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PRINCIPAL INVESTIGATOR: Marjorie Leek, Ph.D.

CONTRACTING ORGANIZATION: Loma Linda Veterans Association for Research & Education, Redlands, CA

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Marjorie R. Leek, Ph.D., Edward J. Walsh, Ph.D.

E-Mail: Marjorie.Leek@va.gov

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Loma Linda Veterans Association for Research and Education
LLVARE
25884 Business Center Drive, Suite A
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14. ABSTRACT
Exposure to noise can cause damage to structures in the inner ear, often resulting in a loss of hearing. Recent findings in noise-exposed animals raise a new specter that even moderate noise exposures may result in damage specifically located in the synaptic region between the sensory cells in the cochlea and primary auditory neurons. There is no way currently that scientists and clinicians can diagnose possible auditory synaptic damage in humans, and diagnosis is critical for the development of innovative treatments. The objective of this project is to develop a statistical model that will accurately predict the likelihood of synaptopathy in humans who have had noise exposures in their lives. The development of the statistical model will be supported by collecting non-invasive measurements in both humans and guinea pigs. Regulatory documents supporting human and animal testing have been approved both locally and through the relevant offices of the USAMRMC. The animal laboratory was outfitted with a new sound booth for auditory testing, and data collection is on schedule to begin shortly. Research technicians have been hired to assist in both the human and animal facilities. However, overall progress has been significantly slowed by the seven months and ongoing international pandemic.

15. SUBJECT TERMS
Human hearing loss, guinea pig, Noise exposures, Neural deficits, Statistical model development

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1.Introduction

Exposure to noise has long been known to cause damage to structures in the inner ear, often resulting in a loss of hearing. This is a significant health concern for millions of people and it will intensify as the population ages and loud noises become a more prevalent feature of the soundscape. Recent findings in noise-exposed animals raise a new specter that even moderate noise exposures may result in damage specifically located in the synaptic region between the inner hair cells in the cochlea and primary auditory neurons. Although this synaptopathy has been demonstrated in several animal models, there is no way currently that scientists and clinicians can diagnose possible synaptic damage in humans, and diagnosis is critical for the development of new, innovative treatment plans. The objective of this project is to develop and populate a statistical model that is designed to accurately predict the presence and degree of synaptic damage resulting from noise exposure in guinea pigs, and subsequently to use that model to predict the likelihood of synaptopathy in humans who have had significant noise exposures in their lives. Auditory function will be assessed using a variety of non-invasive tests following noise exposure in non-human animals, and cochleae will subsequently be analyzed for evidence of synapse loss or damage. Performance on tests of auditory function in non-human animals will be compared with human performance to develop a predictive model that can be refined and extended by adding results from behavioral tests of temporal processing and speech perception in noise by human listeners. Finally, a comprehensive statistical predictive model will be developed to evaluate the likelihood and severity of synaptic damage in individual humans.

2.Keywords

Human hearing loss, guinea pig, Noise exposures, Neural deficits, Statistical model development

3.Accomplishments

What were the major goals of the project?

a. Establish and set up final electrophysiological and acoustic diagnostic tests. Milestone 1 to be completed by end of 4th month (i.e., January 31, 2020). This is ongoing, and about 75% complete in the Walsh (animal) lab, and about 20% complete in the Leek (human) lab.

b. Acquire test data on 12 guinea pigs per quarter. This has not been completed during this year of the award.

c. Perform anatomical assessment of 12 guinea pigs per quarter. This has not been completed during this year of the award
.

d. Acquire physiological and behavioral data on 10 human subjects per quarter. This has not been completed during this year of the award.

What was accomplished under these goals?

a. The research team meets regularly to fine tune stimuli, and work on computer programming to support experiments. Due to the pandemic, all research on human subjects is shut down, and lab members are mostly working from home. We have not been able to recruit and test human subjects, and there is so far no indication about when VA research will resume. We are assuming that the pandemic will not be completely over at that time, so in order to be proactive about protecting our researchers and subjects, we have prepared and submitted to the IRB our Mitigation Plans for changes we can make to our testing protocols to minimize the amount of face-to-face time between staff and subjects. This plan is attached as an Appendix to this report. In addition to continuing attention to handwashing, disinfecting surfaces frequently, mask and glove wearing, and arranging to exercise social distancing as often as possible, we plan to setup a small network within the lab that will permit control of equipment and the software to collect the data from subjects sitting in the sound room, with the experimenter in a separate room. The local VA is putting out guidelines for opening up to patients, which we incorporate into our own planning.

b. Efforts to initiate data acquisition related to non-human animal studies are underway. Essential alterations to the sound attenuating booth were completed, efforts to reconfigure the in-lab lighting and sewage systems are underway, and the installation of essential data acquisition system components has been completed. Equipment system calibration procedures are underway, anticipating data acquisition activities in the very near future. A research assistant was hired and is scheduled to begin work on 11/01/2020 to support non-human animal research activities.

c. No progress has been made on this goal because guinea pig testing will be started only after system testing and initial calibrations are complete.

d. A research assistant was hired for the Leek lab (human) on 09/30/2019, at the initiation of the award. Her training for the specific testing in the human lab is ongoing. A number of organizational materials for setting up confidential files and organizing testing protocols have been completed. In preparation for both animal and human testing, a database has been set up for viewing and potentially limiting the number of variables we will need for the ultimate modeling. No human recruiting, enrollment, or testing has begun because human subject research is on hold until the risk of people coming to the VA hospital for anything other than health care is abated. California (and most other states, unfortunately) is experiencing an increase in CoVid-19 cases, so it is unclear when we will be able to begin recruiting and testing human subjects.

