MATERIALS AND METHODS

Mouse models and immunofluorescence

The Animal Resource Facilities of Creighton University approved protocols on mouse breeding, husbandry and cochlear morphological analysis performed in this study. All *in vitro* and *in vivo* experiments performed at the University of Brighton complied with Home Office guidelines under the Animals (Scientific Procedures) Act of 1986 and were approved by the University of Brighton Animal Welfare and Ethical Review Body.

Mice were housed in a facility with a 12-h light/dark cycle and free access to food and water. All mice used here were purchased from The Jackson Lab: Fgfr3iCreER^{T2} (stock # 025809) and Rosa-CAG-LSL-hChR2(H134R)-tdTomato-WPRE or COP-tdTomato (stock #012567) were crossed to create the Fgfr3-iCreER^{T2}; COP-tdTomato mouse. Tamoxifen was injected intraperitoneally (i.p.) at 250mg/kg on P12 and P13 mice to visualize the expression of COPtdTomato in cochleae at P28. Mice were genotyped as described in protocols by The Jackson Lab.

To induce COP-tdTomato expression specifically in mature DCs and PCs in the organ of Corti, we used a previously characterized Fgfr3-iCreER^{T2} mouse line that displays ~100% inducible Cre activity in cochlear DCs and PCs when tamoxifen was injected at juvenile and adult ages [1,2], no COP-tdTomato was detected in DCs/PCs in Fgfr3-iCreER^{T2-}; COP-tdTomato^{+} control mice at P28, whereas Fgfr3-iCreER^{T2+}; COP-tdTomato^{+} experimental mice showed robust expression of COP-tdTomato fusion protein specifically in both DCs and PCs within the organ of Corti at P28 (Fig. 1b-g).

The cochlea samples were fixed in 4% Paraformaldehyde for overnight at 4 °C (P28 cochleae). Tissues were washed in PBS and decalcified in 120mM EDTA (pH = 7.4) for 48 hrs before microdissection of the organ of Corti. The samples were blocked and permeabilized in the blocking buffer (10% Fetal Bovine Serum serum and 0.2 % Triton X-100 in PBS) for 2 hours at room temperature. They were then incubated at 4°C overnight with primary antibody Rabbit anti-Myosin VI (1:400 Proteus Bioscience). The tissues were incubated with 1:800 diluted secondary antibodies for 2 hours at room temperature, washed with PBS, and incubated with Dapi (1:1000 Thermo Scientific) for 2 mins at room temperature, washed with PBS, and then mounted for imaging using

Fluoromount-G (SouthernBiotech). All samples were imaged with an LSM700 confocal laser scanning image system (Carl Zeiss, Jena, Germany).

In Vitro Methods

Mice from P14 to P24 were culled using the Schedule 1 method. The mouse cochlea was freshly isolated and dissected in an ice-cold solution containing (in mM) NaCl 5.8, KCl 135, $CaCl_2$ 1.3, $MgCl_2$ 0.9, NaH_2PO_4 0.7, d-glucose 5.6, HEPES 10, Sodium pyruvate 2, pH 7.5 (adjusted with NaOH) and osmolarity ~308 mOsm.

The dissected apical coil of the organ of Corti was transferred to a microscope chamber, immobilized using a nylon mesh fixed to a stainless-steel ring and viewed using an upright microscope (Axioskope, Zeiss, Germany). Cells were observed with Nomarski differential interface contrast optics (40× water immersion objectives).

Dieters cells were recorded in the acutely isolated dissected organ of Corti at room temperature (22 °C). Currents and membrane potentials were recording using the same solution as for dissection. Patch pipettes were filled with intracellular solution containing (in mM) KCl 131, $MgCl_2$ 3, Na_2ATP 5, Na_2-phosphocreatine 10, EGTA 1, HEPES 10, Na_2GTP, pH 7.3 (adjusted with KOH) and osmolarity ~293 mOsm. All chemicals were obtained from Sigma Aldrich UK. The patch pipettes were fabricated with dual stage glass micropipette puller (Narishige PC-100) using borosilicate glass with outer diameter 1.5mm (Sutter Instruments) and heat polished with microforge Narishige MF -900. Currents were amplified with an Axopatch 700B amplifier (Molecular Devices, Union City, CA) and filtered at a frequency of 2–5 kHz through a low-pass Bessel filter. The data was digitized at 5–500 kHz using an analogue-to-digital converter (Digidata 1500; Molecular Devices). The whole cell current recordings were done using pCLAMP software (version 10, Axon Instruments, Foster City, CA, USA). The sampling frequency was determined by the protocols used. No online leak current subtraction was made, and only recordings with holding currents less than 50 pA were accepted for analyses.

