TUNING OF THE OUTER HAIR CELL MOTOR BY MEMBRANE CHOLESTEROL
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Running title: Cholesterol modulates OHC function
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Cholesterol affects diverse biological processes, in many cases by modulating the function of integral membrane proteins. We observed that alterations of cochlear cholesterol modulate hearing in mice. Mammalian hearing is powered by outer hair cell (OHC) electromotility, a membrane-based motor mechanism that resides in OHC lateral wall. We show that membrane cholesterol decreases during maturation of OHCs. To study the effects of cholesterol on hearing at the molecular level, we altered cholesterol levels in the OHC wall, which contains the membrane protein prestin. We show a dynamic and reversible relationship between membrane cholesterol levels and voltage-dependence of prestin-associated charge movement in both OHCs and prestin-transfected HEK 293 cells. Cholesterol levels also modulate the distribution of prestin within plasma membrane microdomains and affect prestin self-association in HEK 293 cells. These findings indicate that alterations in membrane cholesterol affect prestin function and functionally tune the outer hair cell.

Cholesterol is an important component of the plasma membranes of most animal cells. It modulates the mechanical properties of the membrane and affects the function of membrane-associated proteins. Recent studies have shown modulation by membrane cholesterol of such diverse membrane proteins as rhodopsin (1,2), the serotonin receptor 1A (3) and serotonin transporter 5HT1 (4), the chloride channel ClC-2 (5), several classes of potassium channels (6,7), the nicotinic acetylcholine receptor (8) and several G-protein coupled receptors (GPCRs) (#, 9,10). These studies indicate that cholesterol may act by: 1) binding directly to and influencing the conformation and dynamics of membrane proteins (11-14); 2) by altering the biophysical and mechanical properties of membranes (15-19); and/or 3) promoting the formation of cholesterol-rich microdomains (9,20,21). These heterogeneous ordered microdomains contain distinctive lipid and protein compositions in which cholesterol may contribute as much as 50% of the membrane lipids (22-24). Cholesterol-rich membrane domains might serve to compartmentalize cellular processes, promote protein-protein and lipid-protein interactions, and thereby regulate protein function (23,25,26).

Cellular cholesterol levels are tightly regulated, and disruption of cholesterol homeostasis leads to a host of disease conditions. Several clinical and experimental
studies carried out using rabbits, chinchillas, guinea pigs, and human subjects have linked sensorineural hearing loss and/or increase of hearing thresholds to hypercholesterolemia (27-31). Reduction of cholesterol by statins or apheresis have been shown to delay hearing loss in mice (32) and improve hearing recovery in humans (33). A study of hypercholesterolemic humans indicated that the effects of cholesterol on hearing might involve effect(s) on non-linear mechanical processes in the cochlea (34).

A piezoelectric-like membrane-based motor in the outer hair cell (OHC) contributes to the exquisite sensitivity and frequency selectivity of mammalian hearing. This motor mechanism is required to counteract viscous damping in the fluid-filled cochlea, which would otherwise impair mechanical tuning. The OHC lateral wall is specialized for electro-mechanical force transduction (35,36). Here, the energy in the transmembrane electric field is converted into mechanical energy. The organization of the OHC lateral wall is unique among hair cells and among all adult mammalian cell types. It is an elegant, nanoscale (~100 nm thick), trilaminate structure. The outer and inner layers are the plasma membrane and subsurface cisternae (SSC), respectively, and sandwiched between them is a layer of cytoskeletal proteins called the cortical lattice (CL). The lipid composition of the plasma membrane and the SSC membranes is unknown but the constituent lipids are in the fluid phase allowing for free diffusion (37-39). Labeling studies suggest that lateral wall membranes contain less cholesterol than the OHC apical and basal plasma membranes (40-43). The relatively low cholesterol level of the OHC lateral wall plasma membrane is unusual among animal cells, and may serve to modulate the function of the membrane proteins that reside there. These proteins include a modified anion exchanger AE2 (44), the Glut5 sugar transporter (45-47), stretch activated ion channels (48-50), and prestin (45).

Prestin (SLC26A5), a critical component of the OHC lateral wall motor, is a polytopic integral membrane protein (45,51,52) and is essential for OHC electromotility and mammalian hearing (53). Prestin greatly increases charge movement into and out of, as opposed to through, the membrane (54,55). Intracellular anions such as chloride and bicarbonate have been shown to be the charge carrier (55) consistent with prestin’s membership in the SLC26A family of anion transporters (56). When transfected into several mammalian cell lines, prestin confers a voltage-dependent non-linear capacitance (NLC), the accepted electrical signature of electromotility (54,55) (see supplemental text for in-depth description).

Motivated by the clinical effects of cholesterol on hearing and the reduced cholesterol levels in the OHC lateral wall, we have explored the effect of cholesterol on hearing at the organ, cellular and molecular levels to clarify its biological basis of action. We observe that cholesterol affects otoacoustic emissions and functionally tunes non-linear mechanical processes in the OHC, most likely through its effects on the OHC membrane protein, prestin.

**EXPERIMENTAL PROCEDURES**

**Materials**

Methyl-β-cyclodextrin, water-soluble cholesterol (MβCD loaded with cholesterol), filipin, and BSA were obtained from Sigma (St. Louis, MO). Primers were obtained from Sigma Genosys. Anti-flotillin-1 antibody (1:250 working dilution) was purchased from BD Biosciences (San Jose, CA). Anti-HA (1:1000) was purchased from Cell Signaling Technology (Danvers, MA). Anti-GFP anti-mouse monoclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). AlexaFluor 594 phalloidin (1:200),
AlexaFluor 594 goat anti-mouse antibody (1:800) and Concanavalin A-AlexaFluor 350 conjugate (working concentration 50–200 µg/ml) were purchased from Molecular Probes (Carlsbad, CA). Peroxidase-labeled horse anti-mouse antibody was obtained from Vector Laboratories (Burlingame, CA). The ECL western blotting detection kit was obtained from Amersham (Piscataway, NJ).

