, $x_2 = 166 \text{ nm}$, $I_{\text{max}} = 355 \text{ pA}$. OHC: $a_1 = 0.092 \text{ nm}^{-1}$, $a_2 = 0.038 \text{ nm}^{-1}$, $x_1 = 8.2 \text{ nm}$, $x_2 = 49.0 \text{ nm}$, $I_{\text{max}} = 462 \text{ pA}$.

where α , expressed in degrees, is the rotation of the hair bundle, d is the displacement of the tip of the hair bundle, and h is the bundle height.

Now it can be seen that, although the maximum conductance is smaller in the VHCs, the rotational operating range is comparable to that of the OHCs. This is even more evident in figure 3*d*, where

conductance normalization has been employed again, and where there was no significant difference between any of the parameters of the Boltzmann functions for VHCs and OHCs. The main remaining difference appears in the fraction of the maximum conductance activated at rest. There is no obvious explanation for this—note that there was no significant difference in

Figure 3. Various transformations of averaged transfer functions of five VHCs (solid lines) and eight OHCs (dashed lines). (a) Transducer conductance as a function of bundle displacement. (b) As above, but transducer conductance normalized with respect to the maximum available conductance (VHCs: 2.8 nS; OHCs: 5.5 nS). (c) Transducer conductance as a function of bundle rotation. (d) As above, but conductance normalized with respect to maximum. Averaged transfer functions were obtained as follows: for each cell, transducer conductance was plotted as a function of bundle displacement or rotation. Second-order Boltzmann functions were fitted, and the parameters $a_{1,2}$, $x_{1,2}$ (with respect to either translation or rotation) and $g_{\max}$ were noted. The arithmetic means of the parameters were determined and used to construct the transfer functions. (a, b) VHC: $a_1 = 0.049 \pm 0.028 \text{ nm}^{-1}$, $a_2 = 0.010 \pm 0.003 \text{ nm}^{-1}$, $x_1 = 67 \pm 39 \text{ nm}$, $x_2 = 92 \pm 55 \text{ nm}$, $g_{\max} = 2.8 \pm 1.2 \text{ nS}$. OHC: $a_1 = 0.092 \pm 0.051 \text{ nm}^{-1}$, $a_2 = 0.025 \pm 0.011 \text{ nm}^{-1}$, $x_1 = 19 \pm 11 \text{ nm}$, $x_2 = 45 \pm 22 \text{ nm}$, $g_{\max} = 5.5 \pm 1.5 \text{ nS}$. (c, d) VHC: $a_1 = 7.8 \pm 3.8 \text{ deg}^{-1}$, $a_2 = 1.7 \pm 0.7 \text{ deg}^{-1}$, $x_1 = 0.39 \pm 0.22 \text{ deg}$, $x_2 = 0.54 \pm 0.30 \text{ deg}$, $g_{\max} = 2.8 \pm 1.2 \text{ nS}$. OHC: $a_1 = 6.8 \pm 3.4 \text{ deg}^{-1}$, $a_2 = 1.9 \pm 0.8 \text{ deg}^{-1}$, $x_1 = 0.25 \pm 0.14 \text{ deg}$, $x_2 = 0.58 \pm 0.27 \text{ deg}$, $g_{\max} = 5.5 \pm 1.5 \text{ nS}$.

this measure in the population of tested cells, when one outlier was excluded (see table 1). This VHC was, however, included in the calculation of the mean transfer functions.

Various measures of the performance of the transduction mechanism of VHCs and OHCs are presented in table 1. The VHCs tested in this study have taller hair bundles than the OHCs. The maximum transducer conductance in the VHCs is only about half that of the OHCs. The operating range of the transducers is

characterized as the translation or rotation at which 50% or 90% of the maximum available conductance is activated. The difference in responsiveness between VHCs and OHCs with respect to translation disappears when rotation is taken as the criterion. The maximum sensitivity (the slope of the transfer function) occurs for small perturbations of the bundle in the excitatory direction ($69 \pm 45 \text{ nm}$ or $0.40 \pm 0.25 \text{ deg}$ for the five VHCs; $31 \pm 20 \text{ nm}$ or $0.39 \pm 0.22 \text{ deg}$ for the eight OHCs). The OHCs appear more sensitive than

Table 1. *A comparison of mechano-electrical transduction properties of VHCs and OHCs*