What opportunities for training and professional development has the project provided?

Although this project was not intended to provide formal training and professional development opportunities, as a matter of course, the more senior members in each lab (Walsh and Leek labs) do encourage professional and scientific growth among the less senior team members. For example, Dr. Venezia, who is an accomplished statistician and modeler, has led our team in understanding the type of modeling we plan to successfully complete this study. He also has led the team in understanding his career development grant, which includes both behavioral testing of human subjects, as well as functional MRI techniques. The Research Audiologist, Dr. Whittle, has assisted us all in understanding and interpreting audiological behavioral and electrophysiology testing, both of which are cornerstones of this project. In the Walsh lab, we have all learned more about working with animal subjects, carrying out the regulatory procedures for animal work, and learning how animal electrophysiology will be performed to mimic (as much as possible) the human testing. It is our practice for the entire Auditory Research Group at the VA hospital (some 13 scientists and technicians) to meet every two weeks (currently virtually on Zoom) to hear about the ongoing research and data from each lab, or review a current article that is of interest to the labs. Because our group has a wide range of interests and expertise, this is an enjoyable and valuable learning experience, and also encourages the social bonding of the group.

How were the results disseminated to communities of interest?

Nothing to Report.

What do you plan to do during the next reporting period to accomplish the goals?

In the Walsh Lab (animal): The lab equipment set-up in the Auditory Neurobiology Laboratory (where non-human animal experiments will be performed) is complete, and calibration and pilot testing will occur in the early part of November. As soon as it is clear that the lab is ready to receive subjects, the first batch of guinea pigs will be purchased, and subsequently data collection and noise exposure protocols will be initiated. We anticipate that this work will begin to make up for the delays we have experienced in beginning the animal work. We plan to teach the newly hired research technician all requisite procedures for conducting testing on non-human animals.

In the Leek lab (human): We are not as close to being able to begin data collection due to delays in recruiting and testing human subjects because of the pandemic. We are also experiencing secondary delays due to an institutional decision to physically move our laboratory during this period, with needed renovations to the new space that are ongoing (see discussion in section on Changes/Problems below). As soon as we can begin to work normally, we will set up the new space, calibrate equipment, and begin recruiting subjects. However, at this time, it is unclear when the pandemic will abate sufficiently to permit studies in the hospital using human participants. As soon as we are able to get back into the human lab, we will program and pilot test all data collection software.

4.Impact

What was the impact on the development of the principal discipline(s) of the project?

Nothing to Report.

What was the impact on other disciplines?

Nothing to Report.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Nothing to Report.

5.Changes/Problems

Changes in approach and reasons for change

Nothing to Report.

Actual or anticipated problems or delays and actions or plans to resolve them

As we described in the previous quarterly reports, we have been delayed in implementing this project during this first year because of delays in installation and setting up of the animal sound booth, obtaining regulatory approvals in a timely fashion, and because of the COVID-19 pandemic. A secondary cause for delay in the human subject work is that the institution decided this was a good time to move our laboratory (including two sound booths and all our equipment, books, files, etc) Taking each of these delays in turn:

Administrative Delays: These delays occurred based on both delays in regulatory approvals as well as delays in purchasing and installing the new animal sound booth. On the regulatory side, IACUC and ACURO approvals came through in December 2019, but the final HRPO approval came in May 2020, well after the hospital was shut down to human subject participation in research due to the pandemic. Booth purchasing was delayed a few months due to delays in developing specifications, and delays in government purchasing. All of these administrative delays have now been alleviated.

Pandemic Although the new animal sound attenuating booth was installed on April 4, 2020, unfortunately, the much larger problem in making significant progress on both sides of this project (both animal and human) is the COVID-19 shutdown during which none of the lab staff were able to work at the VA. Instead, as of March 2020, all staff on this project were teleworking from home. Several members of the research team began working at least part time at the VA hospital starting around August 2020, but some staff who have vulnerabilities to the virus are not back in the laboratories.until the COVID cases in California and specifically at the hospital are reduced. It is not at all clear when that will occur, and we have no resolution to propose for that.

An additional impact of the pandemic for the human work is that the hospital itself was closed to human subject research from mid-March until the present. Until the COVID-19 cases begin to diminish significantly, both at the VA hospital as well as in our part of California, we will not be able to have subjects come to the lab for testing. This is a problem for which we have no resolution.