**DPOAE measurements**

Mice used for DPOAE measurements were of a mixed genetic background derived from two strains, 129SvEv and C57B6/J and were 4–8 weeks old. Healthy mice were anesthetized with ketamine/xylazine and immobilized in a head holder. The pinna was resected and the middle ear bulla opened to expose the round window. An earbar connected to two speakers and a probe tip microphone was inserted into the ear canal to within 2 mm of the tympanic membrane. The cubic distortion product amplitude was measured using an F2 frequency of 20 kHz with F1=F2/1.2 (57). The intensities of the primary tones were equal. First, we ranged the primary tones from 20 dB to 80 dB in 10 dB steps in order to verify that there was no notch in the DPOAE amplitude curve between 50–70 dB. During the experiment, we set the primary tones to 60 dB SPL and the DPOAE amplitude was measured every 9 seconds. After a few minutes, a borosilicate micropipette with a tip diameter of ~ 50 µm containing the treatment solution (either 100 mM MβCD, 200 mM water soluble cholesterol, ~200 mM raffinose, or 10 mM water-soluble cholesterol) was carefully inserted through the round window membrane. The high concentrations of each treatment (in comparison with established in vitro studies) were chosen to compensate for dilution of the solutions in the mouse perilymph. The treatment solutions were allowed to diffuse passively into the perilymph. The middle ear space was monitored for fluid seepage and any fluid was carefully aspirated. DPOAE amplitudes were collected for up to 30 minutes. In some cases, at the conclusion of the experiment, the basilar membrane was perforated to eliminate DPOAEs, thereby verifying the measurements obtained. DPOAE amplitudes were then normalized so that the amplitude after the micropipette was inserted and all middle ear fluid was cleared was 0 dB. This time window is indicated as a gray box in each panel of Fig. 1.

**Outer hair cell isolation**

Albino guinea pigs of either sex weighing 200–300 gm and having a normal startle response to a handclap were decapitated. The temporal bones were taken and the middle ear bullae opened. The otic capsule was removed, and the spiral ligament was peeled off to expose the organ of Corti. The modiolus with the intact organ of Corti was removed from the temporal bone and subjected to mild trypsinization for ~10 minutes at room temperature, and trituration to detach OHCs. OHCs were plated onto the glass bottom of a coated microwell Petri dish (MatTek, Ashland, MA). Isolated cells were selected for study on the basis of standard morphological criteria within 4 hr of animal death. Under the light microscope, healthy cells display a characteristic birefringence, a uniformly cylindrical shape without regional swelling, a basally located nucleus, and no Brownian motion of subcellular cytoplasmic particles (58).

**Prestin constructs and transfection:**

Gerbil prestin was cloned into the pIRES-hrGFP vector (Stratagene, La Jolla, CA) as a HA-tag-fusion protein (HA-prestin), and into the pEGFP, pECFP, and pEYFP (Clontech, Mountain View, CA) vectors as a GFP-, CFP- or YFP-fusion protein (prestin-EGFP), as previously described (59-61). The prestin-ECFP and prestin-EYFP constructs
were modified by site directed mutagenesis (QuickChange mutagenesis kit, Stratagene, La Jolla, CA) to include a single amino acid substitution (A206K) on the CFP/YFP fusion protein, which renders CFP/YFP monomeric. The sequences of the constructs were verified using five overlapping sequencing primers. NLC measurements confirmed that all constructs used in this study are functional in HEK 293 cells. HEK 293 cell lines were transfected 24h after passage with preston-EGFP, preston-ECFP, preston-EYFP or HA-prestin at a 3:1 ratio of DNA with FuGene 6 (Roche Applied Science, Indianapolis, IN).

Cholesterol manipulations

In outer hair cells: Due to the sensitivity of outer hair cells to temperature and their deterioration with time after isolation, cholesterol manipulations were performed differently in OHCs than in HEK 293 cells. Cholesterol depletion was carried out by pipetting MβCD into the external solution in the dish containing hair cells at a final concentration of ~100 µM (one-hundredth that used in HEK cells, see below), and incubating at room temperature (see Fig. 3 for times of incubation). Higher concentrations of MβCD produced drastic morphological changes in OHCs and even cell death due to destabilization of the cholesterol-rich apical and basal membranes, causing the nucleus to be blown out of the cell. Cholesterol was loaded in a similar manner at a final concentration of 1 mM of MβCD containing cholesterol (also referred to as water-soluble cholesterol). In both cases, treatment was carried out after forming a whole-cell patch on an OHC, and capacitance recordings were taken throughout the incubation time.

In HEK 293 cells: Steady-state electrophysiological measurements were performed on preston-transfected HEK 293 cells, 48 hours post-transfection, after treatment with 10 mM MβCD or water-soluble cholesterol (at a 10mM MβCD concentration) for 30 mins at 37°C. The effects of cholesterol manipulations were followed kinetically in HEK 293 cells by pipetting MβCD or water-soluble cholesterol into the external solution at a final concentration of 10 mM after obtaining a whole cell patch. HEK 293 cells did not show morphological changes as seen in OHCs upon cholesterol depletion. Filipin labeling of untreated, cholesterol depleted, and cholesterol loaded HEK 293 cells (Supp. Fig. 1) shows changes in filipin fluorescence signal with cholesterol manipulations, confirming that cholesterol levels are altered by our treatments.

Electrophysiological measurements

Electrophysiological data were obtained from cells using the whole-cell voltage clamp technique. Our recording techniques are fully described earlier (60) a brief description follows. Culture dishes containing transfected cells were placed on the stage of an inverted microscope (Carl Zeiss, Gottingen, Germany) under 100X magnification and extensively perfused with the extracellular solution containing Ca²⁺ and K⁺ channel blockers prior to recording. All recordings were conducted at room temperature (23°C ± 1°C). Patch pipettes (quartz glass) with resistances ranging from 2 – 4 MΩ were fabricated using a laser-based micropipette puller (P-2000, Sutter Instrument Company, Novato, CA), and filled with an intracellular solution, also containing channel blockers. Cell membrane admittance, Y was measured with the patch-clamp technique in the whole-cell mode using a DC voltage ramp with dual-frequency stimulus (62) from -0.14 to 0.14 V with a holding potential of 0 volts, and the cell parameters were calculated from the admittance as described earlier (63). The conductance, b was also determined experimentally with a DC protocol, as described earlier (60).
In all representations, capacitances were normalized with respect to baseline capacitance (taken as the capacitance at 0.1 V), and peak capacitance (differs according to treatment), as follows:

\[
C_{\text{norm}} = \frac{C(V) - C_{\text{baseline}}}{C_{\text{baseline}}}
\]

\[
C_{\text{final}} = \frac{C_{\text{norm}}}{C_{\text{norm}_{pkc}}}
\]

where \( C(V) \) is the capacitance at voltage \( V \), \( C_{\text{baseline}} \) is capacitance at baseline voltage (defined above), and \( C_{\text{norm}_{pkc}} \) is equal to \( C_{\text{norm}} \) at \( V_{pkc} \).

