Rat Auditory Inner Hair Cell Mechano transduction and Stereociliary Membrane Diffusivity Are Similarly Modulated by Calcium

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HIGHLIGHTS
Stereocilia membranes are highly diffusive as compared to the cell body membrane

Stereocilia membrane diffusivity varies with internal and external calcium levels

MET channel resting open probability increases as membrane diffusivity decreases

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Rat Auditory Inner Hair Cell Mechanotransduction and Stereociliary Membrane Diffusivity Are Similarly Modulated by Calcium

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SUMMARY
The lipid bilayer plays a pivotal role in force transmission to many mechanically-gated channels. We developed the technology to monitor membrane diffusivity in order to test the hypothesis positing that Ca2+ regulates open probability \(P_o\) of cochlear hair cell mechanotransduction (MET) channels via the plasma membrane. The stereociliary membrane was more diffusive (9x) than the basolateral membrane. Elevating intracellular Ca2+ buffering or lowering extracellular Ca2+ reduced stereociliary diffusivity and increased MET \(P_o\). In contrast, prolonged depolarization increased stereociliary diffusivity and reduced MET \(P_o\). No comparable effects were noted for soma measurements. Although MET channels are located in the shorter stereocilia rows, both rows had similar baseline diffusivity and showed similar responses to Ca2+ manipulations and MET channel blocks, suggesting that diffusivity is independent of MET. Together, these data suggest that the stereociliary membrane is a component of a calcium-modulated viscoelastic-like element regulating hair cell mechanotransduction.

INTRODUCTION
Mechanotransduction (MET) is a key step in many sensory processes, including touch, proprioception, pain, osmoregulation, cell adhesion, balance, and hearing. A common feature to each of these is the activation of mechanosensitive ion channels. In principle, force gating of mechanosensitive ion channels can occur through the lipid bilayer (“force-from-lipids” principle) or through tether-forming proteins that connect the channel to the cytoskeletal and/or extracellular matrix (“force-from-filament” model) to translate mechanical stimulus into electrical or biochemical signals (Ursell and Blount, 2008; Martinac and Poole, 2018). These mechanisms are not mutually exclusive, and the lipid bilayer can play a role in force transmission in both scenarios.

The lipid bilayer is indirectly implicated in modulating cochlear hair cell MET. GsMTx4, a lipid-mediated stretch-activated channel modifier (Suchyna et al., 2000; Bowman et al., 2007), shifts the MET activation curve rightward, decreasing the resting open probability \(P_o\) of MET channel, and inhibits the leftward shift induced by reducing extracellular divalent ions and membrane depolarization (Peng et al., 2016). Increasing intracellular Ca2+ buffering also shifts the activation curve leftward, increasing the resting \(P_o\), independent of the extracellular effects. PIP2, an endogenous lipid, modulates MET channel conductance, open probability, and kinetics (Hirono et al., 2004; Effertz et al., 2017; Cunningham et al., 2020). And finally, computational models support a role for the lipid bilayer in shaping the force exerted on hair cell MET channels (Powers et al., 2012, 2014; Gianoli et al., 2017). Present work directly assesses membrane diffusivity, one component of membrane mechanical properties.

Hair cells detect mechanical stimuli via displacement of their sensory hair bundle. The apically located hair bundle comprises an array of stereocilia, actin-filled microvilli, that increase in height in a staircase-like manner. An extracellular tether, the tip link, connects stereocilia columns such that deflection toward the tallest stereocilia row pulls the tip link (Pickles et al., 1984), exerting force that is transmitted to MET channels located at the top of the shorter stereocilia (Beurg et al., 2009). MET channels are mechanically coupled to the tip link through several membrane-spanning proteins and the lipid bilayer and likely also with proteins spanning from the plasma membrane to the cytoskeleton (Gillespie and Müller, 2009; Peng et al., 2011). The upper tip link is similarly coupled both to the membrane and cytoskeleton through...
a variety of proteins, likely including myosin molecules. How the lipid bilayer influences mechanical coupling between these molecules and thus modulates force transmission to the MET channel is a focus of the present work.

One method for monitoring membrane properties uses FRAP (fluorescence recovery after photobleaching) to measure lateral diffusivity of fluorescent particles as a measure of membrane properties. Lateral fluorescent particle diffusivity, which we refer to as membrane diffusivity for simplicity, is influenced by factors such as the lipid and protein composition, membrane order (lipid packing efficiency), membrane curvature, membrane tension (stress in the plane of the membrane), and hydrophobic thickness (Butler et al., 2001; Haidekker et al., 2001; Blood et al., 2005; Reddy et al., 2012). FRAP provides an average across a large volume of membrane (optically limited) over a relatively slow time course. FRAP does not provide information regarding fast changes in membrane properties or nanodomains created by individual proteins, but measurements can be influenced by underlying specializations. Changes in these membrane properties might alter diffusivity and indirectly affect membrane protein structure and function (Tillman and Cascio, 2003). Diffusivity is inversely related to membrane viscosity, but the specific relationship is complex and dependent on many of the properties described above.

We explored stereociliary membrane diffusivity using two-photon FRAP of the lipid probe di-3-ANEPPDHQ under conditions known to alter MET channel resting $P_o$ in rat inner hair cells (IHCs) and their stereocilia. We found that the stereociliary membrane is about nine times more diffusive than the IHC soma, consistent with previous findings (Boutet de Monvel et al., 2006). Unlike the soma, stereociliary diffusivity is sensitive to Ca$^{2+}$ and voltage, yet independent of MET channel activity. Our data support a mechanism whereby the stereociliary membrane diffusivity and MET channel $P_o$ are co-modulated such that increases in diffusivity are paralleled by decreases in MET channel $P_o$.

RESULTS
One-Dimensional Diffusion in the Stereociliary Membrane

Two-photon FRAP was used to evaluate membrane diffusivity in rat apical IHCs. The tallest (row 1) and middle (row 2) rows of the sensory hair bundle were evaluated using the membrane dye, di-3-ANEPPDHQ, as illustrated in Figures 1A–1D. The laser beam was focused at the top of a stereocilium which allowed for the average fluorescence intensity of a 1.9 μm long region (axial resolution of our optical system) of that stereocilium to be monitored over time. Figure 1C shows a set of stereocilia before and after photobleaching, with the bleached, reference, and background regions highlighted. Following background subtraction and photobleaching correction, a normalized fluorescence intensity vs time plot (FRAP curve) was generated with $t = 0$ representing the first post-bleach measurement (Figure 1E). To estimate the diffusion coefficient $D$, the FRAP curve was fitted with a stereocilia-specific one-dimensional diffusion model (Figure 1E).

Details of FRAP parameters and diffusion models are presented in Methods and Figures S1–S3. The filled appearance of the stereocilia initially questioned dye localization to the plasma membrane. Further investigations revealed “excitation photoselection effect” (Parasassi et al., 1997; Bagatolli, 2006) that was corrected with a half-wave plate as illustrated in Figure S3. $D$ values were similar with or without the half-wave plate rotation, confirming diffusion was only occurring through the membrane (Figure S3H).

Stereociliary Membrane Is Highly Diffusive Compared to the Soma

We compared FRAP measurements from the stereocilia to those of the IHC’s soma, near the top of the nucleus, as a control for stereociliary specific behavior. Figure 1F presents average FRAP curves from the soma and both rows of stereocilia illustrating differences in recovery time courses. The soma recovered fastest while the row 1 stereocilia recovered slower. In agreement with the FRAP curves, the time constants were significantly faster ($t$ test, $p < 0.001$) for the soma, 0.2 ± 0.05 s ($n = 8$ cells) compared to the row 2 stereocilia, 1.1 ± 0.2 s ($n = 43$ cilia), which are faster again ($t$ test, $p < 0.001$) than the row 1 stereocilia, 4.5 ± 1 s ($n = 21$ cilia) (Figure 1G). However, the time constants of recovery are greatly influenced by the geometry of the investigated system and the bleach extent. Thus, it is critical to develop a morphologically accurate model to correct for these parameters in order to extract a biologically relevant diffusion constant (see Supplemental Information). The estimated diffusion constants between stereociliary rows were not significantly different from each other, with values of 5.1 ± 1.1 μm$^2$/s for row 1 stereocilia and 5.6 ± 0.8 μm$^2$/s for row 2 stereocilia (Figure 1H). However, the stereociliary diffusion constant was about nine times greater than that of the soma of 0.58 ± 0.13 μm$^2$/s (Figure 1H). These results are in agreement with the previous finding of Boutet De Monvel et al. (2006) who report larger diffusion constants for outer hair cell (OHC) stereocilia.
Figure 1. Setup of Two-Photon FRAP of Membrane Dye di-3-ANEPPDHQ in IHC Stereocilia

(A and B) (A) Bright field and (B) two-photon sections of the same IHC bundles stained with di-3-ANEPPDHQ and oriented vertically, focused at the tip of the row 1 (top) and row 2 (bottom) stereocilia. Scale bar represents 5 μm.

(C) Representative FRAP experiment on the row 1 stereocilia with t = 0 indicating the first image taken post-bleaching. The red, blue, and brown circles define the bleached (BL), reference (REF), and background (BK) regions, respectively. Scale bar represents 1 μm.

(D) Depiction of an IHC bundle with red dashed lines indicating the approximate position of the focal plane at the tip of the row 1 and row 2 stereocilia for FRAP experiments. Only one stereocilia was bleached at a time. The gradient filling indicates the degree of bleaching with white representing the highest degree of bleaching.

(E) The experimental FRAP curve is fitted with a one-dimensional diffusion model to estimate the diffusion constant D, yielding a value of 4.6 μm²/s with a fitting error of 1.2%.

(F) The average FRAP curves (mean ± SD) and the corresponding fitted curves (red trace) for the row 1 stereocilia (n = 43, black filled squares), row 2 stereocilia (n = 21, black unfilled squares), and the cell body (n = 8, gray filled squares).
are needed to explain the differences between soma and stereocilia. Modest compared to the difference between soma and stereocilia (x9), suggesting additional factors of di-3-ANEPPDHQ in the stereocilia membrane. And finally, the cholesterol-induced change of measurement implemented at the level of individual stereocilium could detect the reduction in diffusivity addition in both stereociliary rows, with no significant effect (t test, p < 0.001).

This result further demonstrates that FRAP ery (Figure S4). Thus, the membrane enrichment with cholesterol decreases membrane fluidity, consistent with previous reports (Owen, 2015; Ayee and Levitan, 2016). This result further demonstrates that FRAP measurement implemented at the level of individual stereocilium could detect the reduction in diffusivity of di-3-ANEPPDHQ in the stereocilia membrane. And finally, the cholesterol-induced change of ~25% is modest compared to the difference between soma and stereocilia (Figure S2E). The marked difference between stereocilia and soma might imply a functional relevance to the diffusivity.

