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Selective hair cell ablation and noise exposure lead to different patterns of changes in the cochlea and the cochlear nucleus

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Abstract

In experimental animal models of auditory hair cell (HC) loss, insults such as noise or ototoxic drugs often lead to secondary changes or degeneration in non-sensory cells and neural components, including reduced density of spiral ganglion neurons, demyelination of auditory nerve fibers and altered cell numbers and innervation patterns in the cochlear nucleus. However, it is not clear whether loss of HCs alone leads to secondary degeneration in these neural components of the auditory pathway. To elucidate this issue, we investigated changes of central components after cochlear insults specific to HCs using diphtheria toxin receptor (DTR) mice expressing *DTR* only in HCs and exhibiting complete HC loss when injected with diphtheria toxin (DT). We showed that DT-induced HC ablation has no significant impacts on the survival of auditory neurons, central synaptic terminals, and myelin, despite complete HC loss and profound deafness. In contrast, noise exposure induced significant changes in synapses, myelin and CN organization even without loss of inner HCs. We observed a decrease of neuronal size in the auditory pathway, including peripheral axons, spiral ganglion neurons, and cochlear nucleus neurons, likely due to loss of input from the cochlea. Taken together, selective HC ablation and noise exposure showed different patterns of pathology in the auditory pathway and the presence of HCs is not essential for the maintenance of central synaptic connectivity and myelination.

Keywords

selective hair cell ablation; auditory nerve fiber; synapse; myelin; noise exposure; cochlear nucleus

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Introduction

Cochlear insults induced by acoustic overstimulation or ototoxic drug administration cause damage to several types of cochlear cells including HCs, spiral ganglion neurons (SGNs) synapses between HCs and SGNs and supporting cells (SCs) as well as to more central regions of the auditory pathways such as the cochlear nucleus (CN), resulting in sensorineural hearing loss (SNHL) (Lesperance et al., 1995, Aarnisalo et al., 2000, Kujawa and Liberman, 2009, Shibata et al., 2010, Liu et al., 2013). No effective biological treatment is available for human patients with severe or profound SNHL, although some patients with severe SNHL benefit from a cochlear implant, a prosthesis that electrically stimulates the SGNs. Preservation of healthy auditory neurons and their components, including central synapses and myelin, is thus critical for clinical success of cochlear prostheses and functional HC regeneration. Yet, the detailed mechanism of degeneration in SGNs and synapses in SNHL patients remains largely unknown due to the complexity of cochlear damage and inner ear structures.

SGNs are primary auditory neurons involved in signal transmission from the peripheral auditory receptors, the HCs in the organ of Corti, to the CN. About 95% of SGNs are type I SGNs, which are myelinated and synapse with inner HCs (IHCs) via their peripheral processes (Spoendlin, 1975). All known excitatory activity in the type I SGN pathway is glutamatergic (Flores et al., 2015). At the peripheral terminal of type I SGNs, there are numerous postsynaptic puncta that express the glutamate receptor 2 (GluR2), a critical subunit of the AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) type glutamate receptor (Matsubara et al., 1996, Frank et al., 2010, Arbeloa et al., 2012). At the central terminal of type I SGNs in the CN, vesicular glutamate transporter-1 (VGLUT-1) packages the glutamate into synaptic vesicles (Zhou et al., 2007, Zeng et al., 2009). These synaptic components, including GluR2 and VGLUT-1, are required for transmission of sound-induced signals from the cochlea to the CN.

Synaptic degeneration, including decreases in GluR2 and VGLUT-1, and demyelination of auditory nerve fibers (ANFs) after cochlear insults have been reported (Zhou et al., 2007, Zeng et al., 2009, Barker et al., 2012, Tagoe et al., 2014, Wan et al., 2014, Heeringa et al., 2016). These studies used either noise or ototoxic drugs to induce cochlear damage. However, both of these methods can damage other cochlear tissues, including SGNs themselves, and it is not clear whether the loss of HCs alone results in the degeneration of synapses and myelination. To determine whether the loss of HCs without any other direct cochlear tissue injury is sufficient to damage synapses and myelination, we used a novel transgenic mouse model, the DTR mouse (Golub et al., 2012), in which the gene for *human diphtheria toxin receptor* (*hDTR*) was inserted under regulation of the promoter for *Pou4f3*, a HC-specific transcription factor (Keithley et al., 1999). In DTR mice, a single injection of diphtheria toxin (DT) kills all cochlear HCs, leaving the cochlear neural components intact and providing an appropriate model to study responses of the auditory pathway to loss of HCs (Golub et al., 2012, Kaur et al., 2015, Tong et al., 2015). Here we compared the pathology induced by selective ablation of HCs by DT to that caused by noise-induced HC cochlear trauma. Specifically, we examined non-sensory cells in the cochleae, SGNs, myelin, CN, and synapses, including the expression of GluR2 and VGLUT-1, and

determined that following DT-induced HC ablation the changes in the cochlea and the CN are minimal whereas noise exposure induced significant changes. The data suggest that central synaptic connectivity and myelination can be maintained as long as 2 months following the loss of IHCs but noise exposure can change the neural substrate even without loss of IHCs.

Experimental procedures

Animals, groups, and drug administration

All animal experiments were approved by the University of Michigan, Institutional Animal Care and Use Committee, conformed to the NIH Guide for the Care and Use of Laboratory Animals, and were performed using accepted veterinary standards. We used DTR transgenic mice expressing the gene for *hDTR* under regulation of the *Pou4f3* promoter, which were kindly provided by Prof. W. Edwin Rubel. The activity of the *Pou4f3* promoter in the inner ear is restricted to the HCs, in the developing and mature state (Keithley et al., 1999, Golub et al., 2012). The generation and characterization of these mice have been previously described (Golub et al., 2012, Kaur et al., 2015, Tong et al., 2015). The DTR mice were originally made on the C57/BL6 background as described in the Golub paper, and more recently became available on the CBA background, as described in the Kaur and Tong papers and in the study reported here.

The only other tissue where *Pou4f3* is expressed is a sub-type of retinal ganglion cells, likely restricted to developmental time (Badea and Nathans, 2011). Transgenic deletion of *Pou4f3* in early embryos caused only minor changes in vision, unlike deletion of other *Brn* genes. These results suggest DT would not be lethal to retinal ganglion cells in the mature DTR mouse.

Both male (N = 18) and female (N = 25) mice were used. For identification of DTR (*Pou4f3^{DTR/+}*) and WT (*Pou4f3^{+/+}*) mice, we used genotyping methods similar to those described previously (Kaur et al., 2015, Tong et al., 2015). The transgenic mice were generated in a CBA/CaJ mouse strain, which maintains its hearing well into old age (Henry and Chole, 1980, Jimenez et al., 1999). Animals were divided into 3 groups (Fig. 1A): 1) WT mice with DT injection at 3 weeks of age (WT group, N = 12 animals); 2) DTR mice with DT injection at 3 weeks of age (DT group, N = 22 animals); and 3) DTR mice exposed to loud noise at 3 weeks of age without DT injection (Noise group, N = 9 animals). The DT group was further divided into 2 groups based on survival times: 1) mice sacrificed 1 month after DT injection (DT 1M group, N = 11 animals); and 2) mice sacrificed 2 months after DT injection (DT 2M group, N = 11 animals). Mice in both WT and DT groups received a single systemic intramuscular injection of DT (15 ng/g), a dose we found appropriate for eliminating all HCs while being tolerated well by the mice as demonstrated by absence of systemic effects. Mice in both WT and Noise groups were allowed to survive 1 month after DT or noise exposure.

Noise exposure

Noise exposure was performed as previously described (Fairfield et al., 2005, Gong et al., 2012). Briefly, awake mice were placed in individual wire mesh cages in a ventilated sound exposure booth and exposed for 3 hours to a broadband noise (2 to 20 kHz) at 120 dB sound pressure level (SPL).

Auditory brainstem response

We determined the thresholds in the left ear of each animal with auditory brainstem response (ABR) measurements, as described in detail previously (Takada et al., 2014). ABRs were measured at 8, 20, and 32 kHz at least 1 day prior to DT injection or noise exposure (baseline ABRs) and then again 1 month later (N = 5 WT and DT 1M, and N = 9 for Noise group). The first 5 animals in the DT groups were assessed by ABR test and found to have no hearing at any frequency tested. Based on this finding, the remaining DT injected DTR mice (7 in the 1M and 11 in the 2M groups) were assumed to have no hearing, and only tested by Preyer's reflex, which was negative. Histology (lack of HCs) later confirmed these animals were indeed completely deaf.

Cochlear immunohistochemistry

Mice were perfused intracardially with 4% paraformaldehyde (PFA) in phosphate buffer (PB). Cochleae were removed and fixative flow facilitated by opening the round and oval windows and perforating the bone over the apex. Tissues were post-fixed in 4% PFA in PB for 1 hour, and decalcified in 5% ethylenediaminetetraacetic acid (EDTA) for 16 hours at 4°C to facilitate the dissection. Cochlear tissues were then micro-dissected into segments of the auditory epithelium. Samples were blocked in 5% normal goat serum (Vector Laboratories, Burlingame, CA) with 0.3% Triton X-100 (Sigma-Aldrich, St. Louis, MO) in phosphate buffered saline (PBS) for 1 hour, followed by reaction with primary antibodies, rinsed, and incubated with the secondary antibody. As primary antibody we used an anti-myosin VIIa (rabbit anti-myosin VIIa; Invitrogen, Grand Island, NY) diluted 1:200 in blocking buffer for 1 hour at room temperature (RT). Secondary antibody was a goat-anti rabbit Alexa Fluor® 594 (Molecular Probes, Eugene, OR), diluted 1:200 in blocking buffer for 1 hour. To double stain for F-actin, we used Alexa Fluor® 488 phalloidin (Molecular Probes), diluted 1:500 in blocking buffer for 30 minutes. To stain for glutamate receptor (GluR2) and C-terminal binding protein 2 (CtBP2) we used a mouse anti-GluR2 antibody (IgG2a; Millipore, Billerica, MA) diluted 1:2000 in blocking buffer and a mouse anti-CtBP2 antibody (IgG1; BD Transduction Laboratories, San Jose, CA) diluted 1:500 in blocking buffer for 20 hours, respectively. To stain neurons we used an anti-neurofilament 200 antibody (mouse anti-NF200 IgG1; Sigma-Aldrich) diluted 1:500 in blocking buffer for 2 hours at RT. Primary incubations were followed by 2 sequential 1 hour incubations at RT in appropriate Alexa Fluor (Molecular Probes; 1:500 in blocking buffer). Specimens were mounted on glass slides using Prolong Gold anti-fading reagent (Molecular Probes). Whole mounts were observed and recorded using a Leica DMRB epi-fluorescence microscope (Leica, Eaton, PA), with CCD Cooled SPOT-RT3 digital camera (Diagnostic Instruments, Sterling Heights, MI) and/or Leica SP5X confocal microscope.