**Tissue preparation and filipin labeling of mouse OHCs**

P6, P12 and adult ICR mice were sacrificed by cervical dislocation and decapitation. The temporal bone was removed and the bony capsule was stripped in fresh cold HBSS (GIBCO, Invitrogen, CA). The membranous labyrinth was exposed in DMEM containing 10%FBS. The sensory epithelium was isolated and affixed to round glass coverslips coated with Cell-Tak™ (BD Biosciences, San Jose, CA). The tissue was washed twice with PBS, fixed with 4% PFA for 30 minutes, and stained with filipin dye (4mg/ml) and AlexaFluor594 phalloidin for 30 minutes. The samples were then washed twice with PBS, mounted on glass slides with Fluoromount G antifade reagent and sealed with nail polish. Images were captured on a Zeiss Axioplan microscope (Carl Zeiss Optics Company, Jena, Germany) with 63X objective and analyzed with Applied Precision SoftWoRx image restoration software. Images were also obtained using a Zeiss LSM 510 confocal microscope with 63X objective, and analyzed using Zeiss AIM imaging software.

**Membrane Fractionation**

Cell membranes were fractionated as described by Vetrivel, et al. (64). Briefly, HEK 293 cells expressing HA-prestin, treated with or without MβCD, or with water-soluble cholesterol for 30 mins at 37°C. Cells were then washed with PBS, stained with ConA-AlexaFluor 350 conjugate (Molecular Probes, Carlsbad, CA) for 1 hr on ice, washed with PBS again, and then permeabilized with PBS-Triton X-100 before fixing with 4% paraformaldehyde in PBS. The cells were then stained with anti-HA antibody (1:1200; Cell Signaling Technology, Inc., Denver, MA), followed by AlexaFluor594 goat anti-mouse secondary antibody (1:800; Molecular Probes, Carlsbad, CA). Coverslips were mounted inverted on glass slides with Fluoromount G antifade reagent (Electron Microscopy Sciences, Hatfield, PA) and fluorescent images captured on a Zeiss LSM 510 deconvolution microscope (Carl Zeiss Optics Company, Jena, Germany) with 63X objective and analyzed with Applied Precision SoftWoRx image restoration software. Images were also obtained using a Zeiss LSM 510 confocal microscope with 63X objective, and analyzed using Zeiss AIM imaging software.

**Immunofluorescence and Imaging**

HA-prestin transfected cells on coverslips were either treated with or without 10 mM MβCD or water-soluble cholesterol for 30 mins at 37°C. Images were captured on a Zeiss LSM 510 deconvolution microscope (Carl Zeiss Optics Company, Jena, Germany) with 63X objective and analyzed with Applied Precision SoftWoRx image restoration software. Images were also obtained using a Zeiss LSM 510 confocal microscope with 63X objective, and analyzed using Zeiss AIM imaging software.

**Crosslinking**

Forty-eight hours post-transfection with HA-prestin, HEK 293 cells were either
Cholesterol modulates OHC function

treated with methyl-β-cyclodextrin (MβCD) or with water-soluble cholesterol for 30 min at 37°C, or left untreated, before gentle harvesting by scraping into 1 mL of PBS, pH 8.0. The cells were pelleted (2000 x g for 5 min) and incubated with various concentrations of crosslinker bis (sulphosuccinimidyl) suberate (0.078 mM to 5 mM of BS³) or without crosslinker for 30 minutes at room temperature. Reactions were quenched with 50 mM Tris, pH 7.5. The amount of protein in each sample was measured and normalized prior to gel loading. Samples were mixed with 8% 2x SDS sample buffer and incubated for 30 minutes at RT before fractionation by a 4-8% Tris-glycine PAGE and analysis by Western blotting.

**Fluorescence Resonance Energy Transfer**

Fluorescence resonance energy transfer (FRET), implemented on a Zeiss LSM 510 confocal microscope (Carl Zeiss Optics Company, Jena, Germany) was used to measure the degree of prestin self-association following cholesterol perturbations. HEK cells were co-transfected with prestin-CFP (donor) and prestin-YFP (acceptor). Both the CFP and YFP fusion proteins had an engineered mutation, A206K, that prevents CFP and YFP dimerization, to allow easier interpretation of FRET results. Details of the acceptor photobleach technique utilized have been published previously (61). Briefly, a region of interest (ROI) on the cell membrane, exhibiting even, membrane localized fluorescence, was bleached to remove YFP signal. CFP fluorescence intensity in the bleached ROI was measured pre- and post-bleach to arrive at the value of FRET efficiency (Eᵣ) from CFP to YFP. CFP intensity in an adjacent unbleached ROI was measured pre- and post-bleach to derive control (Cᵣ) values for each FRET measurement. For detailed description of methods, refer to Greeson et al., 2006 (61).

**Quantification of Puncta**

Membrane segments used for FRET experimentation were also utilized in puncta quantification. Confocal images of prestin-YFP in living HEK 293 cell membranes were cropped to ~25 by 75 pixel (1 pixel = 0.14 µm) membrane containing regions (slice thicknesses = 3.3 µm) and analyzed using the Matlab (The Mathworks, Natick, MA) edge detection filter. The filter generated an image the same size as the input image composed of ones where edges were detected and zeros everywhere else (Supplementary Fig. 3). Edge detection is based on one of six specific methods, and the most advanced of the available methods, the Canny method, was used in this work. The Canny method identifies local maxima in the images gradients and uses two different threshold values to define both strong and weak edges. A weak edge is only included in the output image if it is connected to a strong edge. The use of two threshold values insures that this method is less likely to detect false positive weak edges. Following application of the filter, the output image was analyzed for the presence of puncta. Puncta identification was guided by the output image of the detection filter and supported by the membrane region image (Supplementary Fig. 3). The results of this analysis are shown in Fig. 5.