FRAP Sensitivity
FRAP sensitivity was evaluated by increasing membrane cholesterol levels, a manipulation previously shown to reduce membrane diffusivity (Nguyen and Brownell, 1998; Organ and Raphael, 2009). Figures 1I and 1J show that cholesterol addition slowed the time course of FRAP curves compared to control, with corresponding reductions in the diffusion constant for both stereociliary rows (t test, p < 0.001), from 5.1 ± 1.1 μm²/s to 4.2 ± 0.8 μm²/s for row 1 stereocilia and 5.6 ± 0.8 μm²/s to 3.8 ± 0.9 μm²/s for row 2 stereocilia. Similarly, the time constant showed significant increases (t test, p < 0.001) with cholesterol addition in both stereociliary rows, with no significant effect (t test, p > 0.2) on the mobile fraction of recovery (Figure S4).

Thus, the membrane enrichment with cholesterol decreases membrane fluidity, consistent with previous reports (Owen, 2015; Ayee and Levitan, 2016). This result further demonstrates that FRAP measurement implemented at the level of individual stereocilium could detect the reduction in diffusivity of di-3-ANEPPDHQ in the stereocilia membrane. And finally, the cholesterol-induced change of ~25% is modest compared to the difference between soma and stereocilia (x9), suggesting additional factors are needed to explain the differences between soma and stereocilia.

Effect of Internal Ca²⁺ Buffering on Membrane Diffusivity
The lipid membrane is implicated in modulating the resting $P_o$ of the hair cell MET channel (Peng et al., 2016; Effertz et al., 2017). Hypothesizing that the viscoelastic properties of the stereocilia membrane impact MET channel properties, we investigated lipid membrane diffusivity as a first step in probing membrane mechanics, under conditions that alter MET channel resting $P_o$. As diffusivity is an indication of viscosity, any correlation between diffusivity and MET channel resting $P_o$ can only suggest a common regulatory site for the two processes as viscosity effects can only manifest themselves in a non-steady state condition, i.e., during stimulation.

Elevated internal Ca²⁺ buffering increases MET channel resting $P_o$ (Crawford et al., 1991; Ricci and Fettiplace, 1997). To examine the effect of internal Ca²⁺ buffering on the stereocilia membrane diffusivity, we compared FRAP results from the stereocilia of cells patched with 0.1 mM BAPTA (1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetra-acetic acid) and 10 mM BAPTA in the internal solution. Increasing internal Ca²⁺ buffering from 0.1 mM BAPTA to 10 mM BAPTA with a holding potential of ~84 mV increased resting $P_o$ significantly (t test, p < 0.001) from 3.8 ± 1.4% (n = 65 cells) to 14.6 ± 4.6% (n = 47 cells) (Figures 2A and 2B), similar to other reports (Fettiplace, 1992; Ricci and Fettiplace, 1997; Peng et al., 2013).

Figures 2C and 2D summarize average FRAP curves measured across the two rows of stereocilia comparing 0.1 mM BAPTA to 10 mM BAPTA with the superimposed model fits for each data set. There is a small but distinct slowing of recovery with higher BAPTA concentrations, similar in scale to that observed with elevating cholesterol. Both stereociliary rows show a similar slowing of recovery with elevated BAPTA. The diffusion constant (Figure 2E) similarly suggests that both stereociliary rows are sensitive to BAPTA concentration in that diffusivity in 10 mM BAPTA (3.9 ± 0.8 μm²/s for row 1 and 3.9 ± 0.5 μm²/s for row 2) and is significantly reduced (t test, p < 0.001) as compared to 0.1 mM BAPTA (4.8 ± 1.1 μm²/s for row 2) and is significantly reduced (t test, p < 0.001).
The time constants also showed a significant increase ($t$ test, $p < 0.001$) with elevated internal Ca$^{2+}$ buffering in both stereociliary rows, with no effect on the mobile fraction of recovery (Figure S5).

Figure 2. Internal Ca$^{2+}$ Buffering Reduces Membrane Diffusivity of the Stereocilia
(A) Voltage-clamped (V) MET current responses of IHCs to fluid jet sinusoidal stimulus (M) with 0.1 mM BAPTA (black) and 10 mM BAPTA (red) internal solutions, with a holding potential of −84 mV.
(B) Summary box plots for the resting open probability ($P_o$) measured for 0.1 mM BAPTA ($n = 65$ cells) and 10 mM BAPTA ($n = 47$ cells) internal solutions. Each data point corresponds to a cell.
(C and D) Average FRAP data points (mean ± SD) and fitted curves from the row 1 (C) and row 2 (D) stereocilia comparing 0.1 and 10 mM BAPTA internal solutions. Insets show slower FRAP curves with 10 mM compared to 0.1 mM BAPTA internal solutions.
(E) Summary box plots show that the diffusion constant was significantly lower with 10 compared to 0.1 mM BAPTA for both stereocilia rows ($t$ test, $p < 0.001$). There was a significant reduction in diffusivity ($t$ test, $p < 0.001$) of row 2 with 0.1 mM BAPTA compared to unpatched cells. Also, application of 1 mM curare had no effect on the diffusivity of row 1 and row 2 with and without patching ($t$ test, $p < 0.001$). Each data point corresponds to a stereocilium.
(F) Summary of diffusion constants from the soma shows no significant difference between unpatched cells and patched with 10 mM BAPTA internal solution. Different symbols correspond to different cells. Boxes in (B), (E), and (F) represent the SD, and the star symbol indicates the mean. 0.1 B, 0.1 mM BAPTA; 10 B, 10 mM BAPTA; NP, not patched; NP CUR, not patched and treated externally with 1 mM curare; 0.1B CUR, patched with 0.1 mM BAPTA internal and treated externally with 1 mM curare. ***$p < 0.001$.

1 and 4.4 ± 0.6 μm$^2$/s for row 2). The time constants also showed a significant increase ($t$ test, $p < 0.001$) with elevated internal Ca$^{2+}$ buffering in both stereociliary rows, with no effect on the mobile fraction of recovery (Figure S5).
Role of MET Channel in Modulating Diffusivity

We observed a difference in D values between rows when comparing hair cells prior to obtaining a whole-cell recording to those with 0.1 mM BAPTA internal solution, with row 2 having a statistically significant reduction in D (t test, p < 0.001) while row 1 remained unaffected (Figure 2E). One possible interpretation of this difference is that row 2 has a higher Ca2+ concentration because of the functional MET channels located in them, making row 2 more sensitive to BAPTA. Similarly, the reduced relative change between 0.1 and 10 mM BAPTA for row 2 (19% in row 1 and 11% in row 2) may reflect local buffer saturation in row 2 with 0.1 mM BAPTA because of open MET channels in row 2. To determine whether the difference between the 0.1 mm BAPTA and the non-patched cell was due to changes in MET channel Po, we applied curare (1 mM) externally to non-patched hair bundles to block the MET channels (Glowatzki et al., 1997; Farris et al., 2004; Kirkwood et al., 2017). Curare had no effect on membrane diffusivity of stereocilia, arguing MET channels were not driving the difference. We also tested the row 2 membrane sensitivity to Ca2+ entry through MET channels by applying curare after obtaining whole-cell mode (with 0.1 mM BAPTA internally); curare addition again had no effect on the enhanced BAPTA sensitivity of row 2, further arguing that adjustments to MET channel Po were not responsible for changes in diffusivity. The 10 mM BAPTA condition similarly affects both stereociliary rows relative to non-patched cells (24% in row 1 and 30% in row 2), potentially by removing Ca2+ away from the lipid bilayer and altering lipid packing (Ito and Ohnishi, 1974; Melcrova et al., 2016). Thus, we find stereociliary membrane diffusivity to be sensitive to internal Ca2+ buffer but to be independent of MET channel activity.

Effect of Extracellular Ca2+ on Membrane Diffusivity

Lowering extracellular Ca2+ increases MET channel resting Po (Crawford et al., 1991; Peng et al., 2016; Effertz et al., 2017) and its effect on Po is blocked by GsMTx4, a compound that interferes with force transmission through the bilayer to MET channels (Suchyna et al., 2000; Bowman et al., 2007; Peng et al., 2016). We investigated the effect of extracellular Ca2+ on the diffusivity of di-3-ANEPPDHQ with 0.1 mM BAPTA or 10 mM BAPTA as the internal Ca2+ buffer. Lowering extracellular Ca2+ from 2 to 0.02 mM increased MET channel resting Po (Figures 3A and 3B) from 3.1 ± 1.0% to 9.1 ± 6.2% with 0.1 mM BAPTA (paired t test, p < 0.001) and from 12.7 ± 4.0% to 20.2 ± 10.3% with 10 mM BAPTA (paired t test, p < 0.01). Lowering extracellular Ca2+ to 0.2 nA with 0.1 mM BAPTA internal solution and from 0.7 ± 0.1 nA to 1.2 ± 0.2 nA with 0.1 mM BAPTA internal solution, a result due to removal of Ca2+ block from the MET channel (Crawford et al., 1991; Ricci and Fettiplace, 1998). FRAP measurements were obtained after observing the reported increase in resting Po and maximum MET current which ensured reliable hair bundle perfusion and equilibration of the 20 µM Ca2+.

As this experiment requires repeated FRAP measurements over time, we first demonstrate that the FRAP values are stable over the duration of the experimental time (Figure S6A). Lowering extracellular Ca2+ results in slower recovery in the stereocilia for both 0.1 and 10 mM BAPTA internal solutions as indicated by average FRAP curves (Figures 3D and 3G). The diffusion constants of the stereociliary membrane were significantly decreased in both rows (Figures 3E and 3H) in the presence of low extracellular Ca2+, from 5.7 ± 1.1 µm2/s to 4.2 ± 1.4 µm2/s (paired t test, p < 0.01) and 4.4 ± 0.8 µm2/s to 3.6 ± 0.8 µm2/s (paired t test, p < 0.05) for the row 1 and row 2 stereocilia, respectively, with 0.1 mM BAPTA internal solution. With 10 mM BAPTA internal solution, the diffusivity reduced from 4.6 ± 0.8 µm2/s to 3.4 ± 0.8 µm2/s (paired t test, p < 0.001) and 4.5 ± 0.8 µm2/s to 3.8 ± 0.7 µm2/s (paired t test, p < 0.01) for row 1 and row 2 stereocilia, respectively. Both internal solutions produced similar shifts in the diffusion constant in both rows of stereocilia (Figures 3F and 3I), suggesting that the effector site was external, similar to a previous report on MET channel Po (Peng et al., 2013).

Similarly, the time constants showed significant increase (paired t test, p < 0.001) with low extracellular Ca2+ in both rows of stereocilia, with no significant effect on the mobile fraction of recovery (Figures S6B–S6D). The time course of recovery upon returning the extracellular Ca2+ back to 2 mM appears slower than we accounted for in our measurements such that some stereocilia recovered to a lesser degree than the original measurements. The recovery was not statistically different than the control, and there was no buffer concentration or row-specific
Figure 3. Lowering External Ca^{2+} Reduces Stereociliary Membrane Diffusivity

(A) Representative current responses to sinusoidal fluid jet stimulation from IHCs before, during, and after application of 0.02 mM Ca^{2+} external solution, with 0.1 mM BAPTA internal (top) and 10 mM BAPTA internal (bottom). Note the increase in baseline current and MET current in the presence of low Ca^{2+} external.

(B and C) (B) Resting P_o and (C) peak MET current I_{max} increases significantly when the external Ca^{2+} concentration is reduced from 2 to 0.02 mM (paired t test, p < 0.01). Each symbol indicates a cell for a given internal solution.

