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Lack of hair cell kinocilia Ca^{2+}-responsive mechanosensitivity during mouse hair cell development

In order to accurately measure changes in [Ca^{2+}] in small moving subcellular structures and to control for artifacts due to motion and differential bleaching of ratioing dyes, we first examined a tissue in which mechanosensing is well established. Hair cells in the organ of Corti of the cochlea can detect Angstrom movements and respond within tens of microseconds by opening ion channels that allow permeation of Ca^{2+} [1-3], making them the most sensitive of known Ca^{2+}-responsive mechanosensors (CaRMS). The apical surface of each hair cell carries a hair bundle with staggered rows of specialized actin-based microvillus-like projections called 'stereocilia' (Extended Data Fig. 3). At the apex of the chevron of stereocilia is a single nonmotile, microtubule-based (9+2) true cilium, called a kinocilium. Hair-cell stereocilia are well-established as highly sensitive mechanosensors, responding to force within microseconds by opening Ca^{2+}-permeable non-selective cation channels. Tiny filamentous 'tip links' connect the stereocilia and transmit force to these mechano-electrical transduction (MET) channels, likely encoded by *TMC1 and TMC2* genes [46]. Calcium influx through MET channels raises stereocilia [Ca^{2+}] with a 10-20 ms time constant [4].

In chicken hair cells and likely others, kinocilia are connected to adjacent stereocilia via filamentous links formed by the same proteins as the tip links[47], raising the question of whether kinocilia also have MET channels. Indeed, TMC1 protein has been reported in kinocilia of neonatal mouse hair cells[48]. A mouse line lacking a particular isoform of the tip-link component protocadherin 15 lacks the filamentous links between kinocilia and stereocilia, is deaf and exhibits a planar-cell-polarity phenotype[21]. Together, this evidence suggests that kinocilia may contain both protocadherin-15 and the MET channel that it normally activates. In developing zebrafish hair cells, Ca^{2+} imaging experiments suggest that, prior to the onset of the normal hair cell mechanotransduction, kinocilia mediate a mechanosensitivity elicited by reverse deflections of the hair bundle. Kinocilia and kinocilial links are required for the deflection-dependent rise in cytoplasmic [Ca^{2+}][5,47,49].

Using hair cells from the GECI mouse, we compared [Ca^{2+}] changes in two adjacent structures (stereocilia and kinocilia) of similar geometry (~5 µm length and 300-600 nm diameter) and determined whether the mouse kinocilium is also a CaRMS. In control experiments, IHC and OHC kinocilia and primary cilia of supporting cells (SCs) showed fluorescence ratios significantly increased by digitonin permeabilization (1.0 ± 0.13 to 5.5 ± 1.2, n=6; 1.0 ± 0.12 to 4.2 ± 0.19, n=6 for SC) in E18 and P3 explants, demonstrating that the ciliary Arl13b-mCherry-GECO1.2 sensor reports $[Ca^{2+}]_{cilium}$ without dye saturation (Extended Data Figure 6). At E14 and E15, hair cells were indistinguishable from the sensory epithelial SCs, necessitating measurement of all cilia in the field of view. None of the cilia of inner ear sensory epithelia at E14 (n=95) or E15 (n=41) showed a change in $[Ca^{2+}]_{cilium}$ with deflection, although ~50% of the cilia are expected to be of a hair cell

origin (ratio changes: E14: 1.0 ± 0.02 to 1.1 ± 0.03; E15: 1.0 ± 0.02 to 1.0 ± 0.02; **Fig. 2c**). Data from age E17 IHCs, OHCs and SCs are shown in **Fig. 2d**. At P0 (**Fig. 2 b, e-f**; closest developmentally to that reported in zebrafish[5]), stereocilia bundles of mouse hair cells begin to exhibit MET function; this process is largely complete by P3 across the entire organ of Corti [50]. A pipette was positioned in front of IHC or OHC stereocilia bundles and the kinocilium deflected opposite to the hair bundle's normal axis of mechanosensitivity (the sensitive direction reported in developing zebrafish [5], opposite to that shown in in **Fig. 1e).** After the onset of hair cell MET, such stimuli close any channels open at rest and prevent Ca^{2+} from entering the cell through conventional MET channels in stereocilia. As expected, mCherry and GECO1.2 remained unchanged during and after the deflection (**Fig. 2 e-f**). We also delivered a bidirectional flow stimulus (sinusoidal stimulus of an increasing amplitude), at the ages before the onset of hair cell MET (until P0-P1, data not shown) and found no evidence of CaRMS in mouse hair cell kinocilia.