Assessment of HC survival and GluR2 expression

HC survival was evaluated in whole mounts stained with antibodies to myosin VIIa (red) and phalloidin (green) to stain actin. Tissues were viewed in a Leica DMRB epi-fluorescence microscope with a 50× objective. HC counts were analyzed using the KHRI Cytocochleogram Program, version 3.0 (Sha et al., 2008). In each field of view, a 0.20 mm scale in the 10× eyepiece was superimposed on the centers of the pillar cells (PCs). The percentage loss of HCs was calculated for each 0.20 mm segment; each row was analyzed separately. For the graphic of HC loss (Fig. 2 E-F), the average OHC and IHC losses were calculated for each interval and plotted against percentage of distance from the apex. For statistical analysis, percent HC loss was computed for the entire row of IHCs and for all OHC (all three rows, combined). N = 8 animals for Noise group, N = 4 for each of the other groups.

For quantitative analysis of GluR2 expression, cochlear whole lengths were measured using the ImageJ software (NIH, <http://rsweb.nih.gov/ij/features.html>) and converted to cochlear frequency (Muller et al., 2005). Confocal z-stacks images of the 8, 20, and 32 kHz regions from each ear were obtained in the inner spiral bundle area under the IHCs using a high-resolution oil immersion objective (63×), 3× digital zoom, and a 0.25 μm step size on a Leica SP5X confocal microscope. Images were acquired with the following parameters: pixel resolution of 1024 × 1024 pixels, pixel size of 80.1 × 80.1 nm, and a pinhole adjusted to one Airy unit. The z-stacks were set to span the entire length of the inner spiral bundle so that all the synaptic specializations were included within the image.

Image stacks showing only GluR2 puncta (red) were imported to Adobe Photoshop CC 2015 (Adobe Systems GmbH, Munich, Germany) for auto contrast adjustment and imported to ImageJ for automatic quantification of particles. To separate puncta, the ImageJ “watershed” algorithm was applied to distinguish between closely adjacent spots. To ensure that puncta were counted reliably, visual inspections and manual corrections were always conducted after each automated counting. In the absence of IHCs, GluR2 counts of each z-stack were divided per 10 μm (N = 3 animals).

Cochlear assessment using plastic sections

One hour after cardiac perfusion with 2% glutaraldehyde, cochleae were placed in 3% EDTA with 2% glutaraldehyde for 5 days. Cochleae were then osmicated (1% OsO₄ in dH₂O), dehydrated in ethanol and propylene oxide, embedded in Epon and hardened in a 60°C oven for 2 days. For quantitative assessment of peripheral ANFs and SGNs, the tissues were sectioned at the osseous spiral lamina (OSL) near the habenula perforata and at a near-mid-modiolar plane with a glass knife (1 μm) using a Leica Ultracut R microtome, then stained with toluidine blue. Cross-sections were examined and recorded using a Leica DMRB microscope with oil immersion objectives (40× for SGNs and 63× for ANFs) and a CCD Cooled SPOT-RT digital camera.

Measurements of the stria vascularis (SV) thickness, from the endolymphatic surface of the marginal cells to the spiral ligament (SL) side of the basal cells, were made at the central

thickest portion of the SV, and average SV thickness was calculated (N = 3 animals for each group).

SGN density and cell size measurement were performed as previously described (Fukui et al., 2012). Briefly, SGNs that exhibited a clear nucleus and cytoplasm in Rosenthal's canal of the apex and base were counted (N = 5 for DT 1M, DT 2M and WT, and N = 3 for Noise group). The area of Rosenthal's canal was measured using ImageJ. The density of SGNs per 10,000 μm^2 was calculated for each profile. For the assessment of SGN cell size, soma areas were measured with ImageJ in the same sections used for SGNs counting. Twenty SGN cells were selected randomly in each section for measuring and calculating average cell size (N = 3–5 animals). To evaluate ANF density, axonal counts were made through the OSL at both base (32 kHz region) and apex (8–16 kHz region). All neuronal profiles in the fascicles in each section were counted. The number of ANFs per fascicle was divided by the fascicle area surrounded by the perineurium as measured by ImageJ. The density of ANFs was averaged from 10–15 OSL openings in each ear.

Transmission electron microscopy

Specimens for transmission electron microscope (TEM) were prepared as described in a previous report (Beyer et al., 2000). Briefly, cochlear tissues embedded in Epon were sectioned at the OSL opening near the habenula perforata using a Leica Ultracut R microtome at 80 nm thicknesses and collected onto mesh grids. Each grid was counterstained with uranyl acetate and lead citrate. Samples were viewed on the JEM-1400Plus TEM (JEOL, Tokyo, Japan) with an accelerating voltage of 80 kV. Myelin thickness and axon caliber were measured with ImageJ software using the TEM micrographs. Data were evaluated from 100 myelinated axons for the assessment of myelin thickness, axon caliber, and g-ratio (N = 3 animals for each group). For the assessment of lamella number, data were evaluated from TEM micrographs of 7 myelinated axons (N = 3 animals for each group).

Brain tissue preparation and stereological examination of the CN

Mice were perfused intracardially with 4% PFA in PB and brainstems were removed. Frozen transverse sections (20 μm) were mounted on glass slides. Cresyl violet staining was used for CN stereological study and VGLUT-1 immunostaining for the assessment of glutamatergic central terminals in CN. For stereological analysis of the CN, ventral CN (VCN) volume, and neuron density and size in VCN were quantified using ImageJ. For the measurement of VCN volume, the VCN area of each slide at every 80 μm thickness was recorded using a Leica DMRB microscope (10 $\times$) and a CCD Cooled SPOT-RT digital camera and measured. Each VCN area was multiplied by section thickness and added to calculate the total VCN volume. To assess the density of neurons, the number of VCN neurons per 10,000 μm^2 was measured on the dorsal and ventral side, respectively (N = 6 animals). For assessment of neuron size, cell size in the VCN was measured with ImageJ in the same sections used for counting. VCN was subdivided into dorsal and ventral portions (high- and low-frequency inputs) (Muniak et al., 2013). Twenty neurons in the VCN were selected randomly in each section for measuring and calculating the average cell size in each animals (N = 6 animals).

VGLUT1 immunocytochemistry and image processing

VGLUT1 immunocytochemistry and quantification were conducted as described previously (Zhou et al., 2007, Heeringa et al., 2016). Sections were incubated for 30 minutes in a blocking solution containing 1% normal goat serum in PBS with 0.3% Triton X-100 followed by overnight incubation with primary antibody, anti-VGLUT1 (rabbit anti-VGLUT-1, Synaptic Systems, Germany), diluted in blocking solution 1:2000 for 18 hours at RT. After rinsing in PBS, sections were reacted with the secondary antibody, a goat-anti rabbit Alexa Fluor® 594 (Molecular Probes, Eugene, OR), diluted 1:500 in blocking buffer for 1 hour. After rinsing in PBS, slides were coverslipped using Gel/Mount (Biomedica, Foster City, CA). Negative controls were conducted on sections that were not treated with either primary or secondary antibodies, resulting in no immunolabeling. The VGLUT-1 punctum density was quantified in each of the following CN regions: anteroventral CN (AVCN), posteroventral CN (PVCN), the molecular layer, the fusiform cell layer, and the deep layer of the dorsal cochlear nucleus (DCN1, DCN2, and DCN3, respectively), and granule cell lamina (GCL) (N = 6 animals for each group). For each animal, three pictures (40×) were taken at equal intervals from caudal to rostral for each of the selected regions except DCN1 (i.e., one picture from the 25th percentile, one from the 50th percentile, and one from the 75th percentile). For DCN1, a higher magnification (63×) was used to take three pictures from caudal to rostral in each animal. The photomicrographs were imported to Adobe Photoshop CC 2015 for auto contrast adjustment and were then transferred to ImageJ for automatic quantification. To separate puncta, the ImageJ “watershed” algorithm was applied in order to distinguish between closely adjacent spots. To ensure that puncta were counted reliably, visual inspections and manual corrections were always conducted after each automated (threshold) counting. This ensured that puncta were not merged by the thresholding procedure. The number of VGLUT-1 labeled puncta was divided by the chosen area to calculate punctum density. The average count for a CN region was computed for each animal from the three images of that region and these values were used in tests for differences between groups.

Statistical analyses

For most comparisons, 1-way analysis of variance (ANOVA) was performed to test for group differences. For sets of related and potentially correlated variables, multivariate ANOVA was performed using all variables in the set, followed by univariate post-hoc test to assess the contribution of each variable, with sequential Bonferroni criterion used to judge significance. In multivariate tests, F is estimated using Pillai's trace. When more than two groups were evaluated, a test for the presence of group differences was performed on the data from all groups, followed by tests on pairs of groups to identify which groups were different, with the sequential Bonferroni criterion used to judge significance. Univariate tests were then used to assess the contribution of individual variables to the differences between pairs. Between-group differences in the relationship of myelin thickness to axon diameter were analyzed using a mixed model design, which is a statistical model containing both fixed effects and random effects, to account for random variation of the measure within subjects. Myelin thickness was treated as the dependent and axon diameter as a covariate, group was a second main effect, and their interaction was included to test for differences in

slope between groups. All analyses were conducted in R (version 3.2.1, 2015, R Core Team).