(D) and (G) Average FRAP data points and fitted curves comparing 2 mM (black traces for 0.1 mM BAPTA internal and red traces for 10 mM BAPTA internal) with 0.02 mM Ca^{2+} external (gray traces for 0.1 mM BAPTA internal and light red traces for 10 mM BAPTA internal) for row 1 and row 2 stereocilia.

(E) and (H) Summary box plots of diffusion constants with 0.1 mM BAPTA internal solution (E, black and grey symbols) and 10 mM BAPTA internal solution (H, red and light red symbols) show a significant reduction in diffusivity in the presence of 0.02 mM Ca^{2+} external irrespective of the internal solution and the row of stereocilia (paired t test, p < 0.05). Each symbol in a graph corresponds to a bundle.

(F) and (I) The fractional decrease in D values when the external Ca^{2+} is reduced from 2 to 0.02 mM was not significantly different between the internal solutions i.e., 0.1 mM BAPTA (F) and 10 mM BAPTA (I) and the rows of stereocilia. Boxes represent the SDs, and the star symbol indicates the mean. 0.1 B, 0.1 mM BAPTA; 10 B, 10 mM BAPTA. *p < 0.05, **p < 0.01, ***p < 0.001.
differences in the recovery measurement. We did not compare the stereociliary membrane to that of the soma in low external Ca^{2+} due to the inability to reproducibly expose the basolateral hair cell membrane to low Ca^{2+} solution using the apical perfusion pipette in the semi-intact epithelial preparation.

Our results indicate that extracellular Ca^{2+} modulation of membrane diffusivity is independent of the internal buffer, as the fractional changes in diffusivity were unaffected (t test, p > 0.05) by the internal buffer. Data support the idea of extracellular binding sites for Ca^{2+} that could change membrane mechanics potentially by altering the interaction between adjacent lipids (Ito and Ohnishi, 1974; Melcova et al., 2016). The sensitivity of stereocilia membrane diffusivity to extracellular Ca^{2+} appears independent of the MET channel activity as similar results were obtained in both rows. However, low extracellular Ca^{2+} modulates MET channel resting $P_o$, as well as membrane diffusivity, suggesting a common mode of regulation.

Effect of Voltage on Membrane Diffusivity

Voltage modulates the MET channel $P_o$ in a time-dependent manner in the mammalian OHCs (Peng et al., 2016). Although resting $P_o$ suggests a time independent steady-state measurement reflecting channel open probability when the bundle is not stimulated, with depolarization there is a temporal component where the $P_o$ rapidly increases and then slowly reduces, reflecting a non-steady state condition. We term this $P_o*$, simply to indicate the same measurement (a non-externally stimulated hair bundle) but under non-steady-state conditions. GsMTx4 inhibits the voltage modulation suggesting an involvement of the membrane in regulating $P_o*$. We investigated the effect of voltage on the stereociliary membrane diffusivity of di-3-ANEPPDHQ during prolonged depolarization to +76 mV. Firstly, we characterized how the $P_o*$ changes with voltage in mammalian IHCs by stimulating the bundles with large negative and positive sinusoidal displacements delivered with a fluid jet pipette during prolonged depolarization for 22 s (Figure 4A). We also investigated the dependence of voltage modulation of $P_o*$ on internal Ca^{2+} buffer using 0.1 mM BAPTA or 10 mM BAPTA internally. The MET channel $P_o*$ rose to a maximum rapidly upon depolarization, followed by a slow decrease to a steady-state value during prolonged depolarization. The timing and extent of the response were sensitive to the internal Ca^{2+} buffer (Figures 4B and 4C). The maximum $P_o*$ with depolarization to +76 mV was 26.1 ± 10.7% with 0.1 mM BAPTA, which was significantly lower (t test, p < 0.001) than that with 10 mM BAPTA which peaked at 45.1 ± 6.5% (Figure 4D). Also, the time to peak $P_o*$ was significantly longer for cells with 0.1 mM BAPTA internal compared to 10 mM BAPTA internal, measuring 2.7 ± 1.4 s and 0.38 ± 0.4 s for 0.1 mM BAPTA and 10 mM BAPTA, respectively (Figure 4F).

During prolonged depolarization, $P_o*$ decreased slowly to a steady state of 18.6 ± 9.2% for 0.1 mM BAPTA which was significantly lower (t test, p < 0.001) than that of 10 mM BAPTA internal measured as 25.4 ± 4.5% (Figure 4D). Also, the change in $P_o*$ from the peak to the steady state was significantly lower for 0.1 mM BAPTA (7.4 ± 4.9%) compared to 10 mM BAPTA (19.6 ± 4.1%). Thus, the data suggest that the time-dependent voltage modulation of $P_o*$ is dependent on the internal Ca^{2+} buffering.

We next examined whether voltage affects the stereociliary membrane diffusivity in a time-dependent manner, more specifically during the initial depolarization when we see a rapid increase in the $P_o*$ and during prolonged depolarization which results in a slow reduction of $P_o*$ to a steady state. FRAP measurements were done either during the first 12 s of depolarization (early FRAP) to capture changes responsible for the increase in $P_o*$ or after the first 10 s of depolarization (late FRAP) to capture the reduction of the $P_o*$ over time, as illustrated in Figures 5A and 5B. Figures 5C and 5D summarize average FRAP curves measured across the two rows of stereocilia comparing early FRAP and late FRAP done at +76 mV to FRAP measured at −84 mV either with 0.1 mM BAPTA or 10 mM BAPTA internal. There was no change in the recovery for early FRAP in any condition while there was a slight increase in the recovery with late FRAP in both stereociliary rows and with both internal buffers. FRAP measurements during the early depolarization resulted in no change in the diffusion constant (Figures 5E and 5F). As FRAP is an average measure across a large volume and an extended time course, it is possible that any localized and fast membrane diffusivity changes due to early depolarization response may not be fully detected in these measurements. The stereociliary membrane diffusion constants were significantly increased in row 2 with late depolarization (paired t test, p < 0.01), from 3.9 ± 0.4 μm^2/s to 4.9 ± 1.1 μm^2/s with 0.1 mM BAPTA internal (Figure 5F) but not for row 1. Diffusivity reduced significantly for both stereociliary rows with 10 mM BAPTA internal, from 3.8 ± 0.9 μm^2/s to 5.1 ± 1.3 μm^2/s for row 1 (paired t test, p < 0.01) and 3.7 ± 0.4 μm^2/s to 4.9 ± 0.7 μm^2/s for row 2 (paired t test, p < 0.05) (Figure 5H).
Figure 4. Depolarization Modulates MET $P_o^*$

(A) presents mechanical stimulus (M) and voltage step at the top and a representative current response at the bottom. Depolarization to +76 mV results in an immediate increase in the MET channel $P_o^*$, with $P_o^*$ decreasing and stabilizing with prolonged depolarization in IHCs.

(B) Summary plot showing the change in $P_o^*$ during depolarization for 22 s for 0.1 mM BAPTA (top) and 10 mM BAPTA (bottom) internal solutions. The values measured at −84 mV are represented at time 0. Each symbol indicates a cell for a given internal solution. The peak $P_o^*$ and the steady-state $P_o^*$ (average of $P_o^*$ values after the time point when change in $P_o^*$ is ≤5%) are highlighted with black arrows.

(C) Expanded view of MET current response to a single period of a sinusoidal stimulus (M) from the current plot in (A), highlighted as I, II, and III, for different internal solutions; 0.1 mM BAPTA (black traces) and 10 mM BAPTA (red traces) for −84 mV (top panel), +76 mV at 2 s (middle), and +76 mV at 20 s (bottom).

(D and E) Summary plot of the peak $P_o^*$ and steady state $P_o^*$ in (D) and $\Delta P_o^*$ (peak $P_o^*$ - steady state $P_o^*$) in (E) showing significant differences between 0.1 mM BAPTA (black symbols) and 10 mM BAPTA (red symbols) internal solutions (t test,
To determine whether the differences observed after prolonged depolarization were unique to stereocilia, we also performed FRAP experiments in IHC soma. Using 10 mM BAPTA, we found no difference in diffusivity due to voltage (Figure S7). Thus, we conclude that voltage-dependent diffusivity changes are unique to the stereociliary membrane.

**DISCUSSION**

Stereocilia membrane properties were investigated under conditions known to alter MET channel resting $P_o$ to assess more directly the hypothesis that the lipid bilayer modulates the effect of Ca$^{2+}$ and voltage on mammalian auditory MET channels. We show that the IHC stereocilia membrane is highly diffusive compared to the soma, with both stereociliary rows having similar membrane diffusivity. We further demonstrate that the diffusivity of the stereocilia lipid membrane is sensitive to Ca$^{2+}$ and voltage while the basolateral membrane is not. Membrane diffusivity is not modulated by MET; however, the strong correlation between the resting $P_o$ and diffusivity suggests that membrane mechanics and MET channel resting $P_o$ have a common underlying modulator (Figure 6A).

**High Membrane Diffusivity of the IHC Stereocilia**

IHC stereocilia showed significantly higher diffusion constants (~9 times) than the soma, consistent with reported elevated diffusion constants for guinea pig OHC stereocilia bundles compared to the soma (Bouret de Monvel et al., 2006). Diffusion constants measured for di-3-ANEPPDHQ in the IHC soma ranged from 0.4 to 0.7 μm$^2$/sec, comparable to the previously reported values for di-8-ANEPPS in IHCs and OHCs ranging 0.2–1 μm$^2$/sec (Oghalai et al., 2000, Bouret De Monvel et al., 2006; Chen and Zhao, 2007; Organ and Raphael, 2009). All measured values are within the range typically reported for living cells, i.e., 0.01–10 μm$^2$/sec with most cells exhibiting values from 0.1 to 1 μm$^2$/sec at 18–22°C (Lee and Jacobson, 1994; Almeida and Vaz, 1995). However, the IHC stereocilia values are significantly higher than soma values at 20–22°C using the same lipid dye; similar high values at room temperature are observed in a few cell types such as rat smooth muscle cells and mouse spleen and lymphocytes (Lee and Jacobson, 1994). Membrane diffusivity differences may arise from different lipid composition, protein content, membrane interaction with underlying cytoskeletal structures, mechanotransduction machinery, active lipid transport, and/or membrane curvature (Hoekstra, 1994; Bigay and Antonny, 2012).

**Lipid Composition**

Mass spectrometry of chick vestibular hair bundles suggests that the stereocilia lipid composition does not differ significantly from other cell membranes, except for higher level of sphingomyelin and ceramide (Zhao et al., 2012). Therefore, it is less likely that the high stereociliary diffusivity is due to gross differences in lipid composition. However, compartmentalization of specific lipids or even low levels of some lipids can have dramatic effects on membrane mechanics; therefore, we cannot completely exclude lipid composition as a contributor.

**Protein Content and Cytoskeleton**

The presence of distinct transmembrane proteins, sub-membranous scaffolding proteins, and proteins that link the membrane to the underlying dense actin cytoskeleton would support the stereocilia membrane being less diffusive (Owen, 2015) and so are unlikely responsible for the elevated stereocilia diffusivity. It is possible, though unlikely, that all cell membranes have high levels of proteins embedded and have strong cytoskeletal connections leading to low diffusivity, and it is the lack of proteins and cytoskeletal interactions contributing to the higher stereociliary diffusivity levels.

