Supplementary material

Detailed Methods:

Animals: CBA/CaJ mice of both sexes were obtained from the Jackson Laboratory. Mice were age 3 months at the beginning of each experiment. Mice were euthanized by CO_2 inhalation followed by decapitation. All procedures were approved by the NIDCD Animal Care and Use Committee.

Hearing testing: "Pre-test" auditory brainstem response (ABR) thresholds were measured 24-48 hours prior to the first drug (cisplatin or kanamycin) administration. "Post-test" ABR thresholds and distortion-product otoacoustic emissions (DPOAEs) were measured 15 days after the final cisplatin administration or 21 days after the final aminoglycoside administration. Animals were anesthetized via an intra-peritoneal injection of ketamine (100 mg/kg) and xylazine (20 mg/kg) and placed on a warming pad to maintain body temperature at 37°C.

For ABR testing, subdermal needle electrodes were placed at the vertex and pinna with a ground in the hind leg. For the cisplatin experiments, ABRs thresholds were measured at 4, 8, 11.2, 16, 22.4, and 32 kHz using an Intelligent Hearing Systems SmartEP system (v. 4.0). For the kanamycin experiment, ABR thresholds were measured up to 40 kHz using a Tucker-Davis Technologies TDT System 3 RZ6 ABR and DPOAE Workstation (Tucker Davis Technologies). For both experiments, stimuli were 3 ms tone pips (Blackman-gated, 1.5 ms rise/fall) presented at a rate of 29.9/s. Responses were amplified (100,000x for IHS; 20x for TDT) and filtered (.3-3k Hz), and 512-1024 responses were averaged at each stimulus presentation level. Thresholds were determined by visual inspection of stacked waveforms for the lowest level at which any repeatable peak could be obtained. Stimuli were presented at 80 dB SPL and decreased in 10 dB steps until no response was observed, then increased in 5 dB steps until a

repeatable response was observed. Two waveforms (1024 sweeps each) were obtained at levels at and near threshold to confirm reproducibility of peaks or absence of response. Threshold shifts are reported as the difference between pre-test and post-test ABR thresholds.

DPOAEs were obtained using TDT System 3 hardware and software (BioSigRz, v 5.1) in conjunction with an ER-10B+ microphone (Etymotic). A "DPgram" was measured using fixed primary tone levels of f1 = 65 dB SPL and f2 = 55 dB SPL with f2/f1 = 1.25 and f2 = 4, 8, 11.2, 16, 22.4, 32, and 44.8 kHz. The amplitude of the 2f1-f2 DPOAE and surrounding noise were determined from the FFT of the averaged data (512 averaged responses per data point). The TDT system output is given in dB voltage (dBV); therefore, data were converted to dB SPL offline.

Sound preconditioning: Preconditioning sound consisted of an 8-16 kHz octave-band noise presented for 2 hrs. The noise stimulus was generated using TDT hardware and software (RPvdsEx, v.76). Unanesthetized mice were placed in a custom-built cage that is subdivided into four compartments with one mouse per compartment. The cage was placed on a slowly-rotating (2.7 RPM) turntable in order to ensure a uniform sound exposure over the four-compartment area of the cage. An FF-1 speaker was mounted 10 cm above the cage. Calibration with the cage in place indicated mean stimulus levels of 90.1 ± 2.7 dB within the four compartments. Mice in the cisplatin protocol underwent sound exposures on seven days (1, 3, 10, 14, 16, 23, and 28). Mice in the kanamycin protocol underwent sound exposures on five days (1, 4, 7, 10, and 13). Sound-only mice were exposed to the same sound schedule as cisplatin + sound mice. Mice in the cisplatin-only (no sound) group were placed in the subdivided cage atop the rotating turntable for 2 hours without sound exposure on the same schedule as the cisplatin + sound mice to control for any effects of environmental stress caused

by the handling and cage environment. Each conditioning experiment included *N* = 4-8 mice per experimental group.