Delays associated with the COVID-19 pandemic were compounded by PPE shortages needed for work with the non-human animal subjects. However, we now have the requisite supplies and will begin data acquisition/experimentation on guinea pigs during the next quarter (in November 2020).

We are trying to anticipate some of the later tasks involved with the project beyond data collection, such as beginning a draft of a manuscript, setting up data analysis methods, and keeping up with the literature on synaptopathy in both animals and humans. We are also developing our experimental computer programs at home so that when we can get back into our labs, they will be mostly ready to be verified and implemented.

Moving Human laboratory: The building that housed our human subjects auditory research lab was scheduled for demolition. The administration of the hospital decided this would be a good time to move our lab, in part, because we were already shut down to data collection because of the pandemic. This occurred in July 2020, and we are still without offices or laboratory space due to delays in renovating the new space for our human subjects laboratory. The renovation includes installation of our two sound booths which are necessary for our data collection and which were moved from the old space to the new. It is unknown how long the renovation will take. Right now, this delay is secondary to the delays due to the pandemic, and may not extend beyond the time we are without human subject testing because of the health concerns.

Changes that had a significant impact on expenditures

Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

The only significant change to any of these issues could be the Mitigation Plan for human subjects testing. This has been developed and submitted to the IRB. It includes such issues as doing consenting on the phone rather than asking subjects to come to the lab as usual, scheduling subjects with at least an hour between subjects for sanitizing the testing space, and so on. As with many issues related to the pandemic, this is new to the IRB, but I believe this is only a notification to the IRB, and does not require formal approval. Should it be responded to as a formal approval, I will submit that to the HRPO for their records. The Mitigation Plan is submitted as an Appendix to this Annual Report.

Significant changes in use or care of vertebrate animals

Nothing to Report.

Significant changes in use of biohazards and/or select agents

Nothing to Report.

6.Products

Publications, conference papers, and presentations

Journal publications

Nothing to Report.

Books or other non-periodical, one-time publications

Nothing to Report.

Other publications, conference paper, and presentations

Sargsyan L, Hetrick A, Leek MR, Martin GK, Li H, (under review) “Effects of combined gentamicin and furosemide treatment on cochlear ribbon synapses”, Journal of NeuroToxicology by Elsevier. [Please note: This article is not directly a result of this project, but it has important information about synaptopathy after ototoxicity, and includes as authors two of the key personnel on the present project. Interestingly the results suggest that synaptopathy after ototoxic treatments may be synaptic plasticity rather than a loss of synaptic ribbons. Both Dr. Li’s and Dr. Leek’s DoD grants are acknowledged for funding.]

Website(s) or other internet site(s)

Nothing to Report.

Technologies or techniques

Nothing to Report.

Inventions, patent applications, and/or licenses

Nothing to Report.

Other Products

Nothing to Report.

7.Participants & Other Collaborating Organizations

What individuals have worked on the project?

Name	*Nicole Whittle, AuD*
Project Role	*Research Audiologist*
Researcher Identifier	*None*
Nearest person month worked	*12*
Contributions to Project	*Continued to work on organizational aspects of testing of human subjects, and assisting in development of potentially appropriate predictive models; worked with calibration company to facilitate equipment calibration once we can begin testing; assisted with regulatory submissions; attended and contributed to team meetings; initiated a literature review and outlined an introduction to a first publication from this work; major role in developing Mitigation Plan (see appendix)*
Funding Support	This project

Name	*JoAnn McGee, Ph.D.*
Project Role	*Co-investigator*
Researcher Identifier	*None*
Nearest person month worked	*9*
Contributions to Project	*Established lab preparedness by overseeing the delivery of equipment and supplies to the Veterinary Medical Unit (VMU); Conducted a partial inventory of equipment and supplies to be used in preparation for recording sessions, data acquisition and analyses; Prepared position description for Research Assistant to support animal studies. Searched and reviewed literature relevant to studies proposed in this grant. Set up noise exposure system, data acquisition system, perfusion instrumentation, and began calibration of the systems. Participated in administrative meetings addressing a range of relevant program items.*

Funding Support	*This project*

Name	*Marjorie Leek, Ph.D.*
Project Role	*Principal investigator*
Researcher Identifier	*None*
Nearest person month worked	*4.8*
	Developed and began experimental programming for testing protocols; completed regulatory submissions; attended and contributed to team meetings and discussions
Funding Support	*Institutional funds (salary)*

Name	*Edward J. Walsh, Ph.D.*
Project Role	*Co-Principal investigator*
Researcher Identifier	*None*
Nearest person month worked	*4.8*
Contributions to Project	*Searched and reviewed literature relevant to studies proposed in this grant. Participated in set up of noise exposure and data acquisition systems, and initiated system/equipment calibration process. Identified and hired a full time Research Technician. Participated in administrative meetings addressing a range of relevant program items.*
Funding Support	*This project*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report.

What other organizations were involved as partners?

Nothing to Report.