**Mechanotransduction Machinery**

MET channels are only present in the shorter stereocilia rows (Beurg et al., 2009). The upper tip-link insertion point has a density with selective molecules like harmonin, whirlin, and cadherin 23, while the lower insertion point is linked via protocadherin 15, CIB2 (Calcium And Integrin Binding Family Member 2),
Figure 5. Prolonged Depolarization Increases Stereociliary Membrane Diffusivity

(A and B) Illustration of the FRAP timing with respect to depolarization showing (A) early FRAP, where FRAP recovery phase starts at time 0 of depolarization, and (B) late FRAP, where FRAP recovery phase starts 10 s after depolarization.

(C) Average early FRAP curves (mean ± SD) measured from row 1 (left side) and row 2 (right side) stereocilia, with 0.1 mM (top) and 10 mM BAPTA (bottom) internal solutions, showing no difference between the measurements at −84 mV (black and red traces) and +76 mV (gray and light red traces).

(D) Average late FRAP curves (mean ± SD) show faster recovery with prolonged depolarization to +76 mV (gray and light red traces) compared to that of −84 mV (black and red traces).

(E) and (G) Summary of cells with 0.1 mM BAPTA (E) and 10 mM BAPTA (G) internal solutions showing that early depolarization from −84 mV (black symbols in E and red symbols in G) to +76 mV (grey symbols in E and light red symbols in G) has no significant effect on the diffusion constants in both stereocilia rows as well as with 0.1 and 10 mM BAPTA internal solutions. Each colored/patterned symbol corresponds to a bundle/cell.

(F) and (H) Summary plot of D values with 0.1 mM BAPTA (F) and 10 mM BAPTA (H) internal solutions showing that late depolarization significantly increases the diffusion constant of row 2 irrespective of the internal solution and of the row 1 with 10 mM BAPTA (paired t test, *p < 0.05). Each colored/patterned symbol corresponds to a bundle/cell. Boxes represent SDs, and the star symbol indicate the mean. 0.1 B, 0.1 mM BAPTA; 10 B, 10 mM BAPTA. *p < 0.05, **p < 0.01, ***p < 0.001.
LHFPL5 (Lipoma HMGIC fusion partner-like 5), TMIE (Transmembrane Inner Ear), and TMC (Transmembrane channel-like) molecules (Kazmierczak et al., 2007; Xiong et al., 2012; Kurima et al., 2015; Giese et al., 2017; Pan et al., 2018; Cunningham et al., 2020; Krey et al., 2020). The MET channel at the lower end of the tip links permeates calcium and monovalent ions into this small volume. Yet, our data show that both stereociliary rows have similar baseline membrane diffusivity and similar changes in diffusivity following Ca$^{2+}$ and voltage manipulations, indicating that the observed effects are independent of MET channel activity. Additionally, pharmacologically blocking MET channels had no effect on diffusivity, suggesting that the MET channel complex does not regulate macroscopic membrane diffusivity. Thus, it is unlikely the mechanotransduction machinery is responsible for the differences between soma and stereocilia.

**Active Transport**

High stereociliary diffusivity could also arise from an active lipid transport component coupled with passive diffusion. The stereociliary membrane specifically expresses phospholipid-translocating ATPases such as ATP8B1, which are essential in phospholipid transport to maintain membrane asymmetry and curvature (Sebastian et al., 2012; Coleman et al., 2013), and morphological and functional degeneration of the hair bundles due to the deficiency of ATP8B1 and ATP8A2 indicate the importance of this transport mechanism involved in maintaining the lipid composition and the mechanical stability of the stereocilia (Stapelbroek et al., 2009; Coleman et al., 2014). The functional relevance of this active lipid transport is unclear. Whether this transport can account for the large difference in diffusivity is also unclear, and further experiments are needed to address this possibility.

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**Figure 6. MET Resting $P_o$ Is Correlated to Stereociliary Membrane Diffusivity**

(A) The mean MET resting $P_o$, plotted against the mean diffusion constant $D$ measured across experiments shows a strong correlation ($r^2 = 0.69$, $p < 0.05$), with black symbols representing measurements taken with 0.1 mM BAPTA internal solution/2 mM external Ca$^{2+}$ at −80 mV holding potential, red symbols representing 10 mM BAPTA internal solution/2 mM external Ca$^{2+}$ at −80 mV holding potential, gray symbols representing 0.1 mM BAPTA internal solution/20 mM external Ca$^{2+}$ at −80 mV holding potential, and salmon symbols representing 10 mM BAPTA. (B) presents the relationship in (A) pictorially, suggesting that the MET $P_o$ is sensitive to membrane diffusivity. The stereocilia membrane curvature presented is not scaled as the exact curvature value would depend on the exact position of the MET channel on the stereocilia, which is unknown. (C) Schematic illustrating the difference in membrane curvature between the stereocilia wrapping around its actin core and the basolateral membrane. The white cross sections on the hair bundle and the cell body indicate the approximate location of the represented membrane curvatures for the stereocilia (in red) and basolateral membrane (in blue). The cartoon denotes the need for specialized conical lipids on the outer and inner leaflets to accommodate for the observed curvature. The cartoon is not a scaled representation of the relation between lipid bilayer thickness and curvature as that would make it impossible to see the individual lipids. The lipids are colored to indicate the range of diffusivity with red representing high diffusivity and blue representing low diffusivity. We hypothesize that the inherent curvature alters the sensitivity of the stereocilia to divalent ions which alters diffusivity and can indirectly alter MET channel $P_o$. 

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LHFPL5 (Lipoma HMGIC fusion partner-like 5), TMIE (Transmembrane Inner Ear), and TMC (Transmembrane channel-like) molecules (Kazmierczak et al., 2007; Xiong et al., 2012; Kurima et al., 2015; Giese et al., 2017; Pan et al., 2018; Cunningham et al., 2020; Krey et al., 2020). The MET channel at the lower end of the tip links permeates calcium and monovalent ions into this small volume. Yet, our data show that both stereociliary rows have similar baseline membrane diffusivity and similar changes in diffusivity following Ca$^{2+}$ and voltage manipulations, indicating that the observed effects are independent of MET channel activity. Additionally, pharmacologically blocking MET channels had no effect on diffusivity, suggesting that the MET channel complex does not regulate macroscopic membrane diffusivity. Thus, it is unlikely the mechanotransduction machinery is responsible for the differences between soma and stereocilia.
Membrane Curvature

A simple yet plausible mechanism for elevated stereociliary diffusivity stems from the mechanics associated with high stereociliary membrane curvature. Membrane curvature is generated by the membrane monolayer asymmetry due to the presence of specific lipids such as conical lipids and clustering of shaped transmembrane proteins (McMahon and Gallop, 2005; Jarsch et al., 2016) required for establishing the tight wrapping of the actin core (Figure 6C). In its simplest form, lipid asymmetry is required for the membrane to ensheath the actin core of the stereocilia. Importantly, membrane curvature can mechanically control the spatial organization of the lipid bilayer, with regions of high curvature leading to loose lipid packing (i.e., high diffusivity) due to the preferential localization of disordered lipid domains (Parthasarathy et al., 2006; Bigay and Antonny, 2012; Vamparys et al., 2013). Parthasarathy et al. found a critical curvature of <0.8 μm², where spatial organization of lipids are directly affected by curvature (Parthasarathy et al., 2006). Thus, membranes of IHC stereocilia having curvatures greater than 1 μm² can be mechanically stressed and thus likely impacting membrane diffusivity by the spatial organization of lipid domains, as seen in other membrane protrusions such as filopodia and microvilli (Zhao et al., 2013; Prévost et al., 2015). The observed similar high stereociliary membrane diffusivity in both rows of stereocilia (with similar diameters) may in part be established by the curvature associated with the dimensions of the stereocilia.

FRAP is a slow measurement that averages across a large membrane volume. Stereocilia membranes are described with bumps potentially representing transmembrane proteins. It is also likely that the membrane is not longitudinally uniform, with MET machinery at the tops of the shorter rows and with the tip membrane undergoing deformation via the tip link pulling (either directly or indirectly). FRAP is not reporting on nanodomain differences in diffusivity; alternate technologies are needed for this level of resolution.

How Might the Stereociliary Membrane Be Affected Differently from the Soma?

Data demonstrate Ca²⁺ and voltage effects on stereociliary membrane diffusivity but not on the basolateral membrane. Multivalent ions, especially Ca²⁺, can directly interact with lipids to change the membrane mechanical properties (Ito and Ohnishi, 1974; Shoemaker and Vanderlick, 2003; Melcrova et al., 2016). Ca²⁺ can change lipid packing or membrane order through conformational changes to lipid headgroups, ordering of acyl chains, affecting the hydration shell of lipid headgroups, and altering the repulsive interactions between the lipids. The baseline diffusivity associated with the bilayer wrapping around the stereocilia may simply reflect the above changes better than the soma such that a 10% change is easily detectable in the stereocilia (~0.5 μm²/s) but less so in the soma (0.05 μm²/s). Alternatively, the curvature tension of the stereocilia may create an environment more sensitive to divalent modulation (Figure 6C).

The effects of voltage on stereocilia diffusivity are similarly complex as the effects on MET Pₒ. The differential effect on stereocilia as compared to soma may be as described above, a reflection of membrane curvature impacting lipid distributions. The lack of effect on diffusivity during the initial depolarization where Pₒ is increasing is likely due to the slow temporal resolution of FRAP, and better technology is needed to characterize fast responses. The slower increase in diffusivity correlates with a reduction in Pₒ and suggests a reduction in force transference to the channel. The underlying molecular mechanism for this effect and the physiological relevance remain to be elucidated. The simplest explanation may be a rearrangement of lipid molecules due to charged headgroups being less shielded by calcium ions.

Implications for Hair Cell Mechanotransduction

The nine-fold difference in membrane diffusivity between the stereocilia and soma is likely to provide the necessary lipid environment for modulation of mechanotransduction, the key functional output of this organelle. High membrane diffusivity is often associated with elevated membrane tension and reduced hydrophobic thickness (Butler et al., 2001; Haidekker et al., 2001; Blood et al., 2005; Reddy et al., 2012). Studies of other mechanosensitive channels show that decreased membrane fluidity increases their activation threshold, and thinner membrane favors channel opening (Perozo et al., 2002; Nomura et al., 2012). In contrast, stereociliary membrane diffusivity is generally high while the MET resting Pₒ is low, and the Pₒ increases as diffusivity is reduced (Figures 6A and 6B), at least in the range of measured diffusivity. The opposite polarity for hair cell MET channel sensitivity may be simply due to how force is transmitted to the MET channel. While many mechanosensitive channels sense force transmitted directly through the membrane, hair cell channels are thought to be tethered to both an extracellular and intracellular link (Effertz et al., 2015). Thus, the mechanical contribution of the bilayer may be indirect or in parallel to the protein coupling which in its simplest form would reverse sensitivity. More work is needed to clarify this important point.
Our data support a potential mechanism whereby the stereociliary membrane has a significantly higher baseline membrane diffusivity compared to the soma likely due to the high curvature of the stereociliary membrane. The differential Ca\(^{2+}\) effect on the stereociliary membrane may also be a manifestation of curvature-induced stress, where the stereocilia and the soma are at different curvature tension such that Ca\(^{2+}\) differentially affects lipid packing and thus the membrane diffusivity of these structures. MET channels do not contribute to stereociliary diffusivity but may be directly or indirectly modulated by changes in diffusivity as indicated by the resting open probability. We postulate that the stereociliary membrane contributes to a viscoelastic-like component modulating the hair cell MET channel.