Results

DTR mice (*Pou4f3^{DTR/+}*) and WT mice (*Pou4f3^{+/+}*) exhibited normal appearance, behavior and weight gain before DT injection; individuals segregated for breeding were not injected and had normal fertility. After injection of experimental animals with DT (15 ng/g) at 3 weeks of age, there were no deaths, no apparent loss of body weight, and no other signs of morbidity other than hearing loss and circling. Therefore, administration of low-dose DT could cause targeted ablation of HCs without causing systemic toxicity. Instability and shakiness in the DTR mice were detectable after 3–5 days and persisted for at least 2 months. No apparent differences in balance were observed between male and female mice.

Hearing loss of DTR mice exposed to DT or noise

Baseline ABR thresholds were measured at 8, 20, and 32 kHz in WT and DTR mice (N = 5 for each group) at 3 weeks of age, one day before the DT injection. At baseline (Fig. 1B), there were no significant differences in ABR thresholds between WT and DTR mice in multivariate analyses ($F_{3,6} = 0.614$, $p = 0.63$); in separate tests, even the largest difference, at 32 kHz, was not significant ($F_{1,8} = 2.04$, $p = 0.19$). All animals in WT and DT groups were injected with DT at 3 weeks of age, which is more than a week after hearing onset (Song et al., 2006). WT mice (WT; N = 5) had normal thresholds after DT injection, indicating DT (15 ng/g) had no effect on hearing in WT mice (Fig. 1C). However, DTR mice at 1 month after DT injection (DT 1M; N = 5) exhibited severe threshold shifts such that no measurable ABR could be detected at 100 dB SPL (the limits of our sound system) at all frequencies. The overall difference between DT 1M and WT mice was significant ($F_{6,30} = 2053$, $p < 0.001$), as were the differences at each frequency ($F_{1,8} > 600$, $p < 0.001$).

To examine the influence of noise exposure on ABR thresholds, DTR mice without DT injection were exposed to broadband noise (2–20 kHz) at 120 dB SPL for 3 hours (Noise; N = 9). One month after noise exposure, these mice exhibited ABR threshold elevations with thresholds between 80 and 90 dB SPL (Fig. 1C). Multi-group MANOVA comparing noise exposed DTR mice, DT injected WT mice and DT injected DTR mice found significant differences in hearing loss among groups ($F_{6,30} = 7.57$, $p < 0.001$). There were significant differences between Noise and WT mice ($F_{3,10} = 399.5$, $p < 0.001$) and between Noise and DT 1M ($F_{3,10} = 83.8$, $p < 0.001$). Even the smallest difference, between Noise and DT 1M at 8 kHz, was significant ($F_{1,12} = 28.9$, $p < 0.001$). Furthermore, to investigate the conduction speed of auditory signals, we also measured the latency of ABR peak 1 (P1, Fig. 1D) of WT and noise-exposed mice using a 100 dB SPL signal. The latency was significantly increased in the noise-exposed animals (Fig. 1E) both overall ($F_{3,14} = 50.8$, $p < 0.001$) and at each frequency ($F_{1,16} > 62$, $p < 0.001$). Conduction speed is determined by axon caliber, myelination, and synapse integrity (Cankaya et al., 2003, Lee et al., 2012), thus our result suggests that noise exposure might cause damage to the axon, myelin, or synapses. In the DT 1M mice, ABR P1 latency could not be measured because they had no ABR responses even at 100 dB SPL.

Cochlear pathology after DT or noise

To assess the cochlear morphology and HC loss, whole mounts of cochleae were stained with phalloidin (green) and antibody to myosin VIIa (red), a specific HC marker (Fig. 2A-D). The percentages of missing OHCs and IHCs were assessed in each group (N = 4 for each group, except Noise N = 8; Fig. 2E, F). Multivariate analysis of IHC and OHC found significant differences among groups in severity of HC loss ($F_{6,32} = 138.5$, $p < 0.001$). The cochleae of WT mice demonstrated a well-defined single row of IHCs and three rows of OHCs (Fig. 2A). These ears retained almost all HCs throughout the cochlea (Fig. 2E, F). Noise and both DT groups had significantly more HC loss than WT ($F_{2,5 \text{ or } 2,9} > 90$, $p < 0.001$). The Noise groups had primarily OHC loss, concentrated in the basal portion ($> 40\%$ of distance from apex), whereas the DT 1M and DT 2M groups exhibited $> 98\%$ loss of IHCs and OHCs. The difference in HC loss between DT 1M and Noise was significant ($F_{2,9} > 1000$, $p < 0.001$). The difference between DT 1M and DT 2M also was significant ($F_{2,5} = 23.7$, $p = 0.003$), but univariate tests found a difference only in OHC loss ($F_{1,6} = 49.0$, $p < 0.001$), which increased from 98.4% to 99.9% (Fig. 2C, D). The slightly more rapid and complete demise of IHC might be due to stronger *Pou4f3* expression in the IHCs compared to OHCs (Tong et al., 2015).

SCs in the auditory epithelium do not express *Pou4f3* and therefore were not expected to receive primary damage due to the DT injection. However, we investigated non-sensory regions thoroughly to determine whether they were affected by the loss of HCs. In the Noise group, regions where a large number of OHCs were missing had expanded SCs that displayed a pattern of adherens junctions typical of phalangeal scars (Fig. 2B). Formation of phalangeal scars was seen throughout the organ of Corti in the DT groups.

Cross-sections of the organ of Corti showed the patterns of HC loss described above and also the survival of, DCs, PCs, and other SCs in all groups (Fig. 3A, B). They also showed that 74.5% of DT 2M mice had reduction of SV thickness compared to WT (Fig. 3C), consistent with other models of long-term deafness. Fibrocytes in the SL were unchanged by the loss of HCs, and appeared similar in the WT and DT 2M groups (Fig. 3D). In the Noise group, however, obvious damage to the type IV fibrocytes in the SL was seen, and in 93.0% of mice the SV showed dilated blood vessels and slight thinning compared to WT (Fig. 3C, D). Cross-sections also showed that the Noise group exhibited significant vacuolization under the IHCs (Fig. 3B). Such vacuolization was rarely seen in the WT and DT 2M groups. In previous studies, this change was shown to represent swelling of postsynaptic terminals of afferent fibers associated with glutamate excitotoxicity (Robertson, 1983, Pujol and Puel, 1999).

Glutamate receptor 2 expression in the organ of Corti

To further investigate the fate of postsynaptic terminals after DT-mediated HC ablation and noise trauma, we immunostained cochlear whole mounts with antibodies to GluR2, which recognizes AMPA-type glutamate receptors at the postsynaptic terminal (Matsubara et al., 1996, Liberman et al., 2011), to CtBP2, a presynaptic ribbon structural protein, and to neurofilament (NF), a marker for neuronal processes (8 kHz region of cochlea shown in Fig. 4A-D). CtBP2 immunostaining was absent in the DT group throughout the cochlear duct

(not shown), as expected, since all HCs are missing. The number of GluR2 puncta was assessed in each group at 8, 20, and 32 kHz regions ($N = 3$ animals for each group; Fig. 4E) and was found to differ significantly among groups ($F_{9,24} = 5.89$, $p < 0.001$). Numerous ANF terminals and postsynaptic GluR2 puncta were visible in normal cochleae (Fig. 4A); significantly fewer were seen in DT 1M ($F_{3,2} = 415$, $p = 0.002$) and noise-exposed mice ($F_{3,2} = 46.0$, $p = 0.021$), and the difference between these groups also was significant ($F_{3,2} = 27.7$, $p = 0.035$). The slight additional decline in punctum density between DT 1M and DT 2M was not significant ($F_{3,2} = 5.07$, $p = 0.169$). In the DT groups, we observed a few GluR2 puncta that were not paired with ANF terminals. These could be indicative of slow degradation of these terminals after the loss of the hair cell. Importantly, noise-exposed cochleae exhibited loss of GluR2 puncta even though they did not lose IHCs. Furthermore, GluR2 puncta loss was significant in all frequency regions, including the smallest loss at 20k ($F_{1,4} = 17.9$, $p = 0.013$; Fig. 4B, E).

NF staining revealed a dense meshwork of neuronal processes under the IHCs, which includes radially directed terminals of SGNs and spiraling fibers of the lateral and medial olivocochlear efferent systems (Yuan et al., 2014). Although most of radial and spiraling fibers in the inner spiral bundle remained in the DT groups despite complete IHC loss (Fig. 4A-D), qualitative observations suggest that the number radial fibers crossing the tunnel of Corti appeared to be reduced and the spiraling fibers in the inner spiral bundle seemed to be slightly increased in the DT groups compared to WT. Importantly, noise-exposed cochleae exhibited loss of GluR2 puncta even though they did not lose IHCs. Furthermore, GluR2 puncta loss was significant in all frequency regions, including the smallest loss at 20k ($F_{1,4} = 17.9$, $p = 0.013$; Fig. 4B, E).