(Note: n.s. = not significantly different, i.e. $p > 0.05$. In five VHCs and eight OHCs transducer currents and bundle movement were recorded. Two additional VHCs for which bundle displacement records were not available could be used for some measures. In these cases, n (the number of cells used), is indicated next to the measure.)

	five VHCs:	eight OHCs:	<i>t</i> -test (two-tailed):
bundle height	$9.3 \pm 1.0 \mu\text{m}$ ($n = 7$)	$4.4 \pm 0.4 \mu\text{m}$	$p < 0.001$
g_{max}	$2.6 \pm 1.1 \text{ nS}$ ($n = 7$)	$5.5 \pm 1.5 \text{ nS}$	$p < 0.001$
% g_{max} at rest:	$4.7 \pm 1.4 \%$ ($n = 6$)	$5.8 \pm 1.2 \%$	n.s.
50 % g_{max} at:	$113 \pm 50 \text{ nm}$	$50 \pm 21 \text{ nm}$	$p < 0.05$
	$0.66 \pm 0.27 \text{ deg}$	$0.65 \pm 0.23 \text{ deg}$	n.s.
90 % g_{max} at:	$326 \pm 91 \text{ nm}$	$151 \pm 51 \text{ nm}$	$p < 0.01$
	$2.0 \pm 0.6 \text{ degrees}$	$2.0 \pm 0.7 \text{ degrees}$	n.s.
max. translational sensitivity	$21 \pm 16 \text{ pS nm}^{-1}$	$69 \pm 35 \text{ pS nm}^{-1}$	$p < 0.01$
max. rotational sensitivity	$3.4 \pm 2.0 \text{ nS deg}^{-1}$	$5.1 \pm 2.2 \text{ nS deg}^{-1}$	n.s.
dynamic stiffness	$0.80 \pm 0.56 \text{ mN m}^{-1}$	$5.6 \pm 2.2 \text{ mN m}^{-1}$	$p < 0.001$
	$7.3 \pm 5.1 \times 10^{-14} \text{ Nm rad}^{-1}$	$10.8 \pm 4.2 \times 10^{-14} \text{ Nm rad}^{-1}$	n.s.
steady-state stiffness	$0.67 \pm 0.53 \text{ mN m}^{-1}$	$4.5 \pm 1.9 \text{ mN m}^{-1}$	$p < 0.001$
	$6.2 \pm 4.9 \times 10^{-14} \text{ Nm rad}^{-1}$	$8.6 \pm 3.7 \times 10^{-14} \text{ Nm rad}^{-1}$	n.s.
transducer channels	23 ± 10 ($n = 7$) (36 max.)	49 ± 13 (82 max.)	$p < 0.001$

Figure 4. Force exerted on the bundle versus displacement for a VHC (*a*) and an OHC (*b*; same cell as figure 2*c*, *d*). The relation between force and displacement was determined at 4 ms (dynamic, closed circles) and 45 ms (steady-state, open circles) following the start of the force steps. The slope of this relation equals the stiffness. Note the nonlinear stiffness–displacement relation for the VHC: the excitatory stiffness (dynamic, 1.58 mN m^{-1} ; steady-state, 1.44 mN m^{-1}) is smaller than the inhibitory (dynamic, 2.25 mN m^{-1} ; steady-state, 1.86 mN m^{-1}). The OHC's stiffness was linear (dynamic, 6.78 mN m^{-1} ; steady-state, 5.44 mN m^{-1}). Bundle heights: $8 \mu\text{m}$ (VHC); $4.5 \mu\text{m}$ (OHC).

the VHCs, though not significantly so with respect to rotation.

(c) Stiffness of the hair bundles

Much smaller force steps were required to displace the VHC bundles than we usually employ for OHCs (see figure 2*a*, *c*), indicating the VHCs are considerably less stiff. We have attempted to quantify this difference by using pipettes of similar tip diameters for both cell types, and modelling the shape of the bundles as described in the Methods (§*b*). Stiffness values

corresponding to the slope stiffness (Hudspeth 1992) were calculated from plots of the force exerted by the fluid jet on the bundle versus displacement (figure 4).