Compensation for movement-related artifacts

Initial experiments in mIMCD cells resulted in small ratio changes (maximum: 1.0 ± 0.01 to 1.19 ± 0.03) in 1.3 mM $[Ca^{2+}]_o$. However, the ratio immediately decreased to 1.03 ± 0.01 within 60 ms following the cilia's return to its resting position (τ = 45 ms), an order of magnitude faster than the GECO1.2 response time. This suggests that the ratio change was unrelated to $\Delta Ca^{2+}]$. To further investigate this behavior, we repeated the experiment in low (50 nM) $[Ca^{2+}]_o$, in which $[Ca^{2+}]_i$ (~100 nM) and $[Ca^{2+}]_{cilium}$ (~300-500 nM) are higher than $[Ca^{2+}]_o$, thus preventing $[Ca^{2+}]$ from entering the cilium. When primary cilia were deflected under these conditions, ratios still increased (1.0 ± 0.02 to 1.16 ± 0.04), returning to the baseline (1.03 ± 0.02) within 60 ms following the end of the deflection stimulus (τ = 45 ms). Detailed analysis of decay times for recovery of these small ratio changes, and the similarity of the changes regardless of Ca^{2+} gradient, suggests that this small change is an artifact. The τ for a *bona fide* Ca^{2+} decay from Arl13b-mCherry-GECO1.2 ranges between ~400 ms (HEK293 cells) and ~600 ms (stereocilia, Extended Data Fig. 4). In upright images of primary cilia, recovery τ = 45 ms, ~10 times faster than the intrinsic dissociation rate of GECO1.2. That this fast response is an artifact is confirmed by the lack of change in $F_{GECO1.2}/F_{mCherry}$ ratios in side view images of deflecting cilia. These data show that ciliary $[Ca^{2+}]$ does not measurably change in mIMCD during deflection. We thus emphasize that special care must be taken in evaluating top view $\Delta[Ca^{2+}]_{cilium}$ during movement (see also Extended Data Fig. 5, 6). The most straightforward interpretation of the fast $\Delta[Ca^{2+}]_{cilium}$ artifact in top views is that the changing thickness of fluorescent indicator as the cilium bends (z-volume) changes overall fluorescent light output, as the z-resolution of the objective lens is less than the cilium diameter (~1.15 µm *vs.* ~300 nm). We also note that underlying spatial variation of cytoplasmic autofluorescence will contribute differentially to total fluorescence as the cilium bends over different parts of the cell.

Fluid velocity, shear stress, and forces on cilia in kidney tubules

In hydraulics, 'flow' refers to the movement of fluid. More specifically 'flow rate' (volumetric flow rate, Q) refers to the volume of fluid through a surface per unit of time (e.g. liters or m^3/s). Flow velocity (*v*) is a vector quantity that describes the rate of change of position per unit of time (e.g. meters/s). In a closed system, Q is analogous to electrical current while *v* is analogous to current density. 'Flow' and 'flow rate' are sometimes used interchangeably, and often confused with flow velocity, but the units are the most reliable guides to the meaning. Flow in kidney tubules (proximal tubules, loop of Henle, and collecting ducts) is laminar (low Reynold's numbers) and the flow rate is ~1-10 nl/min, depending on glomerular filtration rate [51]. The Navier-Stokes equation describes unidirectional axial flow in a circular cylinder. The velocity of this flow is highest in the center of the tube, and falls away along a parabola in the x-z axis to zero velocity at the wall (no-slip condition). Fluid velocities in rat proximal tubules have been reported as 400 μm/s to 800 μm/s [52], but have not been measured in individual tubules of the loop of Henle or the adjacent thick ascending limb tubules. Based on Weinstein [9], we estimate these flow velocities as ~300 μm/s. Shear stress arises from the force vector component parallel to the cross section of a material, and in laminar flow is proportional to the strain rate in the fluid. The proportionality constant is the viscosity of the fluid, that is, the resistance to deformation by shear. Shear stress (τ, $dyne/cm^2$) due to laminar flow in cylindrical tubes is calculated as $\tau=4\mu Q/\pi r^3$ where μ is fluid viscosity in Poise, Q the flow rate (as determined by beads in control solutions), and r the internal radius of the tube[53]. Reasonable estimates of shear stress in kidney tubules were calculated to be 0.1-0.5 dyn/cm^2. We used calibrated 'square pipe' flow chambers (IBIDI μ-Slide VI 0.4 flow chamber coated with laminin, see Methods) designed for quantification of flow rate and shear stress. Measurements of forces on the primary cilium itself yield estimates of flexular rigidity as ~10^{-23} N m^2 (~10 fold less stiff than motile cilia), under a fluid drag of 10^{-7}-10^{-8} dyn/cm [10]. In those measurements (as well as ours), velocity, drag, and shear stress was not measured or calculated at each point on the cilium (e.g., tip *vs.* the base) due to the small scale of the cilium, and its continually changing bend, potential torsion, and orientation in flow. Nonetheless our flow rates[9,52], and shear stress significantly exceeded the range of ciliary deflection in those studies (see Figs 2, 7 in ref. [10]) and covered a range from barely detectable movement (~0.4 μm), complete flattening of the cilia against the cell/tissue wall, and finally to cilia disruption at ~10 dyn/cm^2. For quantification of movement, we used centroid displacement, not tip or bent length displacement, as our measure of ciliary movement.

The highest drag at points on the cilium corresponds to the regions of highest flow rates and will be in the middle of a tubule with laminar flow. In most kidney tubules we studied, the cilia were longer than the tubule diameter and thus the cilium's tip did not necessarily experience the highest force (in general, the

outer 2/3 of the cilium experienced the highest velocities). The cilium also passively transmits force from the entire cilium to the base and thus the cilium's base may experience the highest force. We did not observe calcium increases initiated anywhere along the cilium, including the base.

Autofluorescence is high in kidney tubules compared to other cells studied, and thus smaller [Ca^{2+}] changes could be partially masked by the background. However, in no case in which both cilia and cytoplasmic [Ca^{2+}] were measured did we observed a calcium signal propagating from cilium to cytoplasm (see also Discussion in ref. [16]).

Primary cilia orientation in different cell lines

To our surprise, most of the cells from Ocy 454 (~80%), MLO-Y4 (~60%), and, to a lower extend MEF (~20%) cell lines had their cilia located under the cell—between the cell and the coverslip—similar to the MEF cell shown in Extended Data Fig. 8b and Supplementary Video 3. In some experiments in which flow-induced cytoplasm [Ca^{2+}] increases were induced independently of the cilium's deflection, trapped cilia served as an added control (Extended Data Fig. 8b).

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