Survival of auditory nerve fibers and spiral ganglion neurons

To examine the neural degeneration in DT and noise-exposed mice, we counted the myelinated type I ANFs in the OSL and the SGNs in the Rosenthal's canal in both the base and apex ($N = 3-5$ animals for each group; Fig. 5A-D and Fig. 6A-D). No significant differences were observed in the neural density of ANFs ($F_{6,22} = 1.19$, $p = 0.346$; Fig. 5E) and SGNs ($F_{6,28} = 1.67$, $p = 0.166$; Fig. 6E) across the four experimental groups. However, a downward trend in ANF and SGN density could be observed in the Noise group. Although the reduction in neural density was not significant, we did observe a significant difference in cell size of SGNs ($F_{6,28} = 4.06$, $p = 0.005$; Fig. 6F). SGN size was significantly smaller than in controls for both Noise ($F_{2,5} = 6.39$, $p = 0.042$) and DT 1M ($F_{2,7} = 8.67$, $p = 0.013$), but Noise and DT 1M were not different from each other ($F_{2,5} = 0.06$, $p = 0.940$). In addition, DT 2M had significantly smaller cells than DT 1M ($F_{2,7} = 26.8$, $p = 0.001$). In all pairs of groups with a significant difference in SGN size, that effect can be seen in the basal turn ($F_{1,6 \text{ or } 1,8} > 13.0$, $p < 0.008$); but apical SGN size was reduced only between DT 1M and DT 2M ($F_{1,8} = 5.78$, $p = 0.043$). Again, the change in the Noise group occurred despite the absence of IHC loss; and in this group, some SGNs were separated by vacuolated spaces associated with detachments between myelin and neuronal soma (Fig. 6B').

Hypomyelination of auditory neurons

To further explore the effect of DT-induced HC ablation on ANFs, we assessed axon caliber, thickness of the myelin sheath and lamella number of axons in the OSN by TEM ($N = 3$ animals for each group; Fig. 7). We also calculated the g-ratio (estimated by dividing the axon diameter by the myelinated fiber diameter), which is a highly reliable ratio for assessing axonal myelination and functionality (Chomiak and Hu, 2009). After accounting for within-animal variation, axon caliber differed significantly between groups ($F_{3,388} = 8.99$, $p < 0.001$). Compared to controls, axon caliber was significantly smaller in the Noise group ($F_{1,194} = 6.58$, $p = 0.011$), but not in DT 1M ($F_{1,194} = 0.620$, $p = 0.432$). However, axon caliber was smaller in DT 2M even than in the Noise group ($F_{1,194} = 6.07$, $p = 0.015$). Myelin thickness also differed significantly between groups ($F_{3,388} = 34.2$, $p < 0.001$). In the Noise group, myelin thickness was significantly lower than controls ($F_{1,194} = 56.9$, $p < 0.001$). In DT 1M, it was greater than in controls ($F_{1,194} = 7.04$, $p = 0.009$), but was not different from controls in DT 2M ($F_{1,194} = 0.132$, $p = 0.717$).

Lamella number was significantly different among groups ($F_{3,16} = 3.89$, $p = 0.039$), but only the Noise group was significantly different from wildtype ($F_{1,8} = 14.3$, $p = 0.005$). Distributions of the number of lamellae were quite distinct between Noise group and the other groups. Noise group animals never had more than 20 lamellae and all other groups never had fewer than 20 lamellae, except for one DT fiber in one of the DT animals. The hypomyelination in noise-exposed mice might be one of the causes of the increase of ABR P1 latency (Fig. 1D, E).

The changes in axon diameter and myelin thickness produced significant differences in g-ratio ($F_{3,388} = 16.5$, $p < 0.001$). In the Noise group, both axon diameter and myelin thickness decreased and the relatively greater decrease in myelin produced an increase in g-ratio ($F_{1,194} = 7.73$, $p = 0.006$). In the DT 1M group, the slight increase in myelin did not produce a significant change in g-ratio ($F_{1,194} = 0.23$, $p = 0.629$) and in the DT 2M group the large decrease in axon diameter produced a significant decrease in g-ratio ($F_{1,194} = 14.3$, $p < 0.001$).

In addition to changes in the means of axon diameter and myelin thickness, there also were changes in the relationship between them. In WT and Noise groups, there is not a significant correlation ($t < 1.6$, $p > 0.12$; Fig. 7D); large and small axons have similar mean myelin thickness and thus large axons tend to have larger g-ratios. Because the numerator of g-ratio is axon diameter, g-ratio increases at a progressively slower rate with increasing axon diameter and is fit by a log model (Fig. 7E). Compared to WT, the Noise group has a smaller myelin thickness (as described above) and thus a larger g-ratio expected for any value of axon diameter. In the DT 1M and DT 2M groups, myelin thickness increases with axon diameter ($t < 3.4$, $p < 0.001$, Fig. 7D); however, both slopes are less than 1.0, so g-ratios increase with size but less rapidly than in the Noise and WT groups, as indicated by the flatter curves (Fig. 7E).

Stereological changes of VCN after cochlear insults

Previous studies have demonstrated loss of auditory nerve inputs in the VCN after acoustic overexposure (Kim et al., 2004), surgical destruction of the cochlea (Fyk-Kolodziej et al., 2011), and application of ototoxic substances (Zeng et al., 2009, Yuan et al., 2014, Heeringa et al., 2016), but the effects of other types of HC loss on the VCN are not well characterized. To evaluate stereological changes in the VCN after DT-induced HC loss, we prepared transverse sections (N = 6 animals for each group) stained with cresyl violet and assessed the total volume, neuron density, and cell size in the VCN (Fig. 8). VCN volume was significantly different between groups ($F_{3,20} = 3.41$, $p = 0.038$), but only the Noise group was significantly different from WT ($F_{1,10} = 9.95$, $p = 0.010$). Although means for DT 2M and Noise were similar, the larger variance of DT 2M resulted in a non-significant difference from control ($F_{1,10} = 2.12$, $p = 0.176$).

To evaluate whether cell density and size differed between groups and whether that effect differed between dorsal and ventral sides of the VCN, we performed a 2-way MANOVA with group and side as main effects and including an interaction term. We found significant differences between groups ($F_{6,80} = 5.08$, $p < 0.001$) and between sides ($F_{2,39} = 5.90$, $p = 0.006$), but not a significant interaction between group and side ($F_{6,80} = 0.88$, $p = 0.516$). In pair-wise analyses of groups, Noise, DT 1M and DT 2M were all significantly different from WT, but there was a significant difference between sides only for the comparison of Noise to WT. In all deafened groups, cell density did not differ significantly from controls ($F_{1,20} < 2.8$, $p > 0.1$), but cell size decreased ($F_{1,20} > 9.8$, $p < 0.005$). Noise differed from the DT exposed groups in having a smaller decrease of cell size in the ventral region ($F_{1,20} = 11.1$, $p = 0.003$), corresponding to low frequency inputs.

Vesicular glutamate transporter-1 expression in CN

To determine whether DT-induced HC ablation causes synaptic changes at the central terminal of SGNs, we immunostained the CN with an antibody to VGLUT-1 (N = 6 animals for each group; Fig. 9A-D). Quantitative analysis showed that VGLUT-1 puncta density differed significantly among groups ($F_{18,51} = 1.81$, $p = 0.050$), however significant differences from WT were found only for Noise ($F_{6,5} = 6.35$, $p = 0.030$) despite the fact that the DT groups had no HCs and were profoundly deaf and the Noise group had no significant loss of IHC or SGN. Individual regions contributing to the difference between Noise and WT groups were AVCN, PVCN and DCN3 ($F_{1,10} > 11.0$, $p < 0.008$).

Discussion

We showed that within 2 months of DT-induced HC loss, with primary damage restricted to HCs, there was no significant impact on the survival of SGNs, central synaptic connectivity, and myelination, despite complete loss of HCs and profound deafness. In contrast, noise-exposed mice exhibited significant degeneration of synapses and myelin even though they had no loss of IHCs or SGNs. Therefore, selective HC ablation and noise exposure produced different patterns of pathology in the auditory pathway. Loss of neural activity resulting from HC loss caused decreases in neuronal size in the cochlear nucleus regardless of the type of cochlear insult. Consequently, nearly all of the typical changes seen in the cochlea

and the CN after cochlear insults are not secondary effects of the loss of hair cells and loss of input to the auditory nerve, but rather, are due to primary trauma to cells other than hair cells.

Selective HC ablation in DTR mice

Experimental animal studies designed to assess the outcome of HC loss on the neural components of the auditory system have used lesions induced by acoustic overstimulation or ototoxic drug administration. Using these experimental injury models, many cochlear components are damaged in addition to HC, including synapses, SGNs, myelin, and SCs. In contrast, in adult humans, the loss of HCs is not necessarily accompanied by a significant loss of SGNs (Glueckert et al., 2005, Teufert et al., 2006). Therefore, the pathology of these animal models is markedly different from that associated with human deafness, where the pathology can be restricted to the HCs. The DTR mouse, where the insult is specific to HC by design, may represent a better model of the human SGN response to a peripheral lesion.

Initial work with the DTR mouse (Golub et al., 2012, Kaur et al., 2015, Tong et al., 2015) described the pattern of changes in the cochlear sensory epithelium after DT injection. Our results confirm the original characterization of the mouse. We found that DT injection (15 ng/g) leads to complete HC loss and abolition of ABR thresholds by 1 month after the injection. The cochlear pathology, hearing deficits and CN cell size changes in the DTR mice reported here agree with the published data (Golub et al., 2012, Kaur et al., 2015, Tong et al., 2015). We then extended the study to examine the outcomes of the selective HC loss on neural components including synapses, axonal diameter and myelination, and to compare these parameters with changes that occur after noise exposure in the same type of mouse.

Selective loss of specific cell types in the cochlea has been shown using other transgenic technologies in mice. For instance, a low thiamine diet in mice with a targeted deletion of the high-affinity thiamine transporter gene (Slc19a2) resulted in loss of most IHCs and some OHCs. Hair cell-specific Cre-ER alleles to drive expression of DT fragment A (DTA) (Lieberman et al., 2006, Burns et al., 2012, Zilberstein et al., 2012) resulted in a complete elimination of HCs, but the procedure could only be accomplished during development. The DTR mouse can be used to induce a complete loss of HCs at any age. This ability makes these mice powerful tools for assessing the response of the neural network to the loss of all HCs and for studies on restorative strategies such as HC regeneration or cochlear implantation, in young as well as old mice.