**Statistical analysis**

Vpkcs and capacitance gains in OHCs and prestin-transfected HEK 293 cells subjected to different treatments (untreated, cholesterol loading, cholesterol depletion) were compared using two-tailed t-tests. Statistical significance (in comparison to untreated) is indicated in Table 1. Two-way ANOVA was also used to evaluate statistical significance of FRET values in comparison to control FRET for each treatment, as well as to FRET values of untreated cells. Statistical
significance is indicated in Fig. 7D and Table 1 using asterisks.

**RESULTS**

Changes in cholesterol alter the amplitude of Distortion Product Otoacoustic Emissions (DPOAEs). To study the effects of cholesterol on hearing at the organ level, we evaluated cochlear function in vivo by measuring DPOAE amplitudes during cholesterol alteration (Fig. 1). Cholesterol depletion resulted in a 20 dB decrease in DPOAE amplitudes. Cholesterol loading, on the other hand, resulted in an initial 2-3 dB increase in DPOAE amplitude, followed by a decrease of up to 20 dB (Fig. 1). These results confirm that cholesterol modulates hearing. Since DPOAE amplitudes reflect OHC electromotility, the level of cholesterol in the OHC lateral wall may be functionally relevant.

**OHC lateral wall cholesterol content decreases with maturation.** To visualize cholesterol in the OHC lateral wall, we used filipin labeling of mouse OHCs (Fig 2). Filipin labeling clearly indicates membrane cholesterol content, as shown by filipin-labeling of untreated, cholesterol-depleted and -loaded HEK 293 cells (Supplementary Fig. 1). OHCs from P6 and P12 mice showed distinct filipin labeling of the lateral wall, compared to cytoplasm. However, OHCs from adult mice showed intracellular filipin staining surrounded by a region of lower staining corresponding to the membrane (Fig. 2, right panels). The filipin staining patterns are clearly visible in the pixel intensity graphs (Fig. 2, bottom row), which plot pixel intensities along the diameter of a single OHC in each case. These data indicate that the cholesterol in the OHC lateral wall is initially high, and decreases during development. This time frame parallels the onset and maturation of OHC electromotility (65), which includes a depolarizing shift in preasin-associated charge movement.

Alterations in cholesterol content modulate non-linear capacitance in isolated OHCs. We directly evaluated the effect of cholesterol on preasin-associated charge movement at the cellular level by altering cholesterol content in isolated guinea pig OHCs. We observed characteristic bell-shaped voltage-dependent capacitance (NLC) with a peak, $V_{pkc}$, at about -0.050 V in untreated OHCs (Fig. 3A), similar to that reported earlier (66). Depletion of cholesterol shifted $V_{pkc}$ in the depolarizing direction to about +0.080 V, while loading excess cholesterol shifted $V_{pkc}$ towards more hyperpolarizing voltages (< -0.130 V, Fig. 3A). Kinetic studies of these phenomena indicated that the shift in $V_{pkc}$ occurred within minutes of adding water-soluble cholesterol (loading) or MβCD (depletion) (Fig. 3B). Further, the effects of depletion and loading were reversible, and the reversal of the $V_{pkc}$ shift was equally rapid (Fig. 3C). These data (statistics represented in Table 1) indicate a direct and dynamic correlation between OHC membrane cholesterol content and $V_{pkc}$.

Changes in membrane cholesterol reversibly alter $V_{pkc}$ in preasin-transfected HEK 293 cells. We next investigated preasin-specific effects of cholesterol in HEK 293 cells. The $V_{pkc}$ of preasin-transfected HEK 293 cells was approximately – 0.070 V (Fig. 4A). Upon cholesterol depletion, the $V_{pkc}$ shifted towards depolarized voltages, with an average peak at +0.004 V (Fig. 4A). On the other hand, upon cholesterol loading, the $V_{pkc}$ shifted towards hyperpolarized voltages, with an average value of about – 0.116 V (Fig. 4A). Importantly, these effects are reversible. Cholesterol depletion followed by loading, as well as cholesterol enrichment followed by depletion, both shifted the peak voltage...
Cholesterol modulates OHC function

The kinetics of these processes were similar to those measured for OHCs, with changes in the Vpkc occurring within minutes of addition of cholesterol or MβCD (Fig. 4C) and the process was rapidly reversible (Fig. 4D). Comparisons of changes in Vpkc in HEK 293 cells and OHCs are shown in Table 1. Our results demonstrate that changes in cholesterol alter prestin-associated charge movement in HEK 293 cells, as in the native environment of the OHC, indicating direct molecular level effects of cholesterol on prestin function.

Cholesterol modulates the distribution of prestin in membrane microdomains. We explored the possibility of cholesterol modulating prestin’s presence in membrane microdomains by analyzing membrane localization and distribution of prestin in HEK 293 cells (Fig. 5). Prestin colocalizes with the plasma membrane (Fig. 5A) consistent with earlier observations (59,60) and is expressed in foci suggestive of localization to membrane microdomains (Fig. 5A & 5D). Alterations in cholesterol content change the distribution of the foci: upon cholesterol depletion (Fig. 5B & 5E), prestin showed a less punctate (more uniform) membrane distribution, while upon cholesterol loading (Fig. 5C & 5F), the number of foci increased (Fig 5G). Quantification of puncta (Fig. 5G) was based on multiple images of membrane segments from different batches of treated HEK 293 cells (Supp. Fig. 3). In addition, time-lapse images of a single transfected cell taken during the course of depletion or loading show clear decrease and increase in puncta (Supp. Fig. 4). These results demonstrate that cholesterol influences prestin distribution within the membrane.

In order to assay prestin localization in membrane microdomains, we characterized detergent-resistant membrane extracts isolated from prestin-transfected HEK 293 cells. As expected, prestin was detected in the dense ER membrane fractions (lanes 8-10, Fig. 6A), which co-fractionated with the ER markers (59). Prestin was also seen in the less dense plasma membrane fractions and predominantly localized in membrane microdomain fractions (fractions 4 and 5, Fig. 6A), where it co-fractionated with flotillin-1, a microdomain marker and structural component (67). Upon cholesterol depletion prestin, but not flotillin, redistributed out of the microdomain fractions (Fig. 6B). Upon cholesterol loading, prestin remained in microdomain fractions (Fig. 6C) with higher intensities of all bands (Fig. 6C). These data indicate that prestin can localize to membrane microdomains, and that cholesterol modulates its distribution in these domains.