Measurement of ABR wave I amplitudes: ABR wave I amplitudes were measured for the sound-only mice in the kanamycin experiment. These mice were exposed to the sound protocol 5 times over 13 days (Figure 3A). ABRs were recorded prior to the first kanamycin injection and again 21 days after the final kanamycin injection. Peak-to-peak wave I amplitude was calculated from the waveforms at 50 dB SPL for both the pre-test and post-test ABRs (30).

4x4 Cisplatin Administration Protocol: Mice were prehydrated with 2-3 mL of saline (0.9% NaCl, Hospira) administered subcutaneously 24 hrs before the initial cisplatin injection. Cisplatin (100 mg/mL; PCH Pharmachemie) was administered intraperitoneally at a dose of 4 mg/kg each day for 4 days. Mice also received subcutaneous saline each day they received cisplatin. Mice recovered for 10 days following this 4-day cisplatin injection period, during which they were given 1.0-2.0 mL subcutaneous saline for 5 days (or longer if needed). This 14-day injection-plus-recovery protocol was repeated twice more for a total of 3 cycles of cisplatin administration. Mice had an additional 4-5 days recovery following the final cisplatin injection period before the post-test ABR.

8X2 Cisplatin Administration Protocol: Mice received the same prehydration treatment as in the 4X4 protocol (above). Cisplatin was administered at 8 mg/kg/day for 2 days, and mice recovered for 10 days between cisplatin administration periods. The entire 12-day administration-plus-recovery protocol was repeated twice more for a total of 3 cycles, with an additional 4-5 days of recovery following the final cisplatin administration. Thus this protocol resulted in the same cumulative dose of cisplatin (48 mg/kg) as the 4X4 protocol. Control

animals received intraperitoneal and subcutaneous saline injections at the same volumes and on the same schedules as the cisplatin-treated animals.

Kanamycin administration: Kanamycin sulfate (Sigma) was dissolved in sterile saline at a final concentration of 45 mg/ml, after considering the kanamycin activity (78.2% of the product's weight) and corrections for sulfate and water. Sterile-filtered kanamycin (750 mg/kg) was injected subcutaneously twice daily (8 hours apart) for 17 days. Mice recovered for 21 days after the final kanamycin injection to allow the hearing loss to stabilize prior to the ABR post-test (31-33).

Care and husbandry of cisplatin-treated mice: Cisplatin-treated mice were monitored several times a day. Body weight (BW) and body condition (BC) scores (34) were recorded daily prior to the administration of any fluids. Mice received 2-3 mL of subcutaneous saline (0.9% NaCl, Hospira) 24 hrs before the initial cisplatin injection. On each day of cisplatin administration and for the first 5 days of each 10-day recovery period, mice received 1.0-2.0 mL of subcutaneous saline (0.9% NaCl, Hospira). Additional saline injections were provided on the last 5 days of recovery if needed. Mice falling below 75% of their pre-cisplatin BW were hand-fed a nutrient-rich high calorie liquid food supplement (STAT®, PRN, Pharmacal) daily until their BW rose to above 75%. In addition to their regular diet (Wafer, NIH-07, Zeigler Bros, Inc.), mice were also provided with grapes, mini treats (BioServ) and bacon softies (BioServ). The survival rate for cisplatin-treated mice was 90.5%. Control mice that did not receive cisplatin had their body weight and body condition scores recorded weekly.

Isolation of cochlear RNA: For the *Hsp32* and *Hsp70* mRNA induction timecourse experiment (Figure 1C), mice were exposed to sound in the sound chamber as described above. After sound exposure, mice (N = 3-9 per timepoint) were returned to their home cages and remained