8.Special Reporting Requirements

Collaborative Awards

Quad Charts

Quad chart is attached

9.Appendices

a. **Auditory Research Group COVID-19 Risk Mitigation Plan**

b. **Manuscript:** Sargsyan L, Hetrick A , Leek MR, Martin GK, Li H, (under review) “Effects of combined gentamicin and furosemide treatment on cochlear ribbon synapses”, Journal of NeuroToxicology by Elsevier

Auditory Research Group COVID-19 Risk Mitigation Plan

Due to the current pandemic with COVID-19, in order to keep both research participants and staff safe the following actions have been taken involving the use of personal protective equipment (PPE) and current CDC recommendations for sanitization and reduced transmission during human subject testing with the Auditory Research Group.

Personal Protective Equipment

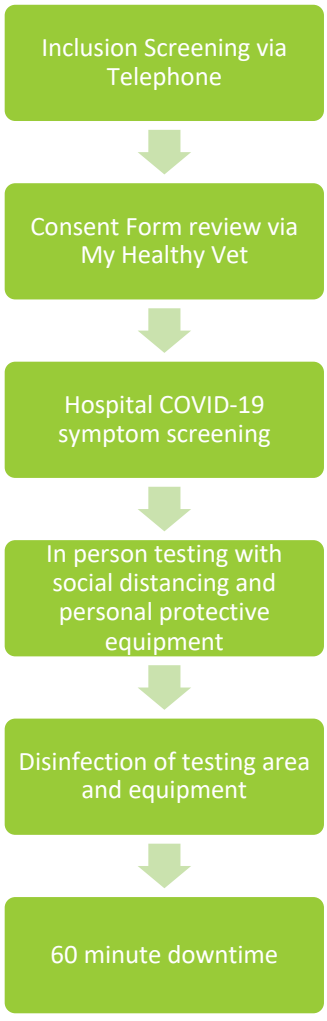
Per VA Loma Linda hospital requirements, all potential and enrolled subjects must go through a formal screening process and temperature check before entering the hospital. If an individual passes the initial daily screening, masks will be required at all times while in the testing facility. Masks must cover both the mouth and nose, if an individual does not have a mask one will be provided for them. Research staff maintain the right to turn away any potential participant who refuses to comply with mask policies.

Research staff will additionally wear masks in addition to full face shields, white lab coats, and latex gloves. For disinfection or disposal, latex gloves will be disposed of after every physical contact with the participant, face shields will be disinfected with hospital grade sanitation wipes at the end of each participant session, surgical masks will be worn for the full day then placed into an isolation bag for the remainder of the week, and white lab coats will be washed after daily use or no less than once per week according to the Society for Healthcare Epidemiology of America (SHEA) guidance on laundering. Hand sanitizer and a handwashing station will be available to both research staff and participants to utilize after immediate contact and at the end of each session.

Testing Accommodations

The following alterations have been made to our testing protocol and will be implemented into all studies

- All **study inclusion screening will be conducted over the phone**. If an individual has passed the screening, a copy of the study's consent form will be sent via My Healthy Vet for them to review and a follow-up call will be scheduled to go through the consent form with a research staff member to answer any questions or concerns related to the study. On the first day of in-person testing, a prefilled copy of the consent form will be provided for the participant to initial, sign, and date.
- **Capacity limits** will be placed on the direct testing area in a 1:1 ratio, one research staff to one participant. A participant may see more than one research staff in a session but not more than one in the sound booth or room where testing is being conducted.
- All **psychoacoustic testing will be conducted with the participant in the sound booth independently** while the research staff performing the testing is in a separate room able to communicate with the participant through microphone, headphones, and cameras for visual monitoring.
- **Audiologic testing poses the highest risk for infection transmission** due to otoscopy, tympanometry, and insert earphone placement all requiring the research staff and participant to be in the sound booth together within 6 feet. To minimize droplets, all instructions will be provided to the participant over microphone so there is no communicating when face to face. Extra care will be taken during any testing that may provoke a cough reflex.
- Functional Magnetic Resonance Image Testing continue to be conducted at the **Loma Linda Health University Radiology Department**, and all staff and participants will be asked to follow their COVID-19 testing protocols while on their premises.
- Single-use equipment such as insert earphone tips, tympanometry eartips, and otoscopy specula will be **disposed of immediately after use** into a waste container with a lid. Testing equipment that cannot be disposed of will be disinfected with hospital grade sanitation wipes.
- A **downtime of 60 minutes** will be implemented between test sessions to allow for disinfection of equipment and for air purification in subject testing areas.



Attached to this document are COVID-19 mitigation plans for both the VA Loma Linda Healthcare System and Loma Linda University Medical Center that will be followed depending on which facility testing is being conducted at.