Survival of SGNs after selective HC loss

We have shown that SGNs can survive for at least 2 months after a selective toxic exposure that completely and exclusively eliminates HCs. These results are consistent with previous reports that selective loss of HCs does not result in significant SGN loss (Zilberstein et al., 2012, Tong et al., 2015, Ding et al., 2016). SGNs in the DT 1M group appeared to be more densely packed compared to those of WT, but the difference was not significant. This clustering change of SGNs might contribute to the SGN survival after HC loss through ephaptic cross transmission and pro-survival signaling. Other experimental models of HC loss result in retraction of the peripheral processes and progressively affect more central

regions of the SGNs (Terayama et al., 1979, Leake and Hradek, 1988). In contrast, complete loss of HCs in the DTR mouse involves no significant loss of peripheral ANFs (Fig. 5). Our noise-exposed mice also showed survival of SGNs and peripheral ANFs at 1 month after noise exposure (Fig. 5, 6) but their IHCs did not degenerate, consistent with previous studies using this model. Some neural degeneration might occur very slowly in noise-exposed mice, but the amount of neural loss is relatively small. Consistent with our results, prior reports of noise-induced neural degeneration showed that the death of SGNs was delayed by months and can progress over years (Kujawa and Liberman, 2006, 2009). Because the time course of our study was limited to 2 months, we cannot determine if changes in SGN density occur in the long term, with either noise or DT ablation of HCs.

Previous studies suggested that the loss of SGNs might also result from associated loss of SCs that surround the IHCs (Sugawara et al., 2005). These SCs, including the inner border and inner phalangeal cells, are in intimate contact with the type I SGN terminals in the neuropil underneath the IHCs and play a critical role in the survival of SGNs through secretion of neurotrophins. Alterations in ErbB receptor signaling in SCs result in reduced expression of neurotrophins accompanied by dramatic SGN degeneration (Stankovic et al., 2004), suggesting that SGN survival in the adult cochlea may be mediated by SCs. Consistent with these reports, our DTR mice showed no loss of SCs and no loss of SGNs. Taken together, these two studies, one eliminating HCs and the other eliminating SCs, demonstrate that the presence of SCs is necessary and sufficient for preserving SGNs and their peripheral fibers, at least for the short term. Studies with longer survival times following HC ablation with DT are needed to determine whether the SCs and neurons are stable throughout the life of the mouse. In particular, further investigation of detailed morphological changes of SCs and cell signaling pathways after selective HC ablation will be necessary.

Expression of GluR2 after HC loss

In recent years, increasing attention has been paid to the role of primary synaptic degeneration without HC loss (Kujawa and Liberman, 2015). IHC-SGN synaptic transmission is excitatory and glutamatergic (Eybalin, 1993). This synapse is susceptible to excitotoxicity; intracochlear perfusion of glutamate agonists results in degeneration of the peripheral synaptic terminals on IHCs (Pujol et al., 1985, Puel et al., 1994). Acoustic trauma results in similar damage to synapses (Puel et al., 1998), presumably as a result of excessive glutamate release from IHCs (Puel et al., 1998, Hakuba et al., 2000). The loss of presynaptic ribbons and postsynaptic terminals results in functional silencing of the affected neurons. In the current study, noise-exposed mice showed swelling of postsynaptic terminals (Fig. 3B) and decreases of postsynaptic GluR2 expression at all frequencies (Fig. 4). This is consistent with our ABR results showing that the threshold at 8 kHz was significantly elevated after noise exposure despite survival of all IHCs in this frequency region. Moreover, we found that postsynaptic GluR2 expression was dramatically decreased after selective HC ablation. It is not surprising that presynaptic ribbons diminished after selective HC loss, because they are components of the IHC. However, GluR2 is a component of the postsynaptic glutamate receptor and its expression was altered after selective HC loss despite the lack of a toxic effect on SGNs. This result suggests HCs are indispensable for the expression of

postsynaptic GluR2 or that neurotransmitter release from the IHC regulates GluR2 expression. These changes in the synaptic regions should be considered when the DTR mouse is used for HC regeneration studies, because their reversal would be necessary for effective restoration of hearing.

VGLUT-1 expression in CN of DTR mice after noise or DT

Selective HC ablation resulted in no significant change in density of VGLUT-1 puncta in the CN despite deafness and complete HC loss (Fig. 9). Moreover, although there was no significant loss of SGNs and IHCs in noise-exposed mice, VGLUT-1 expression was significantly decreased in the AVCN, PVCN, and DCN3 regions that receive type I SGN projections. Our noise exposure results are consistent with previous reports that cochlear damage, such as kanamycin injection or acoustic overstimulation, leads to decrease of VGLUT-1 expression in CN (Zeng et al., 2009, Barker et al., 2012). Furthermore, it was recently reported that SGN degeneration is not necessary for down-regulation of VGLUT-1 punctum density in the CN regions innervated by SGNs (Heeringa et al., 2016). Together, these data indicate that central synaptic changes occur either when the insult affects the SGN directly as may be the case with kanamycin, or due to excitotoxicity, as is the case with noise exposure.

Our results also suggested that presence of HCs and normal hearing are not necessary for the maintenance of central connectivity, at least at the structural level. Furthermore, previous studies suggested that VGLUT-2, which co-labels with non-auditory terminals, is up-regulated in the CN to compensate for cochlear damage, reflecting a reactive re-innervation of the CN by inputs from the non-auditory system (Zeng et al., 2009, Barker et al., 2012, Zeng et al., 2012, Heeringa et al., 2016). This cross-modal plasticity in the CN after cochlear damage might be important for the development of tinnitus (Dehmel et al., 2012, Koehler and Shore, 2013).

Hypomyelination and decrease of axon caliber

Loss or changes in myelination in the ear can lead to hearing dysfunction as sometimes seen in patients with multiple sclerosis, a chronic demyelinating disease of the CNS; Charcot-Marie-Tooth disease, a hereditary disorder caused by genetic mutations related to peripheral nerve myelin; and Guillain-Barré syndrome, an acute immune-mediated neuropathy (Nelson et al., 1988, Starr et al., 2003, Lassmann et al., 2012). In the current study, we found that acoustic overstimulation resulted in significant increase of g-ratio and hypomyelination of the ANFs (Fig. 7), consistent with a previous report (Tagoe et al., 2014). However, selective HC ablation resulted in no significant change of myelination despite complete HC loss and profound deafness (Fig. 7). These results suggest HC survival and normal hearing are not required for the maintenance of myelin on the SGN. Therefore, hypomyelination of ANFs after acoustic overstimulation might be caused by primary damage to the Schwann cells caused by the insult. Moreover, we demonstrated decreases in peripheral axon caliber in both noise-exposed and DTR mice. This suggested that loss of neural activity in these mice might result in the decrease of axon caliber despite the different types of cochlear damage and histopathology. The functional implication of reduction in axon caliber is a decrease in the conduction velocity of the associated neurons (Miller et al., 2002). Since ANFs in DT-

deafened animals did not receive input from HCs anymore, and thus no ABR was recordable, we could not assess conduction velocity in these ears. Future experiments will be needed to determine whether caliber of the central axon also changes after DT injection.

Decrease of cell size in SGNs and VCN

In cases where SGNs degenerate after cochlear damage (Ylikoski, 1974, Spoendlin, 1975, Shepherd and Colreavy, 2004), or when SGN cell size is reduced (Staecker et al., 1996, Leake et al., 1999, Richardson et al., 2005), the cause of these changes is thought to be loss of neurotrophic support (Ernfors et al., 1995, Fritzsch et al., 1999, Schimmang et al., 2003). The mean SGN size in DT-treated DTR mice was found to be significantly smaller than that of WT SGNs, but SGN density did not decrease, suggesting that neurons can survive without connectivity to the HCs for at least 2 months. Considering that decrease in average SGN size can be followed by a delayed decrease in SGN packing density (van Loon et al., 2013), it remains to be determined whether long-term SGN survival can be sustained after selective HC loss. The decrease of SGN size in the base of the cochlea of our noise-exposed mice despite survival of IHCs suggests that multiple factors may influence SGN survival and size. Similarly, the decrease in cell size in the VCN after either selective HC loss or noise exposure is consistent with previous reports that blockade of SGN electrical activity can lead to changes in CN cell size that are independent of IHC loss (Sie and Rubel, 1992, Tong et al., 2015).

Implications for regenerative therapy

Regenerative studies to restore the population of HCs and reverse sensorineural hearing loss often use models where a full depletion of the original HCs is induced, allowing for more conclusive identification of new HCs. However, the insults induced by severe overstimulation or neomycin also lead to neural changes. Another limitation of these insults is the variability that often exists in the extent of HC loss between animals and between right and left ears of the same subject. For these reasons, the DTR mouse is an attractive model, even though it does not represent an actual human pathology. This model provides a cochlea in which the entire population of HCs is depleted reliably and symmetrically in both ears, leaving the SGNs and the CN area nearly intact.

When technology for efficiently and consistently inducing HC regeneration is in place, it will be necessary to also induce restoration or regeneration of synapses between the new HCs and the SGNs. Neurotrophin-3 (NT-3) might be a strong candidate for synaptic regeneration because it has been reported that NT-3 plays critical roles in the restoration of inner ear synapse density after injury (Wan et al., 2014). It is also possible that new HCs will secrete sufficient NT-3 to attract nerve endings and synapse restoration. In addition to serving as a useful model for HC regeneration and synapse formation, DTR mice can also be utilized for designing strategies for regulating neurofilament activity in order to maintain axon caliber or for restoration of myelination.

Conclusions

We have demonstrated that selective HC ablation and noise exposure showed different patterns of pathology in the auditory system, from the cochlea to the CN. Following a complete ablation of all HCs with DT, changes in the cochlea and the CN are minimal. In contrast, noise exposure induced significant changes in the SGN despite survival of IHCs. These results indicate that presence of IHCs is not a prerequisite for the maintenance of central synaptic connectivity and myelination and that noise exposure can change the neural substrate even without loss of IHCs.