in a quiet room for 2, 4, 8, or 12 hours before being euthanized. For the examination of sound-induced *Hsp70* mRNA levels in brain, cochlea, heart, and kidney (Figure 1D), mice (*N* = 8-10 per group) were exposed to sound as above. Control mice were not exposed to sound. Both cochleas from an individual animal were dissected and pooled together in RNALater (Ambion) overnight at 4°C. RNALater was removed and cochleas were homogenized in TRIzol reagent (Invitrogen) using a Polytron Homogenizer (Kinematica). The homogenate was subjected to RNA isolation, and then treated with a Qiagen RNeasy Mini spin column with a 15 min RNase-free DNase I treatment. RNA quality was assessed by capillary electrophoresis using an Agilent RNA 6000 Nano chip on an Agilent Bioanalyzer 2100 (Agilent Technologies); only samples with a RIN score of >8.0 and with distinct 28S and 18S bands at or near 2:1 ratios were used for RT-qPCR analyses. Yields ranged from 200-900 ng RNA per sample. RNA samples from brain, heart and kidney were isolated in a similar fashion. RNA yields were higher for these tissues than cochlear total RNA, so these were diluted to less than 2 µg/ml for RT-qPCR experiments.

RT-qPCR: First strand complementary DNA (cDNA) was synthesized from total RNA. 20 µL RT reactions were prepared using a master mix of Taqman RT-PCR Reagents (Applied Biosystems) containing 1X Reaction Buffer, 5.5 mM $MgCl_2$, 500 µM dNTPs, 2.5 µM random hexamer primers, 0.4 U/µL RNase Inhibitor, and 1.25 U/µL Multiscribe Reverse Transcriptase. Each RT reaction was run on a thermocycler with the following parameters: 25°C for 10 mins, 48°C for 30 mins, 95°C for 5 mins and hold at 4°C. Resulting cDNA underwent real-time qPCR in an Applied Biosystems StepOne RT-PCR System (Applied Biosystems) using the SYBR Green (Applied Biosystems) detection system and primers designed to amplify *Hsp32*, *Hsp70* and *18S* RNAs using the following primer sets (5'-3'):

Hsp32 (Hmox1): (f) CTCACAGATGGCGTCACTTCGTCA (r) TTGCCAACAGGAAGCTGAGA;

Hsp70: (f) AGGCCAGGGCTGGTATTACT (r) AATGACCCGAGTTCAGGATG;

Hsp70.1 (f) TTGTCCATGTTAAGGTTTTGTGGTATA (r) GTTTTTTTCATTAGTTTGTAGTGATGCAA; *18S* (f) TTCGGAACTGAGGCCATGATT (r) TTTCGCTCTGGTCCGTCTTG. Average Cq values were normalized to the 18S ribosomal RNA signal. Thresholds for amplification curves were generated using the StepOne software version 2.2.2 and 7500 software V2.0.1. Fold changes relative to control mice were calculated using the ΔΔCt method assuming 100% efficiency. At least three biological replicates were analyzed per time point (2, 4, 8, and 12 hrs post noise exposure), and each reaction was performed in triplicate. A similar protocol was used for brain (n=10 biological replicates), cochlea (n=9 biological replicates), heart (n=10 biological replicates) and kidney (n=4 biological replicates) for comparison of *Hsp70* mRNA levels in different organs.

Cochlear whole mount preparations and immunofluorescence: Cochleas (*N* = 3-6 per condition) were perfused with ice-cold 4% paraformaldehyde through a small hole in the apex and also through the round window. They were then fixed in 4% paraformaldehyde overnight at 4^{o}C before being decalcified in 0.5 M EDTA for 3-4 days. Cochleas then washed 3 times in 1x PBS. Each cochlea was micro-dissected into three turns (apical, middle, basal) and stained with an antibody against myosin7a (rabbit anti-Myo7a at 1:200, #25-6790, Proteus BioSciences), which was detected using an Alexa Fluor 546 conjugated goat anti-rabbit IgG secondary antibody (1:500, Invitrogen). Each cochlear turn was mounted on a glass slide using Fluoromount G (Electron Microscopy Sciences). 10x images of each cochlear turn were taken using an LSM 780 confocal microscope (Carl Zeiss Microscopy) with 0.8X optical zoom, and the length of the cochlea was measured using Zen 2010 software (Carl Zeiss Microscopy). Using this length estimate, frequency regions were mapped onto the cochlear turns based on tonotopic mapping data for the mouse cochlea (35). Based on this estimation, OHC counts were performed per 200 μm or per 30 IHC in each frequency region tested by ABR.

References

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