Neurotoxicology

Effects of combined gentamicin and furosemide treatment on cochlear ribbon synapses

--Manuscript Draft--

Manuscript Number:	
Article Type:	Full Length Article
Keywords:	Aminoglycosides; loop diuretics; cochlea; hair cells; synaptopathy; hiddlen hearing loss
Corresponding Author:	Hongzhe Li, Ph.D. VA Loma Linda Healthcare System Loma Linda, CA UNITED STATES
First Author:	Liana Sargsyan
Order of Authors:	Liana Sargsyan
	Alisa Hetrick
	Marjorie R. Leek
	Glen K Martin
	Hongzhe Li, Ph.D.
Abstract:	It is well-established that aminoglycoside antibiotics are ototoxic, and the toxicity can be drastically enhanced by the addition of loop diuretics, resulting in rapid irreversible hair cell damage. Recent research also indicated that the combined treatment could lead to the loss of spiral ganglion neurons prior to the loss of hair cells, reminiscent of the disease termed as auditory neuropathy (AN). Using both electrophysiologic and morphological approaches, we investigated whether this treatment affected the cochlea, at the region of ribbon synapses consequently resulting in auditory synaptopathy representing a unique form of AN. A series of varied gentamicin and furosemide doses were applied to C57BL/6 mice, and auditory brainstem responses (ABR) and distortion product otoacoustic emissions (DPOAE) were measured to assess ototoxic damage within the cochlea. In brief, the treatment effectively induced cochlear damage and promoted a certain reorganization of synaptic ribbons, while a reduction of ribbon density only occurred after a substantial loss of outer hair cells. In addition, both the ABR wave I amplitude and the ribbon density were elevated in many treatment conditions, but a correlation between the two events was insignificant for individual cochleae. In sum, combined gentamicin and furosemide treatment, at titrated doses below those that produce hair cell damage, typically triggers synaptic plasticity rather than a permanent synaptic loss.
Suggested Reviewers:	Andew Forge, PhD Prof, King's College London a.forge@ucl.ac.uk Expertise in aminoglycoside-induced ototoxicity, and auditory biology at large.
	Ke Liu, MD PhD Beijing Friendship Hospital, Capital Medical University liuke@ccmu.edu.cn Expertise in drug-induced synaptic damage.

VA LOMA LINDA HEALTHCARE SYSTEM

A Division of VA Desert Pacific Healthcare Network

July 15th, 2020

Hongzhe Li, PhD
Research Scientist
VA Loma Linda HealthCare System
Research (151)
11201 Benton Street
Loma Linda, CA 92357
Email: Hongzhe.Li@va.gov
Office: (909) 825-7084 x2816
Lab: (909) 825-7084 x2088
Fax: (909) 796-4508

Dear Journal Editor,

It is an exciting opportunity to submit our recent work, titled "Effects of combined gentamicin and furosemide treatment on cochlear ribbon synapses", to the Journal of NeuroToxicology by Elsevier.

Neurotoxic damage to the ribbon synapses in the inner ear could result in a unique type of human hearing loss, which has been recently termed as hidden hearing loss. In this type of hearing loss, while the hearing sensitivity maintains, the speech perception is affected. To date, factors including noise over-exposure and the aging process have been implicated in hidden hearing loss. Our laboratory started investigating the effect of ototoxic aminoglycosides on synaptic damage in the inner ear. The major findings are documented in this manuscript.

I have been serving as an *ad hoc* reviewer for NeuroToxicology since 2015, and this is the first time submitting our work to the journal. We appreciate your consideration of this work and look forward to your position response.

All authors have declared no conflict of interest for this manuscript. We confirm that the work has not been published previously (except as conference abstracts). This manuscript will not be submitted elsewhere until we receive your decision.

Yours sincerely,

Hongzhe Li

Research Scientist, Research Service
VA Loma Linda Healthcare System
1 (909) 825-7084 x2816
Hongzhe.Li@va.gov

Associate Professor
Department of Otolaryngology, Head & Neck Surgery
Loma Linda University, School of Medicine

Jerry L. Pettis Memorial VA Medical Center
11201 Benton Street
Loma Linda, CA 92357
(909) 825-7084
www.lomalinda.va.gov

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Author Agreement

All authors have seen and approved the final version of the manuscript being submitted. They warrant that the article is the authors' original work, hasn't received prior publication and isn't under consideration for publication elsewhere.

Effects of combined gentamicin and furosemide treatment on cochlear ribbon synapses

Liana Sargsyan[1], Alisa Hetrick[1], Marjorie R. Leek[1,2], Glen K Martin[1,2], Hongzhe Li[1,2,*]

1. *Research Service, VA Loma Linda Healthcare System, CA 92357, USA*
2. *Department of Otolaryngology - Head and Neck Surgery, Loma Linda University Health, Loma Linda, CA 92354, USA*

*Corresponding author: Hongzhe Li, PhD

Research Service (151), VA Loma Linda Healthcare System, 11201 Benton Street, Loma Linda, CA 92357, USA. *E-mail:* Hongzhe.Li@va.gov. *Tel:* 1(909)825-7084 Ext 2816. *Fax:* 1(909)796-4508

Running Title: Synaptic damage by gentamicin and furosemide

Keywords: Aminoglycosides, loop diuretics, cochlea, hair cells, synaptopathy, hidden hearing loss.