Limitations of the Study
FRAP measurements are both spatially and temporally limited given that recovery times are a function of the volume bleached and the ability to image at high rates. The technology does not allow for monitoring rapid changes in membrane properties. Additionally, the volume is typically dictated by the z-resolution of the objective (as set by the numerical aperture of the objective) and so local changes will be filtered or averaged into the larger volume being monitored. Thus, the technology is unlikely to detect changes associated with MET channel gating, for example, because gating is both very local and fast. An example is that FRAP does not detect a change during the rapid change in MET open probability with depolarization. These data cannot be interpreted as there is no membrane effect but simply that FRAP does not detect a change. Related to the spatial and temporal limitations is the fact that the relationship between MET open probability and membrane diffusivity is a correlation; additional technologies are needed to generate causal links between open probability and membrane mechanics. Data presented here provide the impetus to follow-up on developing technologies to investigate causality.

Resource Availability
Lead Contact
Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Prof. Anthony Ricci, aricci@stanford.edu.

Materials Availability
This study did not generate new unique reagents.

Data and Code Availability
The original unprocessed data of live cell imaging and electrophysiological recordings are contained in very large files. These are available upon request from the corresponding author. All codes have been uploaded to Mendeley Data “Mendeley Data:https://doi.org/10.17632/3wrd9xp4gc.1.” and are also available through the authors.

METHODS
All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101773.

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AUTHOR CONTRIBUTIONS
S.S.G. and A.J.R. designed the experiments; S.S.G. performed the experiments; C.R.S. developed and implemented the diffusion model for stereocilia and soma; S.S.G. and A.J.R. analyzed the data, interpreted the results, and wrote the manuscript.
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Supplemental Information

Rat Auditory Inner Hair Cell Mechanotransduction and Stereociliary Membrane Diffusivity Are Similarly Modulated by Calcium

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Supplemental Information
Figure S1. Characterization of FRAP parameters. Related to Figure 1. (A) Power dependence of the normalized average fluorescence intensity for di-3-ANEPPDHQ excited at 860 nm. The laser power which marks the apparent onset of excitation saturation is highlighted in red. The bleaching power of 7 mW was selected to avoid excitation saturation. (B) Dependence of bleach level on the bleach duration. For this study, bleach duration of 100 ms resulted in ~70% bleaching. (C) Sequence of FRAP images (right) illustrating irreversible bleaching of di-3-ANEPPDHQ following complete photobleaching of pure DOPC vesicles. The bright field (BF) images of the bleached vesicle before and after FRAP is shown on the left. (D) Summary of FRAP curves from vesicles (n = 10) shows no recovery in the fluorescence signal after photobleaching, confirming irreversible bleaching of di-3-ANEPPDHQ for the duration of 12 sec. Each data point in (A), (B) and (D) is the mean ± SD.
Figure S2. Diffusion model and parameters. Related to Figure 1. (A) One-dimensional diffusion model for cylindrical stereocilia. The schematic uses the length for row1 stereocilia. The lipid pool is assumed to be at 4 μm from the taper on the cuticular plate. (B) Degree of bleaching normalized to its maximum (mean ± SD) measured at different positions along a stereocilium’s or cell body’s z-axis. Stereociliary rows were either vertical or at an angle of >10°. The black and red dotted arrows indicate the axial bleach extension values used for row1 and row2 stereocilia respectively in our diffusion model. (C) Length measured for stereocilia rows from live P9 rat organ of Corti stained with di-3-ANEPPDHQ. (D) Summary plot illustrating the dependency of the diffusion constant $D$ on the length $L$ of the stereocilia rows. Boxes represent the SDs and the star symbols indicate the means. Each data point is a stereocilium. R1 = row1, R2 = row2. (E) Sample recovery curves from the cell body comparing our data from IHC to previous data obtained from isolated OHCs (Boutet De Monvel et al., 2006, Chen and Zhao, 2007). By accounting for the experimental
differences using accurate diffusion model, we extracted similar average diffusion constants of 0.58 µm²/s compared to 0.3 - 0.46 µm²/s reported by (Boutet De Monvel et al., 2006) and 0.13 - 0.2 µm²/s reported by (Chen and Zhao, 2007), even though the time constants are very different between studies.

|                          | Diffusivity $D$ (µm²/s) |
|--------------------------|-------------------------|
|                          | Monvel, et al. (2006)    | Chen & Zhao (2007) | Organ and Raphael (2009) | Oghalai et al. (2000) | Present |
| IHC lateral wall         | 0.46                    |                  |                          |                      | 0.58    |
| OHC lateral wall         | 0.30                    | 0.13 - 0.2       | 1.67                     | 0.13 - 0.7           |         |
| Cuticular plate          |                         |                  | 5.26                     |                      |         |
| OHC stereocilia          | 0.7 - 1.1               |                  |                          |                      |         |
| IHC stereocilia          |                         |                  |                          |                      | 5       |

**Table S1**: Related to Figure 1. The table lists the mean diffusivity $D$ measured for various cellular membranes of organ of Corti by several studies.
Figure S3. Excitation photoselection. Related to Figure 1. (A) Schematic representation of excitation photoselection. The inset shows the relative position of di-3-ANEPPDHQ in the membrane. (B) Two-photon images of a pure DOPC vesicle stained with di-3-
ANEPPDHQ without a λ/2 plate (top) showing the photoselection effect (orange arrowheads) and with a λ/2 plate at 10° (bottom) where the photoselection effect is reduced. (C) Schematic illustration of excitation photoselection effect on a stereociliary row when the laser polarization plane is approximately parallel to the row. In this case, adjacent edges of two stereocilia appear as a filled circle, as do strongly excited semicircular regions without a neighbor, resulting in the number of filled circles in the two-photon image (C iii) being greater than the actual number of stereocilia (C i) by 1. (D) When the rows are perpendicular to the laser polarization, the photoselection effect results in an extra row of filled circles in the two-photon image (D iii). (E) and (F) Bright field and two-photon images with the focus plane at the top of row1 (left panels) or row2 (right panels). The rows are either parallel (E) or perpendicular (F) to the laser polarization. The excitation photoselection effect results in adjacent stereociliary membranes and strongly excited semicircular regions without neighbors to appear as filled circles (2nd panels). With the λ/2 plate at 10° in the laser path, the photoselection effect is reduced and the stereociliary membranes appear as unfilled circles (3rd panels). (G) Airy scan confocal images of the hair bundle stained with di-3-ANEPPDHQ focusing on the row1 (top panel) and row2 (bottom panel) stereocilia excited at 480 nm showing no photoselection effect. (H) Comparing the diffusion constant $D$ measured at the bright center of a filled circle, which represents the edges of two adjacent stereocilia, to that of the darker region between two filled circles, which represents the center of a stereocilium.

**Figure S4.** Cholesterol loading results in slower recovery. Related to Figure 1. The time constants $\tau$ derived from the Fourier series solutions, showing significantly slower recovery after cholesterol addition in (A) row1 and (B) row2 stereocilia. (C) The mobile fraction of dye is the same after cholesterol addition in both stereocilia rows. Boxes
represent SDs and the star symbol indicates the mean. Each data point is a stereocilium.

**p < 0.001.**

Figure S5. Internal Ca$^{2+}$ results in slower recovery. Related to Figure 2. (A), (B) The time constants $\tau$ showing significantly slower recovery with 10 mM BAPTA internal solution compared to 0.1 mM BAPTA internal solution in (A) row1 and (B) row2 stereocilia. (C) Summary plot of the mobile fraction showing no significant difference between 0.1 and 10 mM BATPTA internal solutions in both stereocilia rows. Boxes represent the SDs and the star symbol indicates the mean. Each data point is a stereocilium. 0.1 B = 0.1 mM BAPTA, 10 B = 10 mM BAPTA. **p < 0.001.
Figure S6. Lowering external Ca\textsuperscript{2+} results in slower recovery. Related to Figure 3. (A) $D$ values (mean ± SD) extracted from FRAP measurements repeated over time on different stereocilia within a bundle for both stereocilia rows ($n = 11$ for row1, $n = 8$ for row2) show no significant change with time. (B), (C) Summary box plots of time constant $\tau$ show a
significant reduction in the presence of 0.02 mM Ca\(^{2+}\) for both 0.1 mM BAPTA and 10 mM BAPTA internals in (B) row1 and (C) row2 stereocilia. (D) Summary plot of the mobile fraction showing no significant difference between 2 and 0.02 mM external Ca\(^{2+}\) in both stereocilia rows irrespective of the internal solution. Boxes represent the SDs and the star symbol indicates the mean. Each colored symbol corresponds to stereocilia from a cell. 0.1 B = 0.1 mM BAPTA, 10 B = 10 mM BAPTA. * \(p < 0.05\), ** \(p < 0.01\), *** \(p < 0.001\).

**Figure S7.** Depolarization has no effect on soma FRAP recovery. Related to Figure 5. (A) Average late FRAP curves from the soma with fitted two-dimensional diffusion model show no change with prolonged depolarization to +76 mV compared to that of -84 mV with 10 mM BAPTA internal. Each data point is the mean (\(n = 8\)) (B) Summary of diffusion constants from the soma shows no significant difference between the measurements at -84 mV and late depolarization to +76 mV. Different symbols correspond to different cells. Boxes represent the SDs and the black star symbol indicates the mean. 10 B = 10 mM BAPTA.
TRANSPARENT METHODS

Sample Preparation

*Cochlear Explants*: Sprague Dawley rats of both sexes were sacrificed by decapitation using methods approved by the Stanford University Administration Panel on Laboratory Animal Care. The apical turn of isolated organ of Corti was dissected from pups at postnatal day 8 (P8) to P9 and placed in a recording chamber (Beurg et al., 2009). Dissections were done in extracellular solution containing (in mM): 142 NaCl, 2 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 10 HEPES, 6 Glucose, 2 Ascorbate /Pyruvate, 2 Creatine monohydrate, at pH 7.4 and a final osmolality of 304 - 307 mOsm. After removing the tectorial membrane, the tissue was incubated with 6 µM of di-3-ANEPPDHQ (D36801, ThermoFisher Scientific) at room temperature for 5 mins. The tissue was then transferred to the recording chamber with dye-free extracellular solution and held in place with single strands of dental floss while ensuring that IHC bundles were oriented vertically (Ricci and Fettiplace, 1997). During the experiment, tissue was perfused with extracellular solution at rate of 0.3 ml/min maintained at room temperature (19 - 22°C). In addition, extracellular solution was delivered locally to the hair bundle through an apical pipette of tip size ~200 µm (Peng et al., 2016) to wash away any internal solution that might flow from the recording pipette prior to seal formation, without stimulating the hair bundle. For experiments with only electrophysiological recordings, the tissue was not stained with di-3-ANEPPDHQ. No differences were observed in MET properties between cells that were exposed and were not exposed to the dye.