Manipulation of cholesterol content alters prestin associations. Localization to microdomains raises the possibility that prestin may interact with itself or other proteins. In order to determine if prestin-prestin interactions were altered by cholesterol we analyzed prestin complex associations by crosslinking (Fig. 7). In the absence of crosslinker (lane 1), a weak prestin dimer band exists in untreated cell extracts (Fig. 7A). This band disappears upon cholesterol depletion (Fig. 7B), and is stronger upon cholesterol loading (Fig. 7C), indicating cholesterol favors prestin self-association in HEK 293 cells independent of crosslinking. Prestin dimers were present at relatively low BS3 crosslinker concentrations (Lane 2, Fig. 7A) and the dimer band intensified with increasing crosslinker concentrations (Lanes 3-7, Fig. 7A). Cholesterol depletion caused a decrease in prestin crosslinking, with higher concentrations of BS3 required to trap prestin as dimers (compare lanes 2-3 in Fig. 7B with lanes 2-3 in Fig. 7A). In contrast, loading cholesterol into the membrane caused the appearance of oligomeric protein bands even
in the absence of crosslinker (Lane 1, Fig 7C) and bands intensified with increasing crosslinker (lanes 2-8, Fig 7C). In conjunction with PFO-PAGE gels (Supp. Fig. 2A) and co-immunoprecipitation experiments (Supp. Fig. 2B), and in accordance with earlier observations (61,68,69), these data provide strong evidence of prestin self-association, and indicate that prestin self-association increases with cholesterol content.

Further evidence of cholesterol dependent prestin self-association was obtained using acceptor photobleach fluorescence resonance energy transfer (apFRET). We found an average FRET efficiency of 6.7% (Fig 7D) in live, untreated HEK 293 cells co-transfected with prestin-CFP and prestin-YFP. Cholesterol enrichment increased average FRET efficiency to 8.4%, while cholesterol depletion resulted in a marked decrease of FRET efficiency to 0.5% (Fig. 7D), indicating significant loss of prestin self-association. In this case, the measured FRET efficiency is indistinguishable from corresponding control efficiency measurements (p>0.05). To determine whether this elimination of prestin self-association is reversible, cells were first depleted of membrane cholesterol and then reloaded. Upon reloading, FRET efficiency returned to 6.8% (Fig. 7D). These results confirm that modulation of prestin self-association by membrane cholesterol is dynamic and reversible.

Our organ, cellular and molecular level studies greatly expand our understanding of the role of cholesterol in tuning the functionality of the outer hair cell motor.

**DISCUSSION**

Cholesterol is crucial for membrane organization and dynamics, and in the regulation of membrane protein sorting and function. Considering that electromotility is based in the highly specialized and cholesterol-poor OHC lateral membrane, the established link between elevated cholesterol and auditory dysfunction (27,28,34,70,71) suggests direct effects of cholesterol on the OHC membrane and proteins present therein. Models for OHC electromotility based solely on the membrane’s electromechanical transduction capabilities have been proposed (72,73). Following the discovery of the membrane protein prestin, numerous studies have demonstrated alterations of membrane material properties affect prestin function and/or OHC electromotility (67,74-79). These studies point to a dynamic interplay between prestin and the membrane in the generation of non-linear capacitance and electromotility; our study further characterizes this dynamic relationship.

**Cholesterol effects on otoacoustic emissions correlate to effects on NLC:** Cochlear cholesterol alterations influence DPOAE amplitudes (Fig. 1). Manipulating cholesterol in isolated guinea pig OHCs revealed a dynamic and reversible relationship between membrane cholesterol content and prestin-associated charge movement, with similar kinetics (Fig. 3). Cholesterol effects on DPOAEs may result from the observed effects on prestin-associated charge movement as follows. In normal untreated OHCs, the NLC peak is at approximately -0.050 V; therefore in the cell’s range of receptor potential (nominally between -0.060 and -0.080 V) capacitance is sub-maximal. Shifting the NLC peak in the depolarizing direction (as upon cholesterol depletion) would result in a progressive lowering of capacitance and electromotility in the range of the cell’s receptor potential. This would reduce DPOAE amplitudes. On the other hand, shifting the NLC peak in the hyperpolarizing direction would result in an initial increase of capacitance at the cell’s
receptor potential followed by a decrease. Electromotility and DPOAE amplitudes would follow this pattern. This relationship is schematically presented in Fig. 8.

**OHC function may be modulated by cholesterol reduction during maturation:** We show a lowering of membrane cholesterol with maturation (Fig. 2). During the post-natal maturation of rodent OHCs, the distribution of prestin in the lateral wall is initially inhomogeneous (45,65). Concurrent with prestin distribution becoming homogenous, maturation of electromotility and non-linear capacitance is observed, which includes a shift in \( V_{\text{pkc}} \) from an immature hyperpolarized value to the normal adult value (45,65). Both effects may result from a decrease in OHC membrane cholesterol levels with maturation. Our data suggest that the reduction in membrane cholesterol with maturation helps to tune the membrane-based motor to operate at maximal gain in the OHC receptor potential range.

**HEK 293 cells provide a model system for studying prestin function:** Cholesterol manipulations in prestin-transfected HEK 293 cells (Fig. 4) produced qualitatively similar results as in OHCs (Fig. 3; Table 1), validating the use of HEK 293 cells as a model system. The difference between \( V_{\text{pkc}} \) in cholesterol-depleted OHCs and HEK 293 cells may be due to structural differences (membrane tension, turgor pressure), or differences in cholesterol homeostasis mechanisms, between the two cell types, which cause similar trends but different magnitudes in the effects of depletion.

Prestin-transfected HEK 293 cells allowed for histological and biochemical analyses following alterations in cholesterol. Prestin appears to be present in foci characteristic of membrane microdomains in HEK 293 cells (Figs. 5 and 6). Similar foci have not been observed in the adult OHC lateral wall membrane. Perturbing cholesterol content alters the distribution of prestin; prestin shifts out (cholesterol depletion, Fig. 6B) or remains in (cholesterol enrichment, Fig. 6C) the microdomain fractions, suggesting that prestin is capable of localizing to cholesterol-rich microdomains. In addition, a quantitative correlation exists between cholesterol content and the number of prestin puncta in the membrane; cholesterol depletion results in a reduction, while enrichment causes an increase in number of foci (Fig. 5G).

