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Supplementary Information for

**Preventing presbycusis in mice with enhanced medial olivocochlear feedback**

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**Supplementary Information Text**

**Materials and Methods.**

**Animals.** We used a gain of function *knock-in* mouse model carrying an α9 nAChR subunit point mutation that leads to enhanced α9α10 nAChR activity (α9KI). α9KI mice have been previously described (1) and were backcrossed with congenic FVB.129P2-*Pde6bþ Tyrc-ch/AntJ* strain (https://www. jax.org/strain/004828; RRID:IMSR_JAX:004828) for seventeen generations (i.e., N-17). Wildtype (WT) littermates were used as controls at different time points. A similar male/female ratio was used both at 6 months (young) and at 1 year (aged) of age. All experimental protocols were performed in accordance with the American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals (June 2013) as well as Instituto de Investigaciones en Ingeniería Genética y Biología Molecular (INGEBI) and Facultad de Medicina, Universidad de Buenos Aires (UBA) Institutional Animal Care and Use Committee (IACUC) guidelines, and best practice procedures. To isolate the effect of aging from noise exposure, control and genetically modified mice were raised in a sound controlled room in the animal care facility in which the background sound levels were below 50 dB SPL measured at frequencies between 5.6 to 45.25 kHz. Thus, experimental animals were never exposed to high levels of noise. Noise levels were estimated using an electret condenser microphone (FG- 23329-PO7; Knowles). Additional transient sounds were generated during cage cleaning and daily maintenance by animal care staff and never exceeded 70 dB SPL.

**Cochlear function tests.** Auditory brainstem responses (ABRs) and distortion-product otoacoustic emissions (DPOAEs) were performed in mice anesthetized with xylazine (10 mg/kg, i.p.) and ketamine (100 mg/kg, i.p.), placed in a soundproof chamber maintained at 30°C. Recordings were performed at 6 months and 1 year of age. Sound stimuli were delivered through a custom acoustic system with two dynamic earphones used as sound sources (CDMG15008–03A; CUI) and an electret condenser microphone (FG- 23329-PO7; Knowles) coupled to a probe tube to measure sound pressure near the eardrum. For details, see: https://www. masseyeandear.org/research/otolaryngology/investigators/laboratories/eaton-peabody-laboratories/epl-engineering-resources/epl-acoustic-system. Digital stimulus generation and response processing were handled by digital I-O boards from National Instruments driven by custom software written in LabVIEW (kindly provided by Dr. M. Charles Liberman, Eaton-Peabody Laboratories, Massachusetts Eye & Ear Infirmary, Boston, MA.). For ABRs, needle electrodes were placed into the skin at the dorsal midline close to the neural crest and pinna with a ground electrode near the tail. ABR potentials were evoked with 5 ms tone pips (0.5 ms rise-fall, with a $\cos^2$ envelope, at 40/s) delivered to the eardrum at log-spaced frequencies from 5.6 to 45.25 kHz. The response was amplified 10,000X with a 0.3–3 kHz passband. Sound level was raised in 5 dB steps from 20 to 80 dB sound pressure level (SPL). At each level, 1024 responses were averaged with stimulus polarity alternated. Threshold for ABR was defined as the lowest stimulus level at which a repeatable peak 1 could be identified in the response waveform. ABR peak 1 amplitudes were computed by off-line analysis of the peak-to-peak amplitude of stored waveforms. The DPOAEs in response to two primary tones of frequency f1 and f2 were recorded at 2f1-f2, with f2/f1=1.2, and the f2 level 10 dB lower than the f1 level. For DPOAEs recordings, we use an ER10C amplifier system from Etymotic Research. Ear-canal sound pressure was amplified and digitally sampled at 4 μs intervals. DPOAE threshold was defined as the lowest f2 level in which the signal to noise floor ratio is >1.