*Liposome preparation*: Artificial lipid vesicles were used as a control for testing dye properties as well as the optical system. Liposomes were prepared with 100% 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC, Avanti Polar Lipids) on the day of the experiment. The solvent of a 0.1 ml aliquot of 10 mg/ml DOPC in chloroform was evaporated to leave
a dried lipid film. Dried lipids were hydrated in 0.25 ml of 100 mM KCl, 10 mM HEPES, pH 7.4 for an hour. The lipid solution was then extruded (Mini Extruder, Avanti Polar Lipids) through a 200 nm polycarbonate membrane 11 times. Di-3-ANEPPDHQ was added to the lipid solution in 1:1000 ratio and kept at 4°C, protected from light. For imaging, the liposomes were mounted on a slide in 0.5% agarose gel (Fisher Scientific) to keep them mechanically stable.

**Dye Selection and Preparation**

Di-3-ANEPPDHQ is a potentiometric lipophilic dye that specifically labels cell membranes including the stereociliary membrane. Di-3-ANEPPDHQ, a chimeric product of di-8-ANEPPS and RH795, was selected over di-8-ANEPPS (a commonly used lipid dye) because it was easier to stain the organ of Corti due to its higher water solubility and it provided a larger fluorescence signal, consistent with previous reports (Obaid et al., 2004). Note that the greater water solubility of di-3-ANEPPDHQ over di-8-ANEPPS may alter lateral membrane diffusion through one or both of two possible mechanisms: 1) A faster return of the bleached dye molecules into the bathing media would produce a larger concentration gradient driving the fluorescent dye into the bleached region. 2) Increasing the rate that unbleached dye returns to the bathing media would make it available for return into the unbleached region, increasing the small portion of FRAP that results from out of plane diffusion.

RH795, another lipid dye, was also tested and produced fluorescence recoveries that were in general faster than the di-3-ANEPPDHQ, similar to that described by (Boutet De Monvel et al., 2006). Using di-3-ANEPPDHQ in our study allowed direct comparisons to other published work and the slower time course ensured that we were not under-sampling in our imaging. Although potentiometric, di-3-ANEPPDHQ sensitivity to voltage is limited.
We tested this by varying membrane potential in recorded hair cells from -84 mV to +76 mV and found no change in the fluorescence signal, supporting the argument that changes in intensity reflects dye diffusion as a measure of lipid properties.

Di-3-ANEPPDHQ dye stock solution (30 mM) was prepared in 100% ethanol and stored at -20°C. Working dye solution was made daily by diluting in extracellular solution to a final di-3-ANEPPDHQ concentration of 6 µM in 0.02% ethanol and kept in the dark until used.

**Electrophysiology**

In most experiments, FRAP was combined with whole-cell voltage clamping to measure MET and membrane diffusivity from the same IHC. Tissue was viewed in brightfield using a fixed-stage upright BX 61WI (Olympus) microscope with a digital Rolera-XR camera (QImaging). Whole-cell patch clamp recordings were obtained using a thick-walled borosilicate patch pipettes (WPI) pulled on a P97 micropipette puller (Sutter Instruments) to 1 - 2 µm inner diameters (3 - 3.5 MΩ). Pipettes were filled with an internal solution containing (in mM): 116 CsCl, 3.5 MgCl₂, 3.5 ATP, 5 creatine phosphate, 0.1 Cs₄BAPTA, 10 HEPES, and 20 ascorbic acid, and with a pH of 7.2 and an osmolality of 285 - 290 mOsm. For the 10 mM BAPTA internal solution, CsCl was reduced to balance the increase in BAPTA concentration resulting in the final osmolality of 285 - 290 mOsm.

Voltages were adjusted offline for the liquid junction potential and cells were held at -84 mV unless otherwise noted. Elicited currents were low pass filtered at 10 kHz with an Axopatch 200A patch clamp amplifier (Axon Instruments), digitized at 100 kHz using a Personal DAQ3000 (IOtech), and recorded with jClamp (SciSoft). Cells with more than 80 pA of leak current (at -84 mV) were discarded. Uncompensated series resistance (Rs) was 8.7 ± 1.6 MΩ (n = 196) and the cell capacitance (Cm) was 10.5 ± 1.1 pF (n = 196). After achieving whole-cell mode, cells were allowed to equilibrate for 10-12 minutes to stabilize.
the resting current, as determined empirically, before beginning FRAP experiments (Ricci and Fettiplace, 1998, Schnee and Ricci, 2003).

**Mechanical Stimulation:** Hair bundles were deflected with a custom-built fluid jet system. Thin-walled borosilicate pipettes (10 µm tip diameter) were filled with extracellular solution and positioned across from the hair bundle of interest, within 5 µm. The fluid jet was driven with a 50 Hz sinusoidal wave using a piezo electric disc bender whose input was filtered using an 8-pole Bessel filter (Frequency Devices) at 1 kHz before and then driven by a high voltage/high current amplifier to drive the piezo electric disc.

**Drug Delivery**

**Cholesterol Loading:** In three tissue preparations, after di-3-ANEPPDHQ staining, the tissue was incubated in 50 mM water-soluble cholesterol (methyl-β-cyclodextrin (mβCD) + cholesterol; Sigma) in extracellular solution for 10 mins before mounting the tissue in the recording chamber with cholesterol-free extracellular solution. This treatment increases the membrane cholesterol in hair cells (Nguyen and Brownell, 1998, Organ and Raphael, 2009). FRAP measurements were performed between ~12 and 30 mins after initial exposure to treatment solution.

**Low extracellular Ca\(^{2+}\):** The apical perfusion pipette was used to deliver low Ca\(^{2+}\) extracellular solution. This solution contained (in mM): 143 NaCl, 2 KCl, 3.3 CaCl\(_2\), 4 HEDTA, 10 HEPES, and had pH = 7.4 at 304 - 307 mOsm. Free Ca\(^{2+}\) concentration was measured using a MI-600 Ca\(^{2+}\) electrode (Microelectrodes) calibrated using Ca\(^{2+}\) buffer standards (CALBUF-2, WPI) (Ricci and Fettiplace, 1998). The low Ca\(^{2+}\) extracellular solution was ~25 - 30 µM free Ca\(^{2+}\) (n = 27).

**Curare:** The apical perfusion pipette was used to deliver extracellular solution containing 1 mM tubocurarine (93750, Sigma) to block MET current.
Two-photon FRAP acquisition

To perform FRAP experiments, we used an Ultima two-photon system (Bruker, formerly Prairie Technologies) coupled with a BX 61WI microscope (Olympus). The excitation source was a Titanium: Sapphire tunable ultrafast Chameleon Ultra II pulsed laser (Coherent). Di-3-ANEPPDHQ was excited at 860 nm and the fluorescence emission was collected through a 100x 1.0 NA water dipping objective lens (Olympus) and redirected to a multi-alkali photo multiplier tube using a 575 nm dichroic filter and a 645/65 nm bandpass filter (Chroma Technologies). For the optical system implemented, the microscope’s point spread function (PSF) was calculated using 250 nm fluorescent particles (Spherotech) and the estimated lateral resolution was 0.43 µm and the axial resolution was 1.9 µm.

Each FRAP experiment consisted of a series of time lapse scans of a 2.3 µm by 3.3 µm region that incorporated up to 6 IHC stereocilia; a similar size was used for soma measurements. This region size allowed for a sampling period of 72 msec using a pixel dwell time of 4 µs. During FRAP experiments, the Gaussian laser beam was focused at the top of 5 - 6 stereocilia (Fig. 1A-D). For the soma experiments, the part of the cell membrane slightly above the nucleus was chosen, which allowed separation of the hair cell from the neighboring supporting cell using the patch pipette. The FRAP protocol sequences were as follows: 1) pre-bleach sequence (duration = 5 s, frame rate = 1 fps, laser power at the sample = 1.5 mW), 2) photobleaching pulse (duration = 100 ms, laser power at the sample = 7 mW), and 3) post-bleach sequence (duration = 12 to 20 s, frame rate for first 1 s = 5 fps and frame rate for remaining time = 1 fps, laser power at the sample = 1.5 mW). The laser beam passed through a pockels cell (Conoptics) that regulated the laser power sequence required for FRAP.

FRAP parameters: We calibrated laser power and duration of the photobleaching pulse using di-3-ANEPPDHQ stained stereocilia. Excitation saturation can substantially increase
the size of the effective photobleaching PSF (Braeckmans et al., 2006, Mazza et al., 2008) and leads to higher order triplet states, thus affecting the photobleaching mechanism and the reaction order (Loren et al., 2015). A photobleaching power of ~7 mW at the sample was selected, by plotting the normalized fluorescence intensity as a function of laser power, to avoid excitation saturation (Fig. S1A). To allow for rapid photobleaching, we used a bleach duration of 100 ms that achieves 60 to 70% photobleaching (Fig. S1B). The 100 ms bleach time is short in comparison to the characteristic diffusion time (>1 sec) observed in either the stereocilia or the soma (Fig. 1G) and therefore it is reasonable to assume minimal diffusion during the photobleaching time. To ensure that photobleaching of di-3-ANEPPDHQ was irreversible, DOPC vesicles of size < 0.5 μm were selected that would be completely bleached following our bleaching parameters (Fig. S1C). Our selected parameters demonstrate irreversible bleaching over 12 seconds of fluorescence recording (Fig. S1D). A monitoring power of ~1.5 mW was selected empirically to reduce photobleaching during the monitoring/recovery phase to < 5%.

**FRAP curve generation from image data**

The time series images were analyzed offline using ImageJ software (NIH) to quantify fluorescence intensities. We measured the average fluorescence intensity of all regions of interest (ROI): the bleached region (BL), a reference region (REF) and a background region (BK) as illustrated in fig. 1C. A custom code implemented in Igor Pro (Wavemetrics) was then used to generate a FRAP curve with normalized fluorescence intensity on the y-axis and time on the x-axis. First, the average background intensity \(I_{BK}\) was subtracted from the average fluorescence intensity within the bleached spot \(I_{BL}\) and the reference region \(I_{REF}\). Next, the background-subtracted intensity values at each time point were normalized to the pre-bleached intensity averaged from the first 5 images just preceding the photobleaching, such that average pre-bleach intensity is set to 1.
\[ I_{BL\text{norm}}(t) = \frac{I_{BL}(t) - I_{BK}(t)}{I_{BL\text{prebleach}}} \]

\[ I_{REF\text{norm}}(t) = \frac{I_{REF}(t) - I_{BK}(t)}{I_{REF\text{prebleach}}} \]

To account for the limited bleaching that occurs during recovery, the normalized intensity from the bleached ROI \( I_{BL\text{norm}} \) is then normalized to the intensity from the reference ROI \( I_{REF\text{norm}} \) at each time point to generate the FRAP curve as given below:

\[ I_{FRAP\text{curve}}(t) = \frac{I_{BL\text{norm}}(t)}{I_{REF\text{norm}}(t)} \]

Plots of time, where \( t = 0 \) is the first post bleach measurement, against the normalized intensity are presented for each condition and diffusion constants \( D \) are extracted.