This study included 18 cells with a series resistance (Rs) within a 5–15 MΩ range. After 60–90% compensation of the mean residual, uncompensated Rs was 5.1±0.5 MΩ. The seal resistance was typically 2-5 GΩ. The number of cells (n) is given for each data set. Data were analyzed using

pClamp10 (Molecular Devices), Origin9.1 (OriginLab Corp. Northampton, MA) and Excel (Microsoft).

In voltage clamp mode, cells were held at membrane potential of -80 mV, and 10 mV step potentials were applied from -120 mV to +50 mV. Since the currents elicited showed inactivation, we first determined the full functional recovery from inactivation. Using standard recovery protocol and testing different laser strengths (0.2 and 1.7 mW mm^{-2}, see below) we observed that the full recovery was achieved within 30 seconds (data not shown). The design of the stimulation protocol took this value in consideration, which was set to have 60 seconds between each voltage step. In current clamp mode, cells were injected currents from -70 pA to +60 pA, in 10pA increments.

For in vitro illumination of the preparation we used a 470 nm, 50 mW laser source (Dragon Lasers, Changchun Jilin, China) couple to a fiber optic cable (Thorlabs, Newton, New Jersey,

M63L01,105µm, 0.22NA). The tip was location was adjusted with a micro positioner to illuminate the entire preparation. Laser power was adjusted between 0.2 and 1.7 mW mm^{-2} based on in situ calibration with a photodiode sensor power meter (Thorlabs, Newton, New Jersey, PM16-130). In both voltage and current clamp, cells were stimulated by shining the laser light for about 3 seconds. The tip of the laser source was at approximately 1.5 cm from the preparation.

***In Vivo* Physiological Recordings**

Mice at 3-5 weeks of age were anesthetized with ketamine (0.12 mg/g body weight i.p.) and xylazine (0.01 mg/g body weight i.p.) for nonsurgical procedures or with urethane (ethyl carbamate; 2 mg/g body weight i.p.) for surgical procedures at the University of Brighton. Mice were tracheotomized, and their core temperature was maintained at 38 °C. The auditory sensitivity of mice was assessed before surgery using distortion product otoacoustic emissions (DPOAE, see below) to ensure each mouse was sensitive throughout the 1-70 kHz range of the sound system and especially sensitive to tones in the 50 kHz – 60 kHz range to levels ≤ 30 dB SPL.

To measure BM displacements and organ of Corti cochlear microphonics (CM) a caudal opening was made in the ventro-lateral aspect of the right bulla to reveal the round window [3]. Sound was delivered via a probe with its tip within 1 mm of the tympanic membrane and coupled to a closed acoustic system comprising two MicroTechGefell GmbH 1-inch MK102 microphones for

delivering tones and a Bruel & Kjaer (www.Bksv.co.uk) 3135 0.25-inch microphone for monitoring sound pressure at the tympanum and DPOAEs. The sound system was calibrated in situ for frequencies between 1 and 70 kHz and known sound pressure levels were expressed in dB SPL with reference to 2×10^{-5} Pa. Tone pulses with rise/fall times of 1 ms were synthesized by a Data Translation 3010 (Data Translation, Marlboro, MA) data acquisition board, attenuated, and used for sound-system calibration and the measurement of electrical and acoustical cochlear responses. To measure DPOAEs, primary tones were set to generate 2f1−f2 distortion products at frequencies between 1 and 50 kHz. DPOAEs were measured for f1 levels from 10 to 80 dB SPL, with the levels of the f2 tone set 10 dB below that of the f1 tone. DPOAE threshold curves represented the level of the f2 tone that produced a 2f1− f2 DPOAE with a level of 0 dB SPL where the f2/f1 frequency ratio was 1.23. System distortion during DPOAE measurements was 80 dB below the primary tone levels.