**Cochlear processing and immunostaining.** The cochlear labyrinth was perfused with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), post-fixed with 4% PFA overnight, and decalcified in 0.12 M EDTA. Cochlear tissues were then microdissected and permeabilized by freeze/thawing in 30% sucrose. The microdissected pieces were blocked in 5% normal goat serum with 1% Triton X-100 in PBS for 1 h, followed by incubation in primary antibodies (diluted in blocking buffer) at 37°C for 16 h. The primary antibodies were: 1) anti-C-terminal binding protein 2 (mouse anti-CtBP2 IgG1; 1:200; catalog #612044, BD Biosciences; RRID:AB_399431) to label the presynaptic ribbon, 2) anti-glutamate receptor 2 (mouse anti-GluA2 IgG2a; 1:2000;

catalog #MAB397, Millipore; RRID:AB_11212990) to label the post-synaptic receptor plaques and 3) anti-neurofilament, heavy (chicken anti-NF-H; 1:1000; catalog #AB5539, Millipore; RRID:AB_11212161) to reveal all the unmyelinated nerve fibers. A nuclear dye, DRAQ5 (#65-0880, eBioscienceTM) was added to aid in hair cell counting. Tissues were then incubated with the appropriate Alexa Fluor-conjugated fluorescent secondary antibodies (Invitrogen, Carlsbad, CA; 1:1000 in blocking buffer) for 2 h at room temperature. Finally, tissues were mounted on microscope slides in FluorSave mounting media (Millipore).

**Confocal microscopy and image processing.** For IHCs synaptic counts, confocal z-stacks (0.2 μm step size) of the apical, medial and basal regions from each cochlea were taken using a Leica TCS SPE microscope equipped with 63X (1.5X or 4X digital zoom) oil-immersion lens. Each stack usually contained 10 to 20 IHCs. Image stacks were imported to Fiji software (RRID:SCR_002285) (2) and then a custom plugin was developed to automate counting of synaptic ribbons, glutamate receptor patches and co-localized synaptic puncta. Briefly, each channel was analyzed separately and maximum projections were generated to quantify the number of CtBP2 or GluA2 puncta. Additionally, a composite between the two channels was produced to draw the different ROIs that correspond to each IHC taking the CtBP2 stained nuclei as a reference. The maximum projections from the single channels were multiplied to generate a merged 32-bit image of the two channels. Then, they were converted to binary images after a custom thresholding procedure. Automatic counting of the number of particles on each ROI was performed.

For quantitative volumetric synaptic analysis, different areas from the whole cochlea were imaged and confocal z-stacks were processed in Fiji. It has been shown that blurring can affect high-resolution confocal imaging of synaptic contacts, leading to misinterpretations of volumetric information (3). Thus, z-stacks were deconvolved applying 10 iterations of the Richardson-Lucy algorithm in the DeconvolutionLab2 plugin (4), an algorithm that has been used previously in cochlear whole mounts (5). Then, the 3D Object Counter plugin was used to perform three-dimensional image segmentation and object individualization of the deconvoluted stacks. According to previous works (6–8) we reasoned that synapse would occupy a volume between 0.04 μm3 to 5 μm3, then we set a size range of 20-2000 voxels, with a voxel size of 0.11 μm x 0.11 μm x 0.2 μm. Then, the volumes of presynaptic ribbons (CtBP2 fluorescence) and postsynaptic AMPAR clusters (GluA2 fluorescence) at IHC-afferent synapses were measured using the 3D ROI Manager (9). All exported volumes were checked to ensure that volumes were within instrument resolution limits after threshold adjustments.

The stacks of images immuno-stained with anti-neurofilament antibodies (green channel) were z-projected and slightly saturated at the same fixed ratio to avoid missing nerve fibers. Pixel quantification per area and region evaluated was obtained using ImageJ 1.48v. IHCs and pillar cells' (PC) regions were evaluated. After automatic pixel density estimation per region, pixel numbers were transformed into actual nerve fibers according to the corresponding mean number of fibers per pixels per each region. The correspondence between number of fibers and pixels was manually estimated at the PC' region. The nerve fibers thickness quantification in pixels was also obtained at the PC' region.

**Statistical analysis.** Data are presented as group means ± standard error (SEM), unless stated otherwise, and were analyzed with R Statistical Software (RRID:SCR_001905). Friedman test was used in cases of repeated measures (peak 1 amplitude between ages, ABR and DPOAE thresholds). For group comparisons that do not involve repeated measures (peak 1 amplitude between genotypes, IHC synaptic counts, density and thickness of cochlear nerve fibers and % OHC survival), Mann-Whitney test was used to determine statistical significance. We examined the association between the volumes of presynaptic ribbons and postsynaptic AMPAR clusters, using linear regression analysis. Each set of data was represented in a scatter plot and adjusted to a linear function. The slopes of each regression were compared by an F test. A $p<0.05$ was considered statistically significant at a 95% confidence level.

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