**FRAP model for diffusion in the stereociliary membrane**

To calculate the diffusion constant \( D \), we followed the pure-diffusion model from Axelrod et al. (1976) (detailed in Appendix A). Briefly, we first used a Fourier series (Model 1) for a first approximation of diffusivity \( D \). For the stereocilia, the significant diffusion is axial, i.e. one-dimensional (1D) as in Fig. 1D. As the bleach spot is at the tip of the stereocilia, there is zero flux at the tip with a fluorophore pool at the base. The initial extent of bleaching and the stereocilia length were measured from the live tissue preparation (Fig. S2B, C), which leaves the theoretical recovery with only two parameters – diffusivity \( D \) and the long-time limit \( F(\infty) \) which is the fluorescence intensity as time approaches \( \infty \). These were determined by a best fit to the measured recovery, using ‘NonlinearModelFit’ in Mathematica.

Since the base of the stereocilium is hardly a fluorophore pool as assumed in Model 1, we then used Model 2 for a more realistic determination of the diffusivity \( D \). The long-time limit \( F(\infty) \) determined from the Model 1 was used in Model 2. Model 2 consisted of a
cylindrical body with a tapered rootlet area flaring into a flat plate representing the cuticular plate, with the fluorophore pool placed at the edge of the cuticular plate (Fig. S2A). The diffusion equation remained 1D but required a numerical solution with ‘NDSolveValue’ in Mathematica. The percentage of molecules contributing to the fluorescence recovery termed as “mobile fraction” of recovery \( f \) was estimated from the limit \( F(\infty) \). The numerical solution for Model 2 yielded an improved value for \( D \) that provides a least-square fit of the simulation to the experimental FRAP data (Fig. 1E). The model parameters \((D, \tau \text{ and } f)\) reported in the present study were determined by Model 2, unless stated otherwise.

**FRAP model for diffusion in the basolateral membrane**

To estimate the diffusivity of the soma, we considered the case of uniform lipid-bilayer properties with orthotropic diffusivity in 2D (detailed in Appendix B). The circumferential diffusion is even more important for the present bleaching, which has the small lateral extent of 0.8 µm and an axial extent of 2.2 µm. A double Fourier series is used for a cylindrical surface that represents the basolateral membrane of the IHC. The series is chosen to satisfy the condition of continuity in the circumferential direction and fluorophore pools at the ends in the axial direction. The bleach is a small rectangular region with Gaussian distributions in the \( x \)- and \( y \)- directions. Orthotropic diffusivity is included, with different values \( D_x \) and \( D_y \) in the \( x \)- and \( y \)- directions. However, principal axes of diffusion in off-axis directions is not considered. Our calculations indicate that the orthotropic values cannot be consistently obtained from a single recovery measurement. A second measurement is needed, such as the two-stripe bleaching or recovery from a second point as used in (Boutet De Monvel et al., 2006). However, they find that the ratio of the maximum and minimum diffusivities is generally a factor of only 2, and that the assumption of isotropy yields a diffusivity that is the average. So, we use isotropy \( D_x = D_y \), but accurately model the bleach. The result for the theoretical recovery has only two free
parameters – the diffusivity $D$ and the limit $F(\infty)$. Mathematica with ‘NDSolveValue’ yields the parameters for a best fit to the experimental recovery in short time. Considering the difference in procedure and analysis, the agreement of our diffusion calculations with (Boutet De Monvel et al., 2006) for the IHC is remarkable (Table S1) considering the recovery curves are so much different (Fig. S2E).

**Initial axial extent of bleaching**

The initial bleach extent along the stereocilia and soma, and the length of the live stereocilia are critical model parameters. We measured the initial bleach extent in z-axis by bleaching the stereocilium tip and acquiring fast z-stacks (15 ms frame period) using a 250 μm Z-axis Piezo Drive (Bruker) along the stereocilium length immediately after bleaching. Measurements were taken for both stereociliary rows oriented either standing up almost vertically (<10°) or at an angle >10°, and for the basolateral membrane (Fig. S2B). The degree of bleaching along the z-stack was normalized to that at the plane of bleaching and then plotted against the position along the z-axis relative to the bleach spot. Gaussian fitting was used to measure the effective axial extent of bleaching (Fig. S2B). The initial axial extent of bleaching (i.e., half width of full wave half maximum (FWHM) derived from the gaussian fitting) was dependent on the orientation of the row 1 stereocilia which were measured (mean ± SE) as 1.35 ± 0.06 μm (n = 19) and 0.95 ± 0.07 μm (n = 17) for standing up and angled orientations respectively. The row 2 stereocilia was measured to be 1.84 ± 0.3 μm (n = 21) for both orientations. For the basolateral membrane, the initial bleach axial extent was measured as 1.1 ± 0.1 μm (n = 10), slightly higher than the theoretical value of 0.9 μm estimated for the two-photon excitation wavelength of 860 nm and objective NA of 1.0.

The observed larger axial extent of bleaching in the row 2 stereocilia could be potentially due to the reflection or scattering of high-power laser (Zezell and Ana, 2015) from the
neighboring row 1 stereocilia and the cuticular plate resulting in extended bleaching. These data illustrate the importance of calibrating each component being investigated (row 1, 2 and soma) as we estimate a potential diffusion coefficient errors of up to 30% for a single point calibration that could inadvertently suggest differences between stereociliary rows. The diffusion constants reported in the present study were determined using the values 1.35 μm and 1.84 μm for row 1 and row 2 stereocilia respectively as the bundles were oriented vertically.

**Stereocilia length**

For an accurate estimation of the stereocilia length, the tissue was incubated in 6 μM di-3-ANEPPDHQ and mounted in a recording chamber. Live-cell z-stack images of the hair bundle were taken in Airy-scan mode under LSM 880 confocal microscope (Zeiss) with a water immersion 40x 1.0 NA objective (Zeiss), followed by 3D reconstruction of the hair bundle using Imaris 9.3.1 (Oxford Instruments).

Stereocilia lengths were estimated as 6.6 ± 0.2 μm (n = 56 stereocilia from 3 animals) for row 1 and 3.0 ± 0.2 μm (n = 17 stereocilia from 3 animals) for row 2 (Fig. S2C). Given that stereocilia length is an important parameter, we estimated the diffusion coefficient $D$ for lengths ± 2 standard deviation of the sample (SD) from the mean stereocilia lengths. We found that the 2 SD change in the stereocilia length resulted in a 3% and 11% change in the estimated diffusion constant for row 1 and row 2 respectively (Fig. S2D).

**Excitation Photoselection**

As di-3-ANEPPDHQ is a lipophilic dye that fluoresces only in the presence of lipid, the two-photon microscopy images of individual stereocilia membrane were expected to appear as unfilled circles. However, in the present study, each row of stereocilia appeared as a collection of filled circles with brighter fluorescence intensity in the center of each
circle (Fig. 1B, C). To accurately monitor diffusion within the membrane, dye must only be present in the membrane as replenishment must be from diffusion within the membrane and not entry from extracellular or intracellular fluid. The filled appearance of stereocilia was potentially a problem as it suggested there was dye in the cytoplasm. However, we determined that the observed fluorescence pattern was an artefact due to the orientation of the dye in the stereociliary membrane and the polarization of the excitation laser resulting in the *excitation photoselection effect* as illustrated in Fig. S3A. The *photoselection effect* arises when the fluorophore molecules having transition dipole moments aligned parallel to the plane of polarization of the excitation laser are strongly excited, whereas the fluorophore molecules that are aligned perpendicular to the excitation polarization are weakly excited (Parasassi et al., 1997, Bagatolli, 2006). This results in each stereocilium appearing as two semicircles instead of a circle as illustrated by the di-3-ANEPPDHQ stained DOPC vesicles (Fig. S3B). When a stereociliary row is aligned parallel to the laser polarization, adjacent semicircles of neighboring stereocilia from the same row appear to be a single filled circle, as do strongly excited semicircular regions without a neighbor (Fig. S3C, E). Thus, each row of stereocilia had an extra filled circle compared to the actual number of stereocilia. In the case of rows perpendicular to the laser polarization, two adjacent crescents from the neighboring rows form a filled circle, as do isolated crescents, resulting in an apparent additional row of filled stereocilia, one more than the actual number of stereociliary rows (Fig. S3D, F). We confirmed and corrected for the *photoselection effect* by rotating the laser polarization with a $\lambda/2$ (half-wave) plate, thus converting filled stereocilia to unfilled stereocilia (Fig. S3E, F). Note the excitation photoselection effect was not visible with confocal imaging (Fig. S3G). No difference was found in FRAP recovery with or without the $\lambda/2$ plate rotation and most importantly, diffusion was only occurring through the membrane ((paired *t*-test, $p > 0.05$, Fig. S3H).
Data Analysis

We used Mathematica 12.0 to fit the experimental FRAP data when estimating the diffusion parameters. Whole cell currents were visualized and analyzed using jClamp (SciSoft) and OriginPro 2018 (OriginLabs). Graphs were generated with OriginPro 2018 (OriginLabs) and Adobe Illustrator CS6 (Adobe). All statistical analyses used two-sample Student’s t test performed using OriginPro 2018 (OriginLabs). All p values presented used paired t tests with comparisons within a cell, and unpaired unequal variance tests across cell conditions. Significance (p values) are * p < 0.05, ** p < 0.01, *** p < 0.001. Data are presented as mean and standard deviation of the sample (SD), unless stated otherwise.

Appendix A: One-dimensional diffusion model for cylindrical stereocilia

Following (Axelrod et al.), it is presumed the bleaching of di-3-ANEPPDHQ is a simple irreversible first-order reaction, and that our bleaching time of 100 ms is short in comparison to the characteristic diffusion time (1 - 4 sec) and henceforth there is minimal diffusion during the bleaching time. The movement of fluorophores within the stereociliary membrane after bleaching is modeled to be 1D along the length of the stereocilium.