The BM was illuminated by a 470 nm, 50 mW laser source (Dragon Lasers, Changchun Jilin, China) couple to a fiber optic cable (Thorlabs, M63L01,105μm, 0.22NA). The tip was location was adjusted with a micro positioner to cast a 100 μm diameter circle of illumination on the BM, centered on either the micropipette or the BM displacement measurement beam. The laser beam was calibrated by casting the beam on the surface of the photo diode sensor of a power meter (Thorlabs, PM16-130). The laser power at the level of the OHCs was computed according to [4], who used analysis and estimates provided by [5,6]. Laser on-off was controlled through TTL. Intracellular and extracellular voltage responses were recorded from presumed DCs the fluid spaces of the OC using glass pipettes (20 MΩ- 80 MΩ when filled with 3M KCl) pulled from 1 mm diameter thin walled quartz glass tubing on a Sutter **P-2000 m**icropipette puller (Sutter Instrument Novato, CA 94949, USA) pulled. Signals were amplified with a recording bandwidth of DC - 100 kHz using a laboratory designed and constructed preamplifier (James Hartley). The voltage signals were not capacitance compensated. Presumed DCs with very negative resting membrane potentials (-112.5 ± 8.3 mV, n = 18) were encountered by advancing microelectrodes through the BM in the close vicinity of OHCs in the 50 kHz - 60 kHz region of the basal turn of the cochlea. Further advance resulted in encountering the fluid spaces of Nuel with zero potentials that are adjacent to the OHCs, and scala media with positive EP (+114.3± 3.7 mV, n=11).

Tone-evoked BM displacements were measured by focusing the beam of a self-mixing, laser diode interferometer [7] through the round window membrane to form a 20-μm spot on the center of the BM in the 50-56 kHz region of the cochlea. The interferometer was calibrated at each measurement location by vibrating the piezo stack on which it was mounted over a known range of displacements. At the beginning of each set of BM measurements it was ensured that the 0.2 nm threshold used as the criterion for threshold was at least as sensitive as the 0 dB SPL threshold for the DPOAEs before the cochlea was exposed. BM measurements were checked continuously for changes in the sensitivity of the measurement (due to changes in alignment or fluid on the round window) and for changes in the condition of the preparation. If thresholds of the latter changed by more than 5-10 dB SPL, the measurements were terminated. Tone pulses with rise/fall times of 1 ms were used for BM measurements. Stimulus delivery to the sound system and interferometer for calibration and processing of signals from the microphone amplifiers, microelectrode recording amplifiers, and interferometer were controlled by a DT3010/32 (Data Translation, Marlboro, MA) board by a PC running Matlab (The MathWorks, Natick, MA) at a sampling rate of 250 kHz. The output signal of the interferometer was processed using a digital phase-locking algorithm, and instantaneous amplitude and phase of the wave were recorded.

Measurements were made without knowledge of genotype. Less than 5% of all measurements were terminated because the physiological state of the preparation changed during measurement in which case data from the sample was excluded.

Experimental design and statistical analyses

COP-tdTomato$^{+/-}$ experimental mice were crossed to generate +/+, +/- and -/- genotypes. Male and female mice were studied in approximately equal proportions. No phenotypic differences were observed between males and females. Physiological tests were performed on +/+ and -/- littermates to minimize variations of age, environment or genetic background. Tests were performed on 3 – 5 week-old mice to reduce the possibility of progressive loss of high frequency responses, which is common in many mouse strains. Tests were performed on all mice in a litter without knowledge of genotype. Randomization was not appropriate because we had no foreknowledge of the genotype. Genotypes were determined after an experiment. For statistical analysis of physiological experiments, data were compared for at least 5 +/+ and 5 COP-/- mice, obtained from recordings of 2 or 3 complete litters. For analysis of BM and electrophysiological

measurements from presumed DCs and fluid spaces of the OC, 18 +/+ and 10 -/- COP mice in total were tested. Data were analyzed as mean ± standard deviation (S.D.) and plotted using Fig P (www.figpsoft.com) or Origin (www.originlab.com) software. Statistical tests were performed with GraphPad Prism (www.graphpad.com/quickcalcs) and comparisons made using unpaired *t* tests for unequal variances unless otherwise noted. P values are noted as absolute values with t values and degrees of freedom (df).

Software / code

For physiological recordings, data acquisition and data analysis were performed using a PC with programs written in MATLAB (The MathWorks, Natick, MA). The programs are available upon request from authors ANL and IJR. Please note that the programs were written to communicate with specific hardware (Data Translation 3010 board and custom-made GPIB-controlled attenuators) and will need modification if used with different hardware.

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