Recent studies have suggested that prestin may self-associate and dimerize (61,68,69). We have obtained further evidence of prestin self-association using a crosslinking reagent, which revealed the presence of prestin-prestin interactions that are decreased upon cholesterol depletion and increased upon cholesterol addition (Fig. 7, A-C). Further, FRET measurements provide direct evidence of the significant and reversible effect of cholesterol on prestin self-associations (Fig. 7D). In light of these data, the low cholesterol levels on the mature OHC lateral wall are consistent with the homogeneous distribution of prestin.

**Mechanism of cholesterol effects:** The effects of cholesterol on membrane protein function have been the subject of numerous recent studies, and several mechanisms have been put forth to explain cholesterol effects. In addition to effects on membrane material properties such as viscosity, elasticity, compressibility and stiffness (80), cholesterol levels in the membrane influence the formation of ordered microdomains (81,82), and partitioning of proteins into these domains, by altering the membrane’s bending modulus (16) and thereby influencing hydrophobic mismatch (15). The same factors may explain the effect of cholesterol content on prestin-associated charge movement. Since cholesterol is known to modulate membrane material properties, which in turn affect the dynamics of membrane proteins,
Cholesterol modulates OHC function

Cholesterol-dependent changes in membrane stiffness or curvature could alter the dynamic fluctuations of prestin as would changes in membrane dipole potential and lipid packing density (19). Several studies in the OHC have correlated changes in membrane tension, stiffness and mechanics to changes in the $V_{pkc}$ and electromotility (67,75-77,79). Molecular dynamics simulations suggest cholesterol affects lipid lateral pressure profiles (17,18), and this would impact the prestin conformational change that is assumed to accompany charge movement.

Cholesterol-induced $V_{pkc}$ shifts in both OHCs and HEK 293 cells are larger than those resulting from previous manipulations: these include exogenous chlorpromazine (77), fructose (46) and increasing intracellular pressure (76,83), which shift $V_{pkc}$ towards depolarizing potentials; and decreasing intracellular pressure and exposure to the lipophilic ion, tetraphenylborate (TPB$^-$) (84), which move $V_{pkc}$ in the hyperpolarizing direction. Because the magnitude of the $V_{pkc}$ shifts is significantly greater than in previous manipulations that are known to change the material properties of the membrane, we must consider the possibility that the $V_{pkc}$ is also a function of self-association. This contribution would reflect the relative amounts of monomers versus higher-order oligomers, where the monomeric form shifts $V_{pkc}$ to depolarizing, while higher-order oligomers shifts $V_{pkc}$ to hyperpolarizing voltages. The effect of cholesterol on both prestin self-association and on prestin function is reversible, indicating a dynamic interaction of prestin with membrane components.

Cholesterol also has a propensity to localize to membrane microdomains. Prestin is present in microdomains in HEK 293 cells, and its presence in these localized domains may facilitate its interaction with itself, or with other proteins. It is likely that prestin exists in a dynamic equilibrium between monomeric, dimeric and perhaps higher-order oligomeric forms. The effect of cholesterol might be to ‘cluster’ prestin molecules, shifting the equilibrium towards dimeric or oligomeric species. Our data indicate increased self-association in the presence of increased cholesterol. The low cholesterol level observed in the mature OHC lateral wall suggests a preference for lowered prestin self-association, the functional consequences of which remain to be studied.

In summary, our study integrates systems-level, cellular and molecular data to investigate the role of cholesterol in modulating the mechanical aspects of mammalian hearing. We have characterized interrelationships between prestin-prestin interactions and prestin-membrane interactions. Whether the effect of cholesterol is predominantly through formation of functionally distinct microdomains, changes in membrane material properties, or both, the observable effects of changing the cholesterol content are a change in prestin self-association, a reversible shift in $V_{pkc}$, and changes in otoacoustic emissions. This reinforces the concept of the molecular motor driving electromotility as an interdependent entity with protein and membrane components working cooperatively to achieve non-linear charge movement and mechanical motion.
REFERENCES


Cholesterol modulates OHC function


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# List of Abbreviations: GPCR – G-protein coupled receptor; OHC – outer hair cell; SSC – subsurface cisternae; CL – cortical lattice; MβCD – methyl beta cyclodextrin; NLC – non-linear capacitance; GFP, CFP, YFP – green, cyan or yellow fluorescent protein; HA – haemagglutinin; DPOAE – distortion product otoacoustic emissions; SPL – sound pressure level; FRET – fluorescence resonance energy transfer; ROI – region of interest; EDTA – ethylene diamine tetraacetic acid; PMSF – phenylmethanesulphonylfluoride; BS3 – bis (sulphosuccinimidyl) suberate; SDS - sodium dodecyl sulfate; PFO – perfluoro octanoic acid.
Figure Legends

Figure 1. Effect of cochlear cholesterol loading/depletion on DPOAE amplitude. DPOAEs were measured continuously during the delivery of cholesterol, MβCD or control solutions. The solid orange line indicates average noise and the dotted orange line indicates the noise threshold, calculated as three standard deviations above average noise. The gray box indicates time that the micropipette was inserted through the round window membrane and any middle ear fluid aspirated. During this time, the data demonstrate artifactual changes and changes in middle ear mechanics. After this time, the DPOAEs amplitudes are referenced to 0 dB and changes in the data represent changes in cochlear otoacoustic emissions. Top: Injection of 200 mM raffinose (blue) or a dilute (10 mM) MβCD/cholesterol solution (green) caused no changes in DPOAE amplitudes after the micropipette was inserted into the round window and all middle ear fluid aspirated. Middle: Cholesterol depletion (using 100 mM MβCD) caused progressive decreases in DPOAE amplitudes. Bottom: Cholesterol loading (using 200 mM water-soluble cholesterol) caused initial slight (2-3 dB) increases in DPOAE amplitudes, followed by a progressive decrease. The same traces, on a magnified Y-axis, are shown in the inset. DPOAE recordings during depletion showed higher noise levels (2.4 dB standard deviation from the average trendline) post delivery, when compared to loading (0.34 dB) and control treatments (0.2 dB), or to intrinsic noise in the recordings before delivery (0.42, 0.89 and 0.31 for control, depletion and loading treatments respectively). Arrowheads indicate times at which the round window was perforated to eliminate DPOAEs.