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Abbreviations

ABR	auditory brainstem response
ANF	auditory nerve fiber
AVCN	anteroventral cochlear nucleus
CN	cochlear nucleus
CtBP2	c-terminal binding protein 2
DCN1	molecular layer of the dorsal cochlear nucleus
DCN2	fusiform cell layer of the dorsal cochlear nucleus
DCN3	deep layer of the dorsal cochlear nucleus
DCs	Deiters' cells
DT	diphtheria toxin
DTA	diphtheria toxin fragment A
DTR	diphtheria toxin receptor
EDTA	ethylenediaminetetraacetic acid
GCL	granule cell lamina
GluR2	glutamate receptor 2
HC	hair cell
hDTR	human diphtheria toxin receptor
IHC	inner hair cell

NF	neurofilament
NT-3	neurotrophin-3
OHC	outer hair cell
OSL	osseous spiral lamina
PB	phosphate buffer
PBS	phosphate buffered saline
PCs	pillar cells
PFA	paraformaldehyde
PVCN	posteroventral cochlear nucleus
P1	peak 1
RT	room temperature
SCs	supporting cells
SD	standard deviation
SGNs	spiral ganglion neurons
SL	spiral ligament
SNHL	sensorineural hearing loss
SV	stria vascularis
TEM	transmission electron microscope
VCN	ventral cochlear nucleus
VGLUT-1	vesicular glutamate transporter-1

References

- Aarnisalo AA, Pirvola U, Liang XQ, Miller J, Ylikoski J. Apoptosis in auditory brainstem neurons after a severe noise trauma of the organ of Corti: intracochlear GDNF treatment reduces the number of apoptotic cells. *ORL J Otorhinolaryngol Relat Spec.* 2000; 62:330–334. [PubMed: 11054017]
- Arbeloa J, Perez-Samartin A, Gottlieb M, Matute C. P2X7 receptor blockade prevents ATP excitotoxicity in neurons and reduces brain damage after ischemia. *Neurobiol Dis.* 2012; 45:954–961. [PubMed: 22186422]
- Badea TC, Nathans J. Morphologies of mouse retinal ganglion cells expressing transcription factors Brn3a, Brn3b, and Brn3c: analysis of wild type and mutant cells using genetically-directed sparse labeling. *Vision Res.* 2011; 51:269–279. [PubMed: 20826176]
- Barker M, Solinski HJ, Hashimoto H, Tagoe T, Pilati N, Hamann M. Acoustic overexposure increases the expression of VGLUT-2 mediated projections from the lateral vestibular nucleus to the dorsal cochlear nucleus. *PLoS One.* 2012; 7:e35955. [PubMed: 22570693]

- Beyer LA, Odeh H, Probst FJ, Lambert EH, Dolan DF, Camper SA, Kohrman DC, Raphael Y. Hair cells in the inner ear of the pirouette and shaker 2 mutant mice. *J Neurocytol.* 2000; 29:227–240. [PubMed: 11276175]
- Burns JC, Cox BC, Thiede BR, Zuo J, Corwin JT. In vivo proliferative regeneration of balance hair cells in newborn mice. *The Journal of neuroscience : the official journal of the Society for Neuroscience.* 2012; 32:6570–6577. [PubMed: 22573679]
- Cankaya H, Oner AF, Egeli E, Caksen H, Uner A, Akcay G. Auditory brainstem response in children with iron deficiency anemia. *Acta Paediatr Taiwan.* 2003; 44:21–24. [PubMed: 12800379]
- Chomiak T, Hu B. What is the optimal value of the g-ratio for myelinated fibers in the rat CNS? A theoretical approach. *PLoS One.* 2009; 4:e7754. [PubMed: 19915661]
- Dehmel S, Pradhan S, Koehler S, Bledsoe S, Shore S. Noise overexposure alters long-term somatosensory-auditory processing in the dorsal cochlear nucleus--possible basis for tinnitus-related hyperactivity? *The Journal of neuroscience : the official journal of the Society for Neuroscience.* 2012; 32:1660–1671. [PubMed: 22302808]
- Ding D, Jiang H, Chen GD, Longo-Guess C, Muthaiah VP, Tian C, Sheppard A, Salvi R, Johnson KR. N-acetyl-cysteine prevents age-related hearing loss and the progressive loss of inner hair cells in gamma-glutamyl transferase 1 deficient mice. *Aging.* 2016
- Ernfors P, Van De Water T, Loring J, Jaenisch R. Complementary roles of BDNF and NT-3 in vestibular and auditory development. *Neuron.* 1995; 14:1153–1164. [PubMed: 7605630]
- Eybalin M. Neurotransmitters and neuromodulators of the mammalian cochlea. *Physiol Rev.* 1993; 73:309–373. [PubMed: 8097330]
- Fairfield DA, Lomax MI, Dootz GA, Chen S, Galecki AT, Benjamin IJ, Dolan DF, Altschuler RA. Heat shock factor 1-deficient mice exhibit decreased recovery of hearing following noise overstimulation. *J Neurosci Res.* 2005; 81:589–596. [PubMed: 15952177]
- Flores EN, Duggan A, Madathany T, Hogan AK, Marquez FG, Kumar G, Seal RP, Edwards RH, Liberman MC, Garcia-Anoveros J. A non-canonical pathway from cochlea to brain signals tissue-damaging noise. *Curr Biol.* 2015; 25:606–612. [PubMed: 25639244]
- Frank T, Rutherford MA, Strenzke N, Neef A, Pangrsic T, Khimich D, Fejtova A, Gundelfinger ED, Liberman MC, Harke B, Bryan KE, Lee A, Egner A, Riedel D, Moser T. Bassoon and the synaptic ribbon organize Ca(2)+ channels and vesicles to add release sites and promote refilling. *Neuron.* 2010; 68:724–738. [PubMed: 21092861]
- Fritzsch B, Pirvola U, Ylikoski J. Making and breaking the innervation of the ear: neurotrophic support during ear development and its clinical implications. *Cell Tissue Res.* 1999; 295:369–382. [PubMed: 10022958]
- Fukui H, Wong HT, Beyer LA, Case BG, Swiderski DL, Di Polo A, Ryan AF, Raphael Y. BDNF gene therapy induces auditory nerve survival and fiber sprouting in deaf Pou4f3 mutant mice. *Sci Rep.* 2012; 2:838. [PubMed: 23150788]
- Fyk-Kolodziej B, Shimano T, Gong TW, Holt AG. Vesicular glutamate transporters: spatio-temporal plasticity following hearing loss. *Neuroscience.* 2011; 178:218–239. [PubMed: 21211553]
- Glueckert R, Pfaller K, Kinnefors A, Rask-Andersen H, Schrott-Fischer A. The human spiral ganglion: new insights into ultrastructure, survival rate and implications for cochlear implants. *Audiol Neurotol.* 2005; 10:258–273. [PubMed: 15925863]
- Golub JS, Tong L, Ngyuen TB, Hume CR, Palmiter RD, Rubel EW, Stone JS. Hair cell replacement in adult mouse utricles after targeted ablation of hair cells with diphtheria toxin. *J Neurosci.* 2012; 32:15093–15105. [PubMed: 23100430]
- Gong TW, Fairfield DA, Fullarton L, Dolan DF, Altschuler RA, Kohrman DC, Lomax MI. Induction of heat shock proteins by hyperthermia and noise overstimulation in hsf1 $-/-$ mice. *J Assoc Res Otolaryngol.* 2012; 13:29–37. [PubMed: 21932106]
- Hakuba N, Koga K, Gyo K, Usami SI, Tanaka K. Exacerbation of noise-induced hearing loss in mice lacking the glutamate transporter GLAST. *The Journal of neuroscience : the official journal of the Society for Neuroscience.* 2000; 20:8750–8753. [PubMed: 11102482]
- Heeringa AN, Stefanescu RA, Raphael Y, Shore SE. Altered vesicular glutamate transporter distributions in the mouse cochlear nucleus following cochlear insult. *Neuroscience.* 2016; 315:114–124. [PubMed: 26705736]