Abstract

It is well-established that aminoglycoside antibiotics are ototoxic, and the toxicity can be drastically enhanced by the addition of loop diuretics, resulting in rapid irreversible hair cell damage. Recent research also indicated that the combined treatment could lead to the loss of spiral ganglion neurons prior to the loss of hair cells, reminiscent of the disease termed as auditory neuropathy (AN). Using both electrophysiologic and morphological approaches, we investigated whether this treatment affected the cochlea, at the region of ribbon synapses consequently resulting in auditory synaptopathy representing a unique form of AN. A series of varied gentamicin and furosemide doses were applied to *C57BL/6* mice, and auditory brainstem responses (ABR) and distortion product otoacoustic emissions (DPOAE) were measured to assess ototoxic damage within the cochlea. In brief, the treatment effectively induced cochlear damage and promoted a certain reorganization of synaptic ribbons, while a reduction of ribbon density only occurred after a substantial loss of outer hair cells. In addition, both the ABR wave I amplitude and the ribbon density were elevated in many treatment conditions, but a correlation between the two events was insignificant for individual cochleae. In sum, combined gentamicin and furosemide treatment, at titrated doses below those that produce hair cell damage, typically triggers synaptic plasticity rather than a permanent synaptic loss.

1. Introduction

Cochlear synaptopathy is manifested by a loss of synaptic connections between cochlear inner hair cells (IHCs) and their auditory nerve fiber (ANF) terminals that results in a delayed degeneration of spiral ganglion neurons (SGNs) and their central axonal projections (Moser and Starr, 2016). With noise overexposure, it is possible to create animal models exhibiting a selective and permanent synaptic loss with relatively intact hair cells (HCs) in a phenomenon termed hidden hearing loss (HHL), which has been studied extensively by Kujawa and Liberman (Kujawa and Liberman, 2009, 2015; Liberman and Kujawa, 2017). In the meantime, it is less clear if the HHL-type of synaptopathy can be induced by other cochlear insults, which often result in considerable plasticity in terms of affecting hair-cell synaptic densities. Using moderate dosing, aminoglycoside antibiotics can produce a specific SGN death without much damage to HCs in genetically modified mice (Oishi et al., 2015), thus, suggesting the likelihood of cochlear synaptopathy (Jiang et al., 2017). A chemical model of the acoustic synaptopathy in mice is certainly welcomed by the research community to simplify the

preparation procedure, while taking advantage of the readily available manipulation of the murine genome. Yet, to date, a convincing synaptopathy indicating HHL has not been demonstrated *in vivo* in mice by any known aminoglycoside treatment (Hong et al., 2018; Liu et al., 2015; Liu et al., 2013).

Prior to the raised attention on synaptopathy, cochlear insults, regardless of being produced by acoustic overstimulation or aging, were believed to first target sensory elements in the mouse cochlea, followed by their associated neural components such as the SGNs (Willott et al., 1998). Moreover, the resulting damage appeared to be more biased toward the sensitive outer hair cells (OHCs) on the sensory epithelium (Spongr et al., 1997; Willott et al., 1998). Intuitively, these outcomes were not what was expected to occur in an ideal synaptopathy animal model, in which a considerable reduction of the synaptic density with intact HC survival assessed by morphological or electrophysiological approaches, is ordinarily expected. Intriguingly, Ruan et al. (Ruan et al., 2014) reported that a single intraperitoneal injection of gentamicin, assisted by a loop diuretic, furosemide, induced a SGN loss that could occur before damage to HCs, especially, IHCs, which is reminiscent of the disease termed auditory neuropathy (AN). Here, we asked if the ribbon synapse of the IHC could be compromised ahead of HC loss, regardless of the condition of the SGNs. As aforementioned, this would be significant, because it would replicate the HHL that is typically observed with noise overexposure or in certain aging animal models. Mechanistically, independent synaptic damage, *i.e.*, HHL, is possible because aminoglycosides are capable of inducing the excitotoxic type of injury at the synaptic level, which is considered to be a major underlying mechanism for noise-induced HHL (Basile et al., 1996; Liberman and Kujawa, 2017; Segal et al., 1999).

Combining loop diuretics to aminoglycoside treatment is an effective approach for accelerating ototoxic damage, especially, in mice, which results in immediate cochlear trauma (Hirose and Sato, 2011; Taylor et al., 2008). In this simplified version of a mouse ototoxicity model, the addition of loop diuretics, such as furosemide, disrupts the blood labyrinth barrier thereby greatly elevating the concentration of aminoglycosides, such as gentamicin, in the cochlea (Ding et al., 2016; Taylor et al., 2008). Furosemide, by itself, is not considered directly damaging to the inner-ear components including HCs, ribbon synapses or SGNs (Rybak, 1993). In the present study, the dose-dependent effect of gentamicin and furosemide (G/F) in combination was investigated to: 1) characterize the residual hearing function, 2) distinguish the morphology of OHCs and IHCs, and ribbon synapses at

various cochlear locations, and 3) determine whether there is a change in the number, or the distribution, of synaptic ribbons.