Diffusion equation for simple cylinder

We approximate the plasma membrane of the stereocilium by a straight cylinder. The post-bleach concentration of fluorophores is

\[ C(x, t) = C_0 - B(x, t) \]  

where \( C_0 \) is the uniform concentration of pre-bleach fluorophores and \( B(x, t) \) is the concentration of bleached fluorophores at position \( x \) from the tip of the stereocilium and time \( t \). The latter can be separated into a volume fraction \( f \) that diffuses and a portion \( (1 - f) \) that is immobile:

\[ C(x, t) = C_0 - B(x, 0)(1 - f) - fB(0,0)\tilde{B}(x, t) \]
in which the normalized mobile bleach concentration $\tilde{B}(x,t)$ satisfies the conditions:

$$\tilde{B}(x,0) = B(x,0)/B(0,0) \quad (3)$$

$$\tilde{B}(x,\infty) = 0 \quad (4)$$

and satisfies the simple 1D diffusion equation,

$$\frac{\partial \tilde{B}(x,t)}{\partial t} = D \frac{\partial^2 \tilde{B}(x,t)}{\partial x^2} \quad (5)$$

where $D$ is the diffusion coefficient. We use a Gaussian distribution for the initial bleach intensity

$$I(x) = e^{-x^2/2w^2} \quad (6)$$

in which the half width of FWHM $x_{1/2}$ is related to $w$:

$$x_{1/2} = w\sqrt{2\log 2} = 1.177w \quad (7)$$

The observed fluorescence recovery, normalized to the prebleach value is:

$$F(t) = \frac{\int_0^{L_0} I(x)C(x,t)dx}{\int_0^{L_0} I(x)C_0dx} \quad (8)$$

The fluorescence is integrated from the tip to the distance $L_0$. This can be written as:

$$F(t) = F(\infty) - [F(\infty) - F(0)]A(t) \quad (9)$$

where the mobile bleach is in the term:

$$A(t) = \frac{\int_0^{L_0} I(x)\tilde{B}(x,t)dx}{\int_0^{L_0} I(x)\tilde{B}(x,0)dx} \quad (10)$$

Since $\tilde{B}(x,\infty) = 0$, so that $A(\infty) = 0$, the observed long-time limit $F(\infty)$ is the fluorescence intensity as $t$ approaches $\infty$, and $F(0)$ is the measured value immediately post-bleach. If the fraction of mobile fluorophores $f$ is made explicit, the recovery is:

$$F(t) = (1-f)F(0) + f\left[1 - (1 - F(0))A(t)\right] \quad (11)$$

Therefore, the observed limit is related to the fraction by the relation:
\[ f = \frac{F(\infty) - F(0)}{1 - F(0)} \]  

(12)

The initial post-bleach distribution \( \tilde{B}(x, 0) \) is normalized to the value 1 at the tip and shown in Fig. S2B. This can be approximated by the intensity:

\[ \tilde{B}(x, 0) \sim I(x) \]  

(13)

This is valid when the bleach parameter \( K \) in Axelrod, et al. 1976 is around 1, which is the present case. As the bleach spot is at the tip of the stereocilia, the tip has zero flux, \( \partial \tilde{B}(0, t)/\partial x = 0 \), and there is a lipid pool at the cuticular plate at \( x = L \), i.e., \( \tilde{B}(L, t) = 0 \). With these initial and boundary conditions, the solution of the diffusion equation (Eq. 5) is unique and depends only on the diffusivity \( D \). Consequently, the recovery Eq. 9 has only two free parameters, the long-time limit \( F(\infty) \) and the diffusivity \( D \), which are determined from a fit to the measured recovery.

**Fourier series solution to diffusion equation, Model 1**

The solution to Eq. 5 satisfying the given boundary conditions and the initial condition given by Eq. 13 can be given by a Fourier series in the spatial coordinates:

\[ B(x, t) = \sum_{n=1}^{\infty} B_n e^{-\frac{t}{t_n}} \cos \left( \frac{(2n-1)\pi x}{2L} \right) \]  

(14)

in which the time constants \( t_n = \frac{1}{D} \left( \frac{2L}{(2n-1)\pi} \right)^2 \), so that successive time constants become small i.e., \( t_2 = \frac{1}{9} t_1, t_3 = \frac{1}{25} t_1, t_4 = \frac{1}{49} t_1 \) etc. A similar 1D Fourier series is used by Organ and Raphael (2009) for the cell body with a lipid pool at both ends. With Mathematica, Eq. 9 is used directly in “NonlinearModelFit” to obtain a least-squares fit of the experimental FRAP curve, yielding the diffusivity \( D \) and the limit \( F(\infty) \). The time constant \( \tau \) is used to
characterize the recovery curve, which is the time at which the mobile recovery is at midpoint:

\[
F(\tau) = \frac{1}{2} [F(\infty) - F(0)]
\]

This \( \tau \) is a combination of the time constants in Eq. 14 and is determined numerically from the least squares fit.

**More realistic geometry model, Model 2**

The drawback of the Fourier series solution is the lipid pool at the base of the stereocilium. A direct numerical solution is better in that various effects can be included. So, we used a more realistic geometry which consists of a straight stereocilium with a tapered root, attached to the cuticular plate, and a lipid pool at the edge of the cuticular plate (Fig. S2A). This remains an axisymmetric problem, but the arc length along the meridian must be used instead of the axial coordinate \( x \). The diffusion equation is now:

\[
\frac{\partial \tilde{B}(s, t)}{\partial t} = D \left( \frac{\partial^2 \tilde{B}(s, t)}{\partial s^2} + \frac{d r(s)}{r(s)ds} \frac{\partial \tilde{B}(s, t)}{\partial s} \right)
\]

where \( r(s) \) is the radial distance from axis to surface. Calculations show that the details of the end have some effect on the recovery, but the exact radius of the cuticular plate and edge condition, pool or zero flux, have little effect.

A comparison between the models showed that Model 1 with Fourier series solution Eq. 14 produce similar recovery curves to that of Model 2 if the length used in Model 1 was increased by 10%; the extra length simulates the effect of the cuticular plate. The procedure was to use Model 1 with the effective length (i.e., 10% longer than the estimated stereocilia length) for a fast determination of the limit \( F(\infty) \) and a first approximation for the value of \( D \) that minimizes the difference in the numerical solution and the
measurement. Then, we used the numerical solution for Model 2 (Fig. S2A) using the stereocilia length estimated from the live tissue preparation (see below for details) that yielded an improved value for $D$ that provides a least-squares fit of the simulation to the experimental FRAP data as shown in Fig. 1E.

Boutet De Monvel et al. (2006) measured the bundle of outer hair cell (OHC) stereocilia with a 2D analysis and obtained $D$ in the range $0.7 – 1.1 \, \mu m^2/s$ for the axial direction and the much slower values of $0.16 – 0.3 \, \mu m^2/s$ in the perpendicular direction. So, our neglect of the circumferential diffusion seems justified. Our results for the rat IHC stereocilia in the axial direction are higher with $D ~ 5 \, \mu m^2/s$ (Fig. 1H).

**Appendix B: Two-dimensional diffusion model for soma**

For local bleaching of the soma, the diffusion in the lipid bilayer is two dimensional. Boutet De Monvel et al. (2006) considered orthotropy of the diffusion in the wall of the OHC. They use two stripes of bleaching as well as the usual spot bleaching with monitoring of recovery at a second region to obtain the necessary information to discriminate between the diffusion in the axial and circumferential directions. The axial diffusivity is about twice the circumferential. Of interest is that the principal axes of diffusivity seem to align with the cytoskeleton. In Chen and Zhao (2007), the bleach is a circular spot, and the diffusivity is assumed to be isotropic. The Gaussian spot in an infinite domain has a simple approximation for the recovery, which they use to find that for the OHC, $D \sim 0.2 \, \mu m^2/s$, similar to the $0.3 \, \mu m^2/s$ found by Boutet De Monvel et al. (2006). Organ and Raphael (2009) also use a spot bleach, but in the analysis use a 1D Fourier series for the diffusion in the axial direction and neglect the diffusion in the circumferential direction. Consequently, their result of $D \sim 1.67 \, \mu m^2/s$ is an order of magnitude higher.

The diffusion in the circumferential direction is even more important in our measurements, since the width of intensity in the circumferential direction is narrower than in the axial
direction. We consider the case of constant properties of the bilayer with orthotropic diffusivity, so the equation for the concentration of bleached lipids is the diffusion equation in the form:

\[
\frac{\partial \tilde{B}(x,y,t)}{\partial t} = D_x \frac{\partial^2 \tilde{B}(x,y,t)}{\partial x^2} + D_y \frac{\partial^2 \tilde{B}(x,y,t)}{\partial y^2}
\]  

(17)

The surface is taken as cylindrical with the coordinates consisting of the circumferential distance \(y\) and the axial distance \(x\). The diffusivities in the \(x\)- and \(y\)- directions are \(D_x\) and \(D_y\), i.e., the orthotropic axes are in line with the \(x\)- and \(y\)- directions instead of being skewed as shown by Boutet de Monvel, et al (2007). The bleach is centered at the point \(x = 0\) and \(y = 0\). The axial distance to the end of the cell is \(a\) so the total length is \(2a\), and the circumferential distance to the opposite point is \(b\), so the diameter of the cell is \(2b/\pi\).

The initial bleach is approximated to be Gaussian in both \(x\)- and \(y\)- directions:

\[
I(x,y) = e^{-\frac{x^2}{2w_x^2} - \frac{y^2}{2w_y^2}}
\]  

(18)

which permits a different distribution in the \(x\)- and \(y\)- directions. Therefore, the solution of the diffusion equation is sought that satisfies the initial condition:

\[
\tilde{B}(x,y,0) = I(x,y)
\]  

(19)

and the end conditions of a lipid pool:

\[
\tilde{B}(\pm a, y, 0) = 0
\]  

(20)

and the circumferential condition of continuity:

\[
\frac{\partial \tilde{B}(x,b,t)}{\partial y} = 0
\]  

(21)

*Fourier series solution to diffusion equation*
The differential equation and the boundary conditions are satisfied by a double Fourier series in the form:

\[ \tilde{B}(x, y, t) = \sum_{m=1}^{N_x} \sum_{n=1}^{N_y} B_{mn} e^{-t/t_{mn}} \cos \left( \frac{(2m-1)\pi x}{2a} \right) \cos \frac{n\pi y}{b} \]  \hspace{1cm} (22)

where the time constants are:

\[ \frac{1}{t_{mn}} = D_x \left( \frac{(2m-1)\pi}{2a} \right)^2 + D_y \left( \frac{n\pi}{b} \right)^2 \]  \hspace{1cm} (23)

The number of harmonics used are \( N_x \) and \( N_y \) in the \( x \)- and \( y \)- directions. The Fourier coefficients \( B_{mn} \) are determined from the initial condition Eq. 19 in the standard procedure.

**Recovery calculation**

The recovery is measured by integrating the fluorescence over the rectangular region:

\[ -L_x < x < L_x, -L_y < y < L_y \]  \hspace{1cm} (24)

The recovery curve is then:

\[ F(t) = F(\infty) + \left( F(\infty) - F(0) \right) A(t) \]  \hspace{1cm} (25)

where the normalized bleaching is:

\[ A(t) = \frac{\int_{-L_y}^{L_y} \int_{-L_x}^{L_x} I(x, y) \tilde{B}(x, y, t) \, dx \, dy}{\int_{-L_y}^{L_y} \int_{-L_x}^{L_x} I(x, y) \tilde{B}(x, y, 0) \, dx \, dy} \]  \hspace{1cm} (26)

The initial post bleach measured value is \( F(0) \). If the lengths are known, then there are only three free parameters in this theoretical recovery curve - the diffusivities \( D_x \) and \( D_y \) and the limit \( F(\infty) \). The relation between the mobile fraction and the limit in the recovery is the same Eq. 12. We find that one recovery measurement is not sufficient to discriminate
$D_x$ and $D_y$. Consequently, we use isotropy with $D = D_x = D_y$. It appears that $D$ is the average of the actual values of $D_x$ and $D_y$.

Curve fits using Mathematica

We consider a cell with length 40 $\mu$m and diameter 8 $\mu$m and initial bleach width (FWHM) of around 0.8 $\mu$m and 2.2 $\mu$m in the $x$- and $y$- directions. The theoretical recovery can be written in Mathematica with just the two free parameters $D$ and $F(\infty)$. Then, the command “NonlinearModelFit” determines both parameters for a best fit to the measured recovery curve. A high number of harmonics provides a good approximation to the Gaussian distributions of initial bleach; we find that 40 in each direction is adequate.
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