- Henry KR, Chole RA. Genotypic differences in behavioral, physiological and anatomical expressions of age-related hearing loss in the laboratory mouse. *Audiology*. 1980; 19:369–383. [PubMed: 7436856]
- Jimenez AM, Stagner BB, Martin GK, Lonsbury-Martin BL. Age-related loss of distortion product otoacoustic emissions in four mouse strains. *Hearing research*. 1999; 138:91–105. [PubMed: 10575118]
- Kaur T, Zamani D, Tong L, Rubel EW, Ohlemiller KK, Hirose K, Warchol ME. Fractalkine Signaling Regulates Macrophage Recruitment into the Cochlea and Promotes the Survival of Spiral Ganglion Neurons after Selective Hair Cell Lesion. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2015; 35:15050–15061. [PubMed: 26558776]
- Keithley EM, Erkman L, Bennett T, Lou L, Ryan AF. Effects of a hair cell transcription factor, Brn-3.1, gene deletion on homozygous and heterozygous mouse cochleas in adulthood and aging. *Hearing research*. 1999; 134:71–76. [PubMed: 10452377]
- Kim JJ, Gross J, Potashner SJ, Morest DK. Fine structure of long-term changes in the cochlear nucleus after acoustic overstimulation: chronic degeneration and new growth of synaptic endings. *J Neurosci Res*. 2004; 77:817–828. [PubMed: 15334600]
- Koehler SD, Shore SE. Stimulus timing-dependent plasticity in dorsal cochlear nucleus is altered in tinnitus. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2013; 33:19647–19656. [PubMed: 24336728]
- Kujawa SG, Liberman MC. Acceleration of age-related hearing loss by early noise exposure: evidence of a misspent youth. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2006; 26:2115–2123. [PubMed: 16481444]
- Kujawa SG, Liberman MC. Adding insult to injury: cochlear nerve degeneration after "temporary" noise-induced hearing loss. *J Neurosci*. 2009; 29:14077–14085. [PubMed: 19906956]
- Kujawa SG, Liberman MC. Synaptopathy in the noise-exposed and aging cochlea: Primary neural degeneration in acquired sensorineural hearing loss. *Hearing research*. 2015; 330:191–199. [PubMed: 25769437]
- Lassmann H, van Horssen J, Mahad D. Progressive multiple sclerosis: pathology and pathogenesis. *Nat Rev Neurol*. 2012; 8:647–656. [PubMed: 23007702]
- Leake PA, Hradek GT. Cochlear pathology of long term neomycin induced deafness in cats. *Hearing research*. 1988; 33:11–33. [PubMed: 3372368]
- Leake PA, Hradek GT, Snyder RL. Chronic electrical stimulation by a cochlear implant promotes survival of spiral ganglion neurons after neonatal deafness. *J Comp Neurol*. 1999; 412:543–562. [PubMed: 10464355]
- Lee DL, Strathmann FG, Gelein R, Walton J, Mayer-Proschel M. Iron deficiency disrupts axon maturation of the developing auditory nerve. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2012; 32:5010–5015. [PubMed: 22492056]
- Lesperance MM, Helfert RH, Altschuler RA. Deafness induced cell size changes in rostral AVCN of the guinea pig. *Hearing research*. 1995; 86:77–81. [PubMed: 8567424]
- Liberman MC, Tartaglini E, Fleming JC, Neufeld EJ. Deletion of SLC19A2, the high affinity thiamine transporter, causes selective inner hair cell loss and an auditory neuropathy phenotype. *J Assoc Res Otolaryngol*. 2006; 7:211–217. [PubMed: 16642288]
- Liu K, Jiang X, Shi C, Shi L, Yang B, Shi L, Xu Y, Yang W, Yang S. Cochlear inner hair cell ribbon synapse is the primary target of ototoxic aminoglycoside stimuli. *Mol Neurobiol*. 2013; 48:647–654. [PubMed: 23606282]
- Matsubara A, Laake JH, Davanger S, Usami S, Ottersen OP. Organization of AMPA receptor subunits at a glutamate synapse: a quantitative immunogold analysis of hair cell synapses in the rat organ of Corti. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 1996; 16:4457–4467. [PubMed: 8699256]
- Miller CC, Ackerley S, Brownlees J, Grierson AJ, Jacobsen NJ, Thornhill P. Axonal transport of neurofilaments in normal and disease states. *Cell Mol Life Sci*. 2002; 59:323–330. [PubMed: 11924605]
- Muller M, von Hunerbein K, Hoidis S, Smolders JW. A physiological place-frequency map of the cochlea in the CBA/J mouse. *Hearing research*. 2005; 202:63–73. [PubMed: 15811700]

- Muniak MA, Rivas A, Montey KL, May BJ, Francis HW, Ryugo DK. 3D model of frequency representation in the cochlear nucleus of the CBA/J mouse. *J Comp Neurol*. 2013; 521:1510–1532. [PubMed: 23047723]
- Nelson KR, Gilmore RL, Massey A. Acoustic nerve conduction abnormalities in Guillain-Barre syndrome. *Neurology*. 1988; 38:1263–1266. [PubMed: 3399076]
- Puel JL, Pujol R, Tribillac F, Ladrech S, Eybalin M. Excitatory amino acid antagonists protect cochlear auditory neurons from excitotoxicity. *J Comp Neurol*. 1994; 341:241–256. [PubMed: 7512999]
- Puel JL, Ruel J, Gervais d'Aldin C, Pujol R. Excitotoxicity and repair of cochlear synapses after noise-trauma induced hearing loss. *Neuroreport*. 1998; 9:2109–2114. [PubMed: 9674603]
- Pujol R, Lenoir M, Robertson D, Eybalin M, Johnstone BM. Kainic acid selectively alters auditory dendrites connected with cochlear inner hair cells. *Hearing research*. 1985; 18:145–151. [PubMed: 2864329]
- Richardson RT, O'Leary S, Wise A, Hardman J, Clark G. A single dose of neurotrophin-3 to the cochlea surrounds spiral ganglion neurons and provides trophic support. *Hearing research*. 2005; 204:37–47. [PubMed: 15925190]
- Schimmang T, Tan J, Muller M, Zimmermann U, Rohbock K, Kopschall I, Limberger A, Minichiello L, Knipper M. Lack of Bdnf and TrkB signalling in the postnatal cochlea leads to a spatial reshaping of innervation along the tonotopic axis and hearing loss. *Development*. 2003; 130:4741–4750. [PubMed: 12925599]
- Sha SH, Kanicki A, Dootz G, Talaska AE, Halsey K, Dolan D, Altschuler R, Schacht J. Age-related auditory pathology in the CBA/J mouse. *Hearing research*. 2008; 243:87–94. [PubMed: 18573325]
- Shepherd RK, Colreavy MP. Surface microstructure of the perilymphatic space: implications for cochlear implants and cell- or drug-based therapies. *Arch Otolaryngol Head Neck Surg*. 2004; 130:518–523. [PubMed: 15148170]
- Shibata SB, Cortez SR, Beyer LA, Wiler JA, Di Polo A, Pflingst BE, Raphael Y. Transgenic BDNF induces nerve fiber regrowth into the auditory epithelium in deaf cochleae. *Exp Neurol*. 2010; 223:464–472. [PubMed: 20109446]
- Sie KC, Rubel EW. Rapid changes in protein synthesis and cell size in the cochlear nucleus following eighth nerve activity blockade or cochlea ablation. *J Comp Neurol*. 1992; 320:501–508. [PubMed: 1629400]
- Song L, McGee J, Walsh EJ. Frequency- and level-dependent changes in auditory brainstem responses (ABRS) in developing mice. *The Journal of the Acoustical Society of America*. 2006; 119:2242–2257. [PubMed: 16642839]
- Spoendlin H. Neuroanatomical basis of cochlear coding mechanisms. *Audiology*. 1975; 14:383–407. [PubMed: 1156247]
- Staecker H, Kopke R, Malgrange B, Lefebvre P, Van de Water TR. NT-3 and/or BDNF therapy prevents loss of auditory neurons following loss of hair cells. *Neuroreport*. 1996; 7:889–894. [PubMed: 8724667]
- Stankovic K, Rio C, Xia A, Sugawara M, Adams JC, Liberman MC, Corfas G. Survival of adult spiral ganglion neurons requires erbB receptor signaling in the inner ear. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2004; 24:8651–8661. [PubMed: 15470130]
- Starr A, Michalewski HJ, Zeng FG, Fujikawa-Brooks S, Linthicum F, Kim CS, Winnier D, Keats B. Pathology and physiology of auditory neuropathy with a novel mutation in the MPZ gene (Tyr145>Ser). *Brain*. 2003; 126:1604–1619. [PubMed: 12805115]
- Sugawara M, Corfas G, Liberman MC. Influence of supporting cells on neuronal degeneration after hair cell loss. *J Assoc Res Otolaryngol*. 2005; 6:136–147. [PubMed: 15952050]
- Tagoe T, Barker M, Jones A, Allcock N, Hamann M. Auditory nerve perinodal dysmyelination in noise-induced hearing loss. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2014; 34:2684–2688. [PubMed: 24523557]
- Takada Y, Beyer LA, Swiderski DL, O'Neal AL, Prieskorn DM, Shivatzki S, Avraham KB, Raphael Y. Connexin 26 null mice exhibit spiral ganglion degeneration that can be blocked by BDNF gene therapy. *Hear Res*. 2014; 309:124–135. [PubMed: 24333301]

- Terayama Y, Kaneko K, Tanaka K, Kawamoto K. Ultrastructural changes of the nerve elements following disruption of the organ of Corti. II. Nerve elements outside the organ of Corti. *Acta Otolaryngol.* 1979; 88:27–36. [PubMed: 474117]
- Teufert KB, Linthicum FH Jr, Connell SS. The effect of organ of corti loss on ganglion cell survival in humans. *Otol Neurotol.* 2006; 27:1146–1151. [PubMed: 16885783]
- Tong L, Strong MK, Kaur T, Juiz JM, Oesterle EC, Hume C, Warchol ME, Palmiter RD, Rubel EW. Selective deletion of cochlear hair cells causes rapid age-dependent changes in spiral ganglion and cochlear nucleus neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience.* 2015; 35:7878–7891. [PubMed: 25995473]
- van Loon MC, Ramekers D, Agterberg MJ, de Groot JC, Grolman W, Klis SF, Versnel H. Spiral ganglion cell morphology in guinea pigs after deafening and neurotrophic treatment. *Hearing research.* 2013; 298:17–26. [PubMed: 23361189]
- Wan G, Gomez-Casati ME, Gigliello AR, Liberman MC, Corfas G. Neurotrophin-3 regulates ribbon synapse density in the cochlea and induces synapse regeneration after acoustic trauma. *eLife.* 2014; 3
- Ylikoski J. Guinea-pig hair cell pathology from ototoxic antibiotics. *Acta Otolaryngol Suppl.* 1974; 326:5–22. [PubMed: 4534032]
- Yuan Y, Shi F, Yin Y, Tong M, Lang H, Polley DB, Liberman MC, Edge AS. Ouabain-induced cochlear nerve degeneration: synaptic loss and plasticity in a mouse model of auditory neuropathy. *J Assoc Res Otolaryngol.* 2014; 15:31–43. [PubMed: 24113829]
- Zeng C, Nannapaneni N, Zhou J, Hughes LF, Shore S. Cochlear damage changes the distribution of vesicular glutamate transporters associated with auditory and nonauditory inputs to the cochlear nucleus. *The Journal of neuroscience : the official journal of the Society for Neuroscience.* 2009; 29:4210–4217. [PubMed: 19339615]
- Zeng C, Yang Z, Shreve L, Bledsoe S, Shore S. Somatosensory projections to cochlear nucleus are upregulated after unilateral deafness. *The Journal of neuroscience : the official journal of the Society for Neuroscience.* 2012; 32:15791–15801. [PubMed: 23136418]
- Zhou J, Nannapaneni N, Shore S. Vesicular glutamate transporters 1 and 2 are differentially associated with auditory nerve and spinal trigeminal inputs to the cochlear nucleus. *J Comp Neurol.* 2007; 500:777–787. [PubMed: 17154258]
- Zilberstein Y, Liberman MC, Corfas G. Inner hair cells are not required for survival of spiral ganglion neurons in the adult cochlea. *The Journal of neuroscience : the official journal of the Society for Neuroscience.* 2012; 32:405–410. [PubMed: 22238076]

Highlights

- Auditory neurons can survive for at least two months after selective HC ablation.
- Axon caliber and SGN soma size are significantly smaller after selective HC loss, but their density is unchanged.
- Myelin surrounding auditory neurons is maintained in the absence of HCs.
- HCs are indispensable for the GluR2 expression in the peripheral synapses.
- The VGLUT-1 expression in the CN is unchanged two months after DT injection despite complete loss of HCs and deafness.