The dynamic stiffness was determined 4 ms after the onset of the force step, when the rapid component of the bundle movement was completed and the slow component had hardly developed. Steady-state stiffness values, determined at 45 ms, were smaller than the dynamic stiffnesses because of the slow component of bundle relaxation ($p < 0.01$ for the OHCs, not significant; i.e. $p > 0.05$ for the VHCs; paired-sample *t*-test).

While the translational stiffness (K) was almost an order of magnitude smaller in the VHCs than in the

Figure 5. Activity of a single transducer channel of an OHC. (a) Examples of channel openings (inward currents) during excitatory stimuli. The driver voltage to the fluid jet (dv, 25 V) is shown above; below that is the bundle movement recorded simultaneously with the first of the current records. The ensemble average (from 23 such current records, including those shown) gives p_o as a function of time. (c) The same for inhibitory stimuli (−25 V), which were presented to the cell interleaved with the excitatory steps. (b, d) Amplitude histograms created from the 23 current records (sign inverted). The number of observations in each bin (n) is shown as a fraction of the total number ($n_t = 5750$). Parameters for fitted Gaussians (μ = mean; σ = s.d.): (b) $\mu_1 = 0.7$ pA; $\mu_2 = 10.7$ pA; $\sigma_1 = 1.9$ pA; $\sigma_2 = 2.6$ pA; giving a single channel size of 10.0 pA, or 115 pS; (d) $\mu = 0.3$ pA; $\sigma = 1.8$ pA.

OHCs, conversion to rotational stiffness (K_R , in radians) according to:

$$K_R = K \times \bar{h}^2 \quad (7)$$

(where $\bar{h}$ is the mean bundle height: 9.6 μm for five VHCs, 4.4 μm for eight OHCs) made the difference non-significant, although the VHCs were still somewhat less stiff. This suggests the difference in stiffness was almost entirely due to the difference in bundle height.

In the OHCs, the slope stiffness was constant across the bundles' operating range (figure 4b), but the VHCs were consistently stiffer in the inhibitory than they were in the excitatory direction ($p < 0.05$ for both dynamic and steady-state stiffness, paired-sample t -test). Therefore, for VHCs, regression lines were fitted separately to the inhibitory and excitatory portions of the plots (figure 4a). The stiffness data shown in table 1 apply to the excitatory direction only in the case of the VHCs. The mean dynamic stiffness in the

inhibitory direction was $1.29 \pm 0.80 \text{ mN m}^{-1}$, or $11.9 \pm 7.4 \times 10^{-14} \text{ N m rad}^{-1}$ ($n = 5$). At steady-state, the values reduced only slightly to $1.23 \pm 0.83 \text{ mN m}^{-1}$, or $11.3 \pm 7.6 \times 10^{-14} \text{ N m rad}^{-1}$.

(d) Single-channel conductance

In three OHCs, which exhibited no apparent mechano-sensitivity, quantized excursions from the current baseline were observed which had a unitary size and directional sensitivity consistent with their being due to the activity of a single transducer channel. The currents were inward in direction at -84 mV , like the macroscopic transducer currents, and occurred predominantly during excitatory force steps (figure 5*a*). Inhibitory stimuli, by contrast, induced no channel openings (figure 5*c*).

Amplitude histograms (bin size 1 pA) were constructed for excitatory and inhibitory stimuli, as shown in figure 5*b, d*. Gaussian curves were fitted to the peaks, using the outer slopes and inner two bins surrounding the peaks for the excitatory records. This was done to exclude unresolved openings and closures which fill the trough between the two peaks (see also Crawford *et al.* 1991), presumably limited by the 2.5 kHz filtering of the current records, since the $f_{-3\text{dB}}$ due to the series resistance and membrane capacitance was between 9 and 37 kHz for the single-channel events described here. From the difference in the peaks representing the open and the closed states of the channel (as in figure 5*b*), the single-channel current was calculated as $9.7 \pm 1.2 \text{ pA}$ ($n = 3 \text{ cells}$), at -84 mV . This corresponded to a conductance of $112 \pm 13 \text{ pS}$. The opening probability (p_o) for the channel of figure 5 was 0.39 at a bundle displacement of about 40 nm . This was obtained from the areas under the two Gaussians fitted to the histogram of figure 5*b*. A similar p_o of 0.46 could be estimated directly from the ensemble average plotted above the single traces in figure 5*a*. The model OHC transfer function of figure 3*b* predicts a p_o of 0.44 for a displacement of 40 nm , suggesting the single transducer channel had the normal operating range expected if it had been surrounded by other functioning channels.