Figure 2. OHC lateral wall cholesterol content decreases with maturation. Organ of Corti from P6 (left), P12 (middle) and adult (right) mice. Tissue was stained with phalloidin (red; to visualize actin in stereocilia) and filipin (blue; to label cholesterol) and imaged using deconvolution microscopy. Sections through the cuticular plate (top row), lateral wall (middle row) and infra-nuclear region (bottom row) show marked filipin staining in the OHC lateral wall in P6 and P12 versus adult mice. The single row of inner hair cells (IHC) and the three rows of outer hair cells (1, 2 and 3) are indicated in each set of panels. Individual OHCs shown magnified in the insets are highlighted with asterisks. Pixel intensities along the indicated line in each of these insets are plotted as bar graphs in the bottom row. The gray bar in each of the graphs represents approximately 8 microns, the average diameter of a single OHC. Shown are representative data from a single experiment; the experiment was repeated two times.

Figure 3. Cholesterol levels affect Vpke of non-linear capacitance in outer hair cells. A) The peak (Vpke) of non-linear capacitance (NLC) is at about -0.050 V in control untreated OHCs (black trace). Vpke shifts to depolarized voltages upon cholesterol depletion (100 µm MβCD; red trace) and hyperpolarized voltages upon cholesterol loading (1 mM water-soluble cholesterol; green trace). Traces have been normalized relative to peak capacitance. B) Vpke begins to change within minutes after addition of MβCD (●) or water-soluble cholesterol (●). Untreated cells (●) show no change over a comparable time course. Shown are Vpke readings from the same cell as a function of time post-treatment. C) Reversibility of Vpke shifts: Shown are changes in Vpke of a single patched cell upon depletion of cholesterol (●), followed by loading (●). Arrows indicate time of treatment in panels B and C. Shown are representative data from single cells; sample sizes are indicated in Table 1.
Figure 4. Cholesterol affects membrane capacitance of HEK 293 cells expressing prestin. A) The $V_{pkc}$ is at about -0.070 V in untreated cells (black trace). The $V_{pkc}$ shifts to depolarized voltages upon cholesterol depletion (10 mM MβCD; red trace, red arrow) and hyperpolarized voltages upon cholesterol loading (10 mM water-soluble cholesterol; green trace, green arrow). Linear capacitance from an untransfected cell is shown for comparison (gray trace). B) The effect of cholesterol levels on $V_{pkc}$ shifts is reversible. Upon cholesterol depletion and reloading (red trace, red arrow) the $V_{pkc}$ shifts back to a normal voltage. Similarly, upon cholesterol loading followed by depletion (green trace, green arrow), the $V_{pkc}$ shifts back from hyperpolarized voltages to a normal value. Shown are representative average traces from single cells. Traces have been normalized to the capacitance at $V_{pkc}$. C) $V_{pkc}$ changes after addition of MβCD (●) or water-soluble cholesterol (●). Shown are $V_{pkc}$ readings from the same cell as a function of time post-treatment. D) Reversibility of $V_{pkc}$ shifts: Shown are changes in $V_{pkc}$ of a single patched cell upon addition of cholesterol (●), followed by depletion (●). Arrows indicate time of treatment in panels C and D. Shown are representative data from single cells; sample sizes are indicated in Table 1.

Figure 5. Prestin is expressed in punctate foci in the HEK 293 membrane. Immunofluorescence staining of HA-prestin transfected HEK 293 cells was used to visualize membrane distribution and localization of prestin. Representative deconvolution images (A-C) show prestin fluorescence (red) coincides with that of concanavalin-A (blue), a membrane marker. Enlarged images of the membrane are shown in insets. The bottom row (D-F) contains representative enlarged confocal images of the membrane of prestin-YFP transfected HEK 293 cells, showing puncta (arrowheads). A & D: HEK 293 cells transfected with prestin show punctate foci (arrowheads) of prestin fluorescence in the membrane. B & E: Depletion of cholesterol by 10 mM MβCD causes a less punctate, more uniform prestin labeling. C & F: Loading excess cholesterol (10 mM water-soluble cholesterol) causes an increase in number of punctate foci. G: Quantification of number of foci. Puncta in membrane regions were quantified as described using multiple images of membrane segments from different batches of treated HEK 293 cells.; the graph represents average number of puncta per 10.5 μm of membrane, calculated from several live confocal images of membrane segments. Images used in puncta quantification are shown in Supp. Fig. 3; time-lapse images of a single transfected cell over the course of depletion and loading treatments are shown in Supp. Fig. 4.

Figure 6. Cholesterol affects membrane distribution of prestin A) Sucrose density gradient fractionation of membranes from HA-prestin-transfected HEK 293 cells. HA-prestin colocalizes with flotillin-1, a membrane microdomain marker (lanes 4/5). B) Depletion of cholesterol with 10 mM MβCD causes a redistribution of HA-prestin into heavier membrane fractions. C) Cholesterol enrichment (10 mM water-soluble cholesterol) enhances colocalization into membrane microdomain fractions. The arrowhead and black arrow point to unglycosylated and glycosylated monomeric prestin, respectively. The white arrow points to oligomeric species. Shown are representative data from a single experiment; the experiment was repeated at least three times.

Figure 7. Effect of cholesterol on prestin self-association. A) HA-prestin is crosslinked as dimers and oligomers with increasing concentrations (0, 0.078, 0.16, 0.32, 0.65, 1.25, 2.5 and 5 mM in lanes 1–8, respectively) of the membrane-impermeable agent bis (sulphosuccinimidy)
suberate (BS₃).  B) Cholesterol depletion using 10 mM MβCD causes a reduction in crosslinking; higher concentrations of BS₃ are required for dimer formation.  C) Cholesterol loading using 10 mM water-soluble cholesterol causes an increase in crosslinking; oligomer bands appear even in the absence of crosslinker (Lane 1).  M, D, and T denote monomeric, dimeric and trimeric prestin bands respectively (based on molecular weight).  D) Acceptor photobleach FRET measurements to evaluate prestin self-association in live HEK 293 cells.  apFRET efficiencies (■) and control (unbleached) FRET values (■) measured from untreated (n=22), cholesterol depleted (n=20), cholesterol loaded (n=16), and depleted and reloaded (n=23) prestin-expressing HEK cells.  Statistical significance (in comparison to control FRET for each treatment) is represented; asterisks indicate p-value - *<0.05, **<0.01. Shown are representative data from a single experiment; the experiment was repeated at least three times.