Fig. 1.

DTR mice treated with DT are profoundly deaf. (A) Schedule of the experimental procedures. (B) Before DT injection (baseline), ABR thresholds at 8, 20, and 32 kHz are essentially equivalent in WT (black) and DTR (blue) mice (3 weeks of age); $N = 5$ in each group. (C) At 1 month after DT injection, thresholds are elevated above 100 dB SPL for DTR mice (DT 1M, blue) but DT-injected WT mice (black) have normal thresholds. Noise-exposed DTR mice (Noise, red) have elevated but still detectable thresholds at all frequencies 1 month after noise exposure. All differences between groups are significant. (D) Representative ABR waveforms are shown from responses to 32 kHz, 100 dB SPL in WT (black), DT 1M (blue), and noise-exposed (red) mice. Arrowheads indicate ABR peak 1 (P1). (E) ABR P1 latency is significantly greater in noise-exposed mice (red) than in WT mice (black) at all frequencies. In DT-treated mice, ABR P1 latency could not be measured because they had no ABRs at 100 dB SPL. Error bars indicate 1 standard deviation of the mean. Error bars indicate 1 standard deviation of the mean; brackets indicate groups that are significantly different (***, $p < 0.001$).

Fig. 2.

DTR mice treated with DT lose their HCs. Confocal fluorescence images of F-actin (green) and myosin VIIa (red) in whole mounts of the auditory epithelium in the 32 kHz region (A-D). (A) WT cochleae demonstrate a well-defined single row of IHCs and three rows of OHCs. (B) In cochleae exposed to noise, severe OHC damage is observed, but almost all IHCs survive. (C) DT-treated DTR mice analyzed 1 month after the treatment show a complete loss of IHCs and a nearly complete loss of OHCs (white arrowheads indicate 2 surviving OHC). Phalangeal scars made by DCs and other SCs appear typical. (D) At the 2 month time point, all hair cells are missing in DT-treated DTR mice. Cytocochleograms (E, F) show the average percentage of missing OHCs (E) and IHCs (F); N = 4 in each group. In both the DT 1M (yellow) and DT 2M (blue) groups, nearly all HCs are eliminated throughout the cochlea. In the Noise (red) group, OHCs were almost completely eliminated in the basal half of the cochlea, but nearly all IHCs survived at all locations. DC, Deiters' cell; IHC, inner hair cell; OHC, outer hair cell; PC, pillar cell. Scale bar indicates 20 μ m.

Fig. 3.

Differences in cochlear pathology between DT and Noise are not limited to HCs. (A) Photomicrographs of mid-modiolar cross-section through the organ of Corti at the basal turn of WT mice reveal a highly organized sensory epithelium with the typical array of IHCs, OHCs, and SCs. In DT 2M mice, neither IHCs nor OHCs are detected, but all SCs remain intact and appear typical. In noise-exposed mice, OHCs are not detected, but other cells, including IHCs and most SCs, remain intact. IHC, inner hair cell; OHC, outer hair cell. Scale bar indicates 20 μ m. (B) High magnification images of IHCs at the basal turn. Vacuolization in the IHC area is seen only in noise-exposed mice (white arrowhead). Scale bar indicates 10 μ m. (C) Photomicrographs of the lateral wall at the basal turn. Compared to WT mice, DT 2M mice show obvious thinning of the SV (white arrowheads), whereas 1 month after noise exposure, the diameter appears slightly decreased and vascular spaces are enlarged. Scale bar indicates 100 μ m. (D) High magnification images of the lateral wall of the basal turn reveal loss of type IV fibrocytes in noise-exposed mice (white arrowhead) but not in DT 2M mice. Scale bar indicates 50 μ m.

Fig. 4.

GluR2 expression disappears after IHC loss. Confocal fluorescence microscopy z-stack images from the 8 kHz region showing GluR2 (red) and neurofilament (green) (A-D). (A) WT mice have abundant ANF terminals and postsynaptic GluR2 puncta. (B) Noise-exposed cochlea shows decrease of GluR2 postsynaptic puncta. (C, D) DT-treated mice have a marked loss of GluR2 puncta. There are some orphan puncta (i.e., not paired with ANF terminals, white arrows) in DT 1M mice. ANFs remain in the inner spiral bundle in all groups. Scale bar indicates 20 μm. (E) Mean ($\pm$ SD) for counts of postsynaptic GluR2 puncta in three cochlear regions (8, 20, and 32 kHz) reveal significant differences between WT, Noise and DT 1M (*, $p < 0.04$), but not between DT 1M and DT 2M. GluR2, glutamate receptor 2; ANF, auditory nerve fiber.

Fig. 5.

Density of ANFs in OSL does not change after DT. Light micrographs of ANFs in OSL with quantitative analyses of the density. (A) The OSL of the WT ear appears densely packed with ANFs. (B-D) DT and noise-exposed mice are similar to those seen in WT mice. Images are from the base of the cochlea. Scale bar indicates 20 μm. (E) Quantitative analysis of the ANF density in the OSL indicates no statistical difference among groups in base and apex; $N = 3-5$ in each group. Bars are standard error of the mean and statistical comparisons are performed using one-way ANOVA with Bonferroni post-hoc test. OSL, osseous spiral lamina.

Fig. 6.

Size of SGNs decreases after DT. Light microscopy analysis of SGNs in Rosenthal's canal with quantitative analyses of the density and size. (A) Rosenthal's canal in WT mice appears densely packed with SGNs and cells appear healthy with normal shape. (B-D) DT- and noise-exposed mice had SGN densities that were roughly similar to WT, but the SGN somata appear to be smaller than in WT. Images are from the base of the cochlea. Scale bar indicates 50 μm . (B') Enlarged image of boxed area in B showing the vacuolated region. (E) Quantitative analysis of SGN density indicates no statistical difference among groups in base or apex. (F) Quantitative analysis of SGN size indicates DT and noise-exposed mice are significantly different from WT mice (**, $p < 0.01$). Bars are mean $\pm$ standard deviation; brackets indicate groups that are significantly different (*, $p < 0.05$; **, $p < 0.01$).

Fig. 7.

Myelination changes occur after noise but not after DT. Morphology of the ANFs in the OSL. (A) Transmission electron micrographs reveal that axon caliber is decreased in noise-exposed and DT 2M mice compared to WT. In noise-exposed mice, myelin thickness of the ANFs also appears decreased. Scale bar indicates 5 μm. (B) High magnification images of ANFs show that myelin thickness and lamella number decrease in noise-exposed mice, but not in DT-treated mice. Scale bar indicates 200 nm. (C) Summary of differences among groups in myelin thickness, lamella number, axon caliber and g-ratio. Bars are mean ± standard deviation; brackets indicate groups that are significantly different (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). (D) Myelin thickness and axon caliber for all measured axons show significant correlation only for DT 1M and DT 2M groups (**, $p < 0.001$). (E) G-ratio increases with axon caliber, but at a steadily decreasing rate for all groups. The change in rate is smaller for DT 1M and DT 2M, resulting in flatter curves for those groups. In Noise group, smaller myelin thickness results in larger g-ratios at all axon diameters, shifting the curve upward.

Fig. 8.

Density of neurons in VCN does not change after DT. (A) Low magnification light microscopy images stained with cresyl violet show the area of the VCN and the distribution of neurons. Scale bar indicates 100 μm . (B) High-magnification light microscopy images from the dorsal side of AVCN. Scale bar indicates 20 μm . (C) Quantitative analysis reveals that VCN volume differed only between WT and Noise groups. (D) Density of neurons is similar in each group, for both dorsal and ventral regions of VCN. (E) In the dorsal region of VCN, neuron size is significantly smaller than in WT for Noise, DT 1M and DT 2M groups, but in the ventral region, neuron size is reduced only in the DT-injected mice. Bars are mean $\pm$ 1 standard deviation; brackets indicate groups that are significantly different (**, $p < 0.01$). AVCN, anteroventral ventral cochlear nucleus; VCN, ventral cochlear nucleus.

Fig. 9.

VGLUT-1 expression in CN is unchanged after DT. Micrographs of VGLUT-1 staining in AVCN show that the density of puncta in DT-treated mice (C, D) is similar to WT mice (A), but is lower in noise-exposed mice (B). Scale bar indicates 20 μm . (E) Quantitative analysis shows that VGLUT-1 puncta density in several CN regions is not significantly different between DT and WT, but after noise it is lower in three regions (PVCN, and DCN3, $p < 0.05$; AVCN, $p < 0.01$; $N = 6$ animals in each group). Bars are standard deviation of the mean and statistical comparisons are performed using one-way ANOVA with Bonferroni post-hoc test. DCN, dorsal cochlear nucleus; PVCN, posteroventral cochlear nucleus; AVCN, anteroventral cochlear nucleus; GCL, granule cell lamina; VGLUT-1, Vesicular glutamate transporter-1.