The G/F combination treatment is also frequently used to induce cochlear HC loss prior to testing a specific strategy for regenerating HCs (Izumikawa et al., 2005). Excessive damage toward other cochlear components including the ribbon synapse is considered an unfavorable ototoxic event and certainly complicates the assessment of a HC's regeneration capacity. Thus, whether G/F treatment could induce an HHL-type of synaptopathy is an important scientific question, and valuable to the research community with either outcome. We, therefore, examined in detail the effects on the mouse cochlea of this treatment combination using a series of varied doses of G/F.

2. Methods

2.1 Mice and gentamicin/furosemide (G/F) treatment

C57BL/6 (JAX stock #0664) wild-type mice of both sexes, between 6-8 wk of age, were included in the present study. Animals were housed in a Specific Pathogen Free-modified room. Experimental animals were injected intraperitoneally (*i.p.*) with a single dose of gentamicin (Sigma-Aldrich, G1264, Lot #SLBG7734V) followed within 30 min by furosemide (Fresenius Kabi, Germany). Age-matched mice without any ototoxic treatment served as controls. The dose combination was either a fixed gentamicin injection at 200 mg/kg body weight, with varied furosemide doses (50, 100, 200, 400 mg/kg), or a fixed furosemide dose (200 mg/kg) with varied gentamicin injections (25, 50, 100, 200, 400 mg/kg), as illustrated in Supplemental Fig. 1. The majority of mice underwent distortion product otoacoustic emissions (DPOAE) and auditory brainstem response (ABR) tests prior to, and at several posttreatment time points, *i.e.*, primarily at day 3 and day 7. Seven days after treatment, mice were terminated and cochlear tissues harvested for morphological examination. Cochlear samples from a subset of mice were collected 2 days after the G/F treatment. Data reported here were collected from a total of 60 mice. Only three mice did not survive the treatment, including two, which were treated with 400 mg/kg gentamicin and 200 mg/kg furosemide (400G/200F) doses. All animal work was carried out using protocols approved by the Institutional Animal Care and Use Committee of the Jerry L. Pettis VA Medical Center, Loma Linda, CA. Animal-use procedures conformed with federal regulations regarding personnel, supervision, record keeping, and veterinary care.

2.2 ABR and DPOAE measurement

Individual anesthetized mice (ketamine 65 mg/kg/xylazine 13 mg/kg, *i.p.*) were stimulated with a closed tube sound-delivery system sealed into the outer ear canal. ABRs to tone-burst stimuli (5-ms duration, 1-ms rise/fall times) at 4, 8, 12, 16, 24 and 32 kHz, at 5-dB steps, were recorded using a Tucker-Davis Technologies (TDT) System 3 (Alachua, FL, USA). In particular, thresholds and wave I peak-to-peak amplitudes were determined. A full range of input/output (I/O) functions of wave I amplitudes was typically recorded in response to 12-kHz tone-bursts.

$2f_1$-f_2 DPOAEs were measured to non-invasively assess OHC function (Jimenez et al., 1999). Briefly, the f_1 and f_2 primary tones were generated by a dual-channel synthesizer (Hewlett Packard Model 3326A) and attenuated under the control of in-house customized software. The f_1 and f_2 primary tones (f_2/f_1=1.25) were mixed acoustically and delivered to two separate ear-speakers (Tandy Corp, Fort Worth, TX) to avoid artifactual distortion. Ear-canal sound pressure levels were measured by an emissions microphone assembly (Etymotic Research, ER-10B+, Elk Grove Village, IL), embedded in the probe. Sound levels were sampled, synchronously averaged and Fourier analyzed for f_2's ranging from 4.0-64.0 kHz in 0.1-ocatave steps. Corresponding noise floors (NFs) were computed by averaging the levels of the ear-canal sound pressure for five frequency bins above and below the $2f_1$-f_2 frequency bin. Primary tones were presented at L_1=L_2=55 dB SPL.

2.3 Tissue-processing, image acquisition and processing

Cochlear samples were immersion-fixed overnight in 4% paraformaldehyde (pH 7.4), followed by EDTA (10%, pH 7.4) decalcification at room temperature (RT). To prepare the cochlear whole-mounts, the membranous labyrinth of the cochlea was micro-dissected under a dissecting microscope to remove the softened otic capsule, stria vascularis, Reissner's membrane, and tectorial membrane. After further immersion-fixation in 4% paraformaldehyde, specimens were permeabilized in 1% Triton-X solution for 1 h at RT. Then, specimens were incubated at 37°C overnight with primary antibodies including monoclonal mouse anti-CtBP2 IgG1 (612044, BD Biosciences, 1:200), monoclonal mouse anti-GluR2 IgG2a (MAB397; Millipore; 1:1000), and/or polyclonal rabbit anti-Myo7a (PA-936, Thermo Fisher, 1:200). After rinsing, the specimens were incubated with the Alexa Fluor 594- and 568-conjugated secondary antibodies at 1:1000 at 37°C for 1 h in the dark. Some specimens were also incubated with Alexa Fluor 488 phalloidin at a 1:1000 at RT for 20 min in the

dark. After the final wash, the modiolus was removed from the tissue and the epithelia were divided into segments and mounted on slides with the anti-fade fluorescence mounting media VectaShield (Vector Labs, Burlingame, CA) under uniform 60X magnification. Immunolabeled images were acquired using a laser confocal microscope (Fluoview FV3000, Olympus Corp.).