**Figure 8. Schematic representation of correlation between NLC peak shifts and electromotility.** The nonlinear capacitance of untreated OHCs has a peak at about -0.050 V, slightly depolarized from the cell’s resting potential.  The corresponding capacitance in the operating range (receptor potential) of the cell (indicated by gray box and sinusoidal wave) is therefore slightly sub-maximal.  Upon depletion, the peak shifts further away from this operating range, resulting in a progressive reduction in capacitance in this range.  Upon loading, the peak initially shifts into the operating range, resulting in small increase in capacitance, and then shifts beyond the operating range resulting in a decrease of capacitance in the range.  Electromotility and otoacoustic emissions may be presumed to follow the same pattern.

**Supplementary Figure 1. Filipin labeling and cholesterol quantification of HEK 293 cells.** Untreated (A, B), cholesterol depleted (10 mM MβCD; C, D), and cholesterol loaded (10 mM water-soluble cholesterol; E, F) HEK 293 cells were fixed and labeled with filipin.  The pixel intensities of the images were measured using NIH Image software and graphed (G); intensity of the filipin stain is lower in depleted cells than in untreated or loaded cells.  All samples were processed together and individual panels have not been altered relative to one another.  H: Quantification of cholesterol content in membrane fractions of untreated, depleted and loaded HEK 293 cells was performed using the Amplex Red cholesterol assay (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions.  Depleted cell membranes show an five-fold reduction in cholesterol content, while loaded cells show a 2-fold increase.

**Supplementary Figure 2. Prestin self-associates to form dimers and higher order oligomers.** A) Oligomers of prestin-GFP and HA-prestin (not shown) can be visualized when solubilized with PFO and fractionated on PFO-PAGE gels.  B) Prestin-GFP and HA-prestin associate when co-transfected into HEK 293 cells; cell extracts were immunoprecipitated with anti-HA and the coimmunoprecipitated prestin-GFP was visualized by Western blotting with anti-GFP antibody.  Arrowheads indicate unglycosylated and glycosylated prestin, respectively.  The first three lanes represent co-immunoprecipitated prestin-GFP from HEK 293 cells transfected with increasing ratios of prestin-GFP to HA-prestin, while the fourth lane shows no prestin-GFP is co-immunoprecipitated using anti-HA antibody from control prestin-GFP-transfected cells.

**Supplementary Figure 3. Edge detection of membrane puncta.** The top images in each panel represent pixel intensities of membrane segments of prestin-YFP-transfected HEK 293
cells. The bottom images of each panel represent puncta edges detected by the Matlab edge detection filter. Panels represent membrane segments of untreated (a-c), depleted (d-f) and loaded (g-i) cells. Puncta were quantified based on the edge detection output (bottom images of each panel). The number of puncta detected in each represented panel are as follows, from panels a to i respectively: 2, 3, 0, 0, 0, 5, 4 and 2.

**Supplementary Figure 4.** Effects of cholesterol depletion and loading on a single prestin-transfected cell. (a) Cell before treatment. The white box indicates the membrane shown enlarged in bottom left corner. (b-f) Depletion at 2, 5, 10, 20 and 30 minutes, respectively. (g-k) Subsequent cholesterol reloading at 2, 5, 10, 20 and 30 minutes respectively. Disappearance and reappearance of punctate clusters are clearly seen. The cell shown in representative of seven cells visualized and imaged.

**Supplementary Text 1: The relationship between electromotility and nonlinear capacitance.** The voltage dependence of the capacitance function in OHCs is correlated to voltage dependence of electromotility. The voltage/length-change function is a saturating sigmoidal function in which the OHC length increases for hyperpolarizing potentials and decreases for depolarizing potentials. The voltage/length-change function may be fit with either a Langevin (hyperbolic cotangent) function or a two state Boltzmann. The peak of the capacitance function occurs at or near the maximal gain (steepest slope) of the voltage/length-change function. The voltage/capacitance function describes the displacement current resulting from the movement of a small intracellular anion such as chloride or bicarbonate into and out of the membrane. It is assumed that the anions are moving into (hyperpolarization) and out (depolarization) of prestin because of its membership in an anion transporter superfamily. It is further assumed that the charge movement is associated with a conformational change in prestin.
Table 1: $V_{\text{PKC}}$ of non-linear capacitance in OHCs and prestin expressing HEK 293 cells. Mean values and standard deviations are indicated. Sample sizes for each group are indicated in parentheses. Statistical significance of each group (in comparison to untreated cells of the same type) is also indicated: ** = p<0.0001.

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<td>OHC</td>
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<tr>
<td>UNTREATED</td>
<td>-0.051 ± 0.009 **</td>
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<tr>
<td>DEPLETED</td>
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Cholesterol modulates OHC function

Fig. 1
Cholesterol modulates OHC function
Supplementary Figure 1. Filipin labeling and cholesterol quantification of HEK 293 cells. Untreated (A, B), cholesterol depleted (10 mM MβCD; C, D), and cholesterol loaded (10 mM water-soluble cholesterol; E, F) HEK 293 cells were fixed and labeled with filipin. The pixel intensities of the images were measured using NIH Image software and graphed (G); intensity of the filipin stain is lower in depleted cells than in untreated or loaded cells. All samples were processed together and individual panels have not been altered relative to one another. H: Quantification of cholesterol content in membrane fractions of untreated, depleted and loaded HEK 293 cells was performed using the Amplex Red cholesterol assay (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Depleted cell membranes show an five-fold reduction in cholesterol content, while loaded cells show a 2-fold increase.

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Supp. Fig. 2

A

Prestin

1 2 3 4

% PFO in solubilizing buffer

B

Prestin-EGFP:HA-Prestin

Prestin

- EGFP lysate

kDa

487
389.6
292.2
194.8
97.4