4. DISCUSSION

This study has provided a quantitative description of the relation between forces, bundle movements and transducer currents of mammalian VHCs and OHCs. The cells were situated in cultures of the sacculus, utricle and cochlea of newborn mice. Although the onset of auditory function in mice does not occur before about 11 d after birth (Romand 1983), neonatal cochlear hair cells already respond with large transducer currents to mechanical stimuli (Kros *et al.* 1992). Little is known to date about the functional development of the sacculus and utricle, but semicircular canal afferents in the rat already respond to angular acceleration stimulation at birth (Curthoys 1979), albeit with less sensitivity than in the adult. Given the general similarities between mice and rats in the timing of developmental changes, this suggests that the vestibular system may already function to some

extent in newborn mice. Differentiation into type I and type II hair cells may already have occurred just before birth (Anniko *et al.* 1979), although again this has been studied only in semicircular canals. No distinction between type I and type II hair cells could be made under our experimental conditions.

(a) Maximum transducer conductance and the number of transducer channels

At 2.6 nS , the mean value for the maximum conductance of the VHCs was only about half that of the OHCs. The largest conductance in a VHC was 4.1 nS , compared to 9.2 nS found before for an OHC (Kros *et al.* 1992). Transducer currents in vestibular systems have been reported for the frog's sacculus and the chick's sacculus and utricle. Maximum conductances of up to 4 nS have been reported for frog saccular hair cells (Holton & Hudspeth 1986; Denk *et al.* 1995). In the chick, maximum transducer conductances reported by Ohmori (1987) are typically about 0.7 nS , with a largest value of 2 nS . The size of the transducer conductance of neonatal mouse VHCs is, therefore, similar to the largest values found in non-mammalian vestibular hair cells.

The single-channel conductance for OHCs of 112 pS corresponds closely to previous observations of single transducer channels in turtle cochlear hair cells (106 pS ; Crawford *et al.* 1991). Relating the number of stereocilia responding to a saturating mechanical stimulus with a rise in their calcium concentration to the maximum transducer conductance yielded an estimate of 87 pS for the frog sacculus (Denk *et al.* 1995). This close agreement suggests that the single-channel conductance is independent of class of vertebrates and vestibular or cochlear origin of the hair cells. Therefore, the value of 112 pS was used to estimate the number of channels in VHCs and OHCs. From the mean maximum conductance, assuming the maximum opening probability is close to one, the VHCs had about 23 functioning channels, with a highest value of 36 in one cell; the OHCs had, on average, 49 channels, with a maximum of 82 (table 1). Comparing these values with the number of potential tip link or apposition sites of about 30 for VHCs and 100 for OHCs (see Results, §a), there seems to be on average less than one functioning channel for each such site. For the cells with the largest number of channels, the correspondence approaches one-to-one, for both VHCs and OHCs. This direct comparison, using the same experimental techniques, suggests that the difference in maximum transducer conductance between VHCs and OHCs is simply due to a difference in the anatomy of the hair bundles, with fewer stereocilia and channel sites in the VHCs.

(b) Operating range and sensitivity

OHCs require about 150 nm of displacement at the tip of the bundle to activate 90% of their transducer channels; VHCs, with their taller bundles, need about 330 nm . In terms of rotation, this translates to 2.0 deg for both. The current-rotation relationships of VHCs and OHCs normalized for maximum conductance are

similar (figure 3*d*). In chick vestibular hair cells, bundles of different heights also had similar input–output relations with respect to rotation but not translation (Ohmori 1987). Pickles (1993) modelled the stretch generated by bundle rotation in side-to-side links and tip links. Only stretch in the tip links showed an invariant relationship to bundle rotation for different bundle heights, provided the rootlet separation between adjacent stereocilia in neighbouring rows was constant. Furness *et al.* (1997) concluded the same for the shear displacement of the apposition sites just below the tip links. This means that within a ciliary bundle of graded height but constant rootlet separation between different rows, every transducer channel is subjected to the same force, regardless of which pair of stereocilia it connects. It also explains the very similar rotational operating range encountered in VHCs and OHCs of the neonatal mouse, as their rootlet separations are nearly the same, and suggests that the gating properties of their transducer channels are similar.

The difference in translational sensitivity between VHCs (about 20 pS nm⁻¹) and OHCs (about 70 pS nm⁻¹) arises because of the different bundle heights, and additionally the different numbers of transducer channels. Converting to rotation removes the first but not the second factor, and VHCs show a maximum sensitivity of about 3.5 nS deg⁻¹, compared to 5 nS deg⁻¹ for the OHCs. Neonatal OHCs are the most sensitive hair cells encountered yet, e.g. the mean maximum sensitivity of turtle cochlear hair cells was 7.5 pS nm⁻¹, or 0.75 nS deg⁻¹ (Crawford *et al.* 1989), that of adult OHCs only 1.3 pS nm⁻¹ (Ashmore *et al.* 1993). VHCs of neonatal mice, although less sensitive than neonatal OHCs, have the highest sensitivity among vestibular hair cells studied so far. For the frog sacculus, estimates range from 1 to 2.4 nS deg⁻¹ (Holton & Hudspeth 1986; Howard & Hudspeth 1987). The considerably smaller translational stiffness of the VHCs makes them actually more sensitive to applied force than the OHCs: 26 S N⁻¹ versus 12 S N⁻¹.

(c) *Stiffness of the hair bundles*

Table 1 shows that translational stiffnesses of the VHCs were much smaller than those of the OHCs. Rotational stiffness values were still somewhat smaller in the VHCs, although not significantly so. If the stereocilia of VHCs and OHCs were identical apart from the difference in length, and bundle stiffness was mainly determined by the flexural rigidity of the stereociliary rootlets (Crawford & Fettiplace 1985), we would expect the rotational stiffness of the VHCs to be less than one-third of that of the OHCs, because of the difference in rootlet numbers. Conceivably, the VHC bundles' rootlets may be stiffer, or more of their stiffness may be determined by the gating of their transducer channels, possibly via the tip links (see below), or both.

Stiffness values for OHCs are of the same order of magnitude as previous measurements in the same preparation using calibrated glass probes (around 1.5 mN m⁻¹ steady-state stiffness for a large sample of

OHCs, mainly from the apical coil: Kössl *et al.* 1990; Russell *et al.* 1992). Our measured steady-state stiffness was about three times larger, possibly because not all stereocilia may have contributed to the stiffness measured with a probe attached to the tip of the 'V'-shaped hair bundle, or because our simplifying assumptions overestimate the force exerted by the fluid jet on the bundles. The steady-state translational stiffness of most vertebrate hair bundles investigated is in the order of 1 mN m⁻¹ (Crawford & Fettiplace 1985; Howard *et al.* 1988).

The slow component of bundle relaxation, responsible for the difference in dynamic and steady-state stiffness, was in our experiments not correlated with obvious adaptation of the transducer currents, at least for the OHCs (Kros *et al.* 1995). This is in agreement with previous findings with turtle cochlear hair cells (Crawford *et al.* 1989). We tentatively suggest that the hydrodynamics within the bundle causes this slow relaxation component. It is unlikely to be a property of the fluid jet stimulus, because it was never observed when glass or carbon fibres were used to determine the force exerted by the jet.

There were a number of interesting differences between VHCs and OHCs in the responses of the bundles to force steps. OHCs were equally stiff in the excitatory and inhibitory directions. VHCs were stiffer in the inhibitory direction, regardless of whether the fluid jet was applied from the side of the kinocilium or from the side of the shortest stereocilia. An increase in stiffness in the inhibitory direction has been described before (Russell *et al.* 1992), and has been attributed to an immobilization of part of the gating spring for inhibitory displacements (Markin & Hudspeth 1995). Also note the minimum in the slope of the force–displacement relation in the excitatory direction for the VHC of figure 4*a*, which, contrary to Russell *et al.* (1992), we never observed for OHCs. This may correspond to the dip in stiffness attributed to the 'gating compliance' of the transduction mechanism (Howard & Hudspeth 1988). These findings suggest that the gating of the transducer channels contributes a larger fraction of the total bundle stiffness in the VHCs than it does in the OHCs, whose stiffness may be dominated by that of the stereociliary rootlets. In this respect it may be relevant that the average number of functioning channels per stereocilium was 0.61 for the VHCs and 0.38 for the OHCs. Further experiments are required to substantiate this suggestion.

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