Data were analyzed using Prism (GraphPad Software, La Jolla, CA, USA) software for Windows. Statistical methods included a 2-way ANOVA with Sidak multiple comparisons, and unpaired t-tests with Welch's correction. All tests were two-tailed, and a p value of <0.05 was considered statistically significant.

3. Results

3.1 Gentamicin/furosemide dose response and inter-animal ototoxicity variation

To test whether a single combined dose of ototoxic treatment influenced the ribbon synapse in mouse models of acquired sensorineural hearing loss, we tested *C57BL/6* wild-type mice as an established animal model of HC loss.

In *C57BL/6* mice, when gentamicin dosage was fixed at 200 mg/kg, a dose effect of furosemide was evident in terms of group ABR thresholds measured at 3 and 7 days after G/F treatment, from 0 to 400 mg/kg as illustrated in Figs. 1A-D. For lower furosemide dosages including 50 (200G/50F) and 100 (200G/100F) mg/kg, as shown in Figs 1B and 1C, respectively, average ABR thresholds were elevated at the higher tested frequencies of 24 and 32 kHz. The elevation was statistically significant for the 200G/100F treatment (2-way ANOVA, 7-day *vs.* pre, $F(1,60)=8.31$, $p=0.0055$), and barely insignificant for the 200G/50F treatment (2-way ANOVA, 7-day *vs.* pre, $F(1,60)=3.22$, $p=0.078$). For the higher 400 mg/kg furosemide dosage (200G/400F) illustrated in Fig. 1D, the average ABR thresholds increased across the entire frequency-test range (2-way ANOVA, 7-day *vs.* pre, $F(1,60)=79.9$, $p<0.0001$), indicating severe cochlear injury. Although a dose response of furosemide did exist from group data as indicated in Figs. 1A-D, it is noteworthy that the large inter-animal variation made it impossible to reversely predict G/F dose based on individual ABR thresholds. For instance, an ear with 200G/400F treatment (purple lines in Figs. 1E and 1F) could present normal ABR thresholds, while an ear with 200G/50F treatment (green line in Figs. 1E and 1F) presented undetectable ABR thresholds at higher frequencies. When undetectable, a threshold value of 95 dB

was assigned for group-averaging purposes. ABR thresholds measured from other G/F dose combinations are illustrated in Suppl. Fig. 2.

DPOAEs were also measured in most G/F-treated *C57BL/6* mice, thus, providing a more sensitive and reliable injury assessment in individual animals, while the assessment was restricted at the level of the OHCs. DPOAE responses to 55-dB SPL primary tones, from representative ears, comparing G/F pretreatment (blue) and 7-day posttreatment (red) conditions shown in Figs. 2A-D, indicated that OHC injury preferably occurred for the high-frequency region, and the injury expanded to lower frequency regions as the overall ototoxic severity escalated. Similar to the aforementioned inter-animal variability observed in ABR thresholds, OHC injury was also highly variable according to DPOAE measures. For example, for one mouse treated with the same 200G/200F dose, severe OHC damage as indicated in Fig. 2F was observed, while the other mouse might exhibit normal DPOAE responses at 7 days posttreatment. Based on the severity of OHC injuries, we categorized G/F-induced cochleotoxicity into three types. In the mild type, OHC damage represented, for example, in Fig 2B, was limited to the extreme high-frequency range (>30 kHz). In the moderate type shown in Fig. 2C, OHC damage spread into the mid-frequency range (~20 kHz or lower). In the severe type indicated in Fig. 2D, OHC damage was seen throughout the cochlea. Among five mice exhibiting severe DPOAE deficits, four of them involved bilateral injuries as shown for mouse #248 in Fig. 2D, and mouse #387 in Fig. 2F. This observation was in accordance with the consensus that aminoglycoside-induced ototoxicity typically affects both ears equally (Forge and Schacht, 2000). Yet, unilateral mild high-frequency OHC injury was not rare in the present study, as the contralateral showing no sign of DPOAE deficit. Here, the upper-frequency limitation of f_2 stimuli was 64 kHz, whereas DPOAE levels were frequently indistinguishable from the NF as observed for the data present in Fig. 2. This technical upper-frequency boundary was very close to, but not adequately reaching the extremity of the basal cochlear location. As a result, OHC injury at the extreme basal cochlear region could not be detected, and an ear could be mis-identified as normal by DPOAE measurements.

The results from ABR thresholds and DPOAE measures, *in toto*, indicated that we have generated a broad spectrum of cochlear injuries based on various G/F dosing combinations, which allowed us to interrogate the ribbon synapse for possible synaptic damage that is independent of HC injuries.

3.2 The wave I I/O curve of ABR responses

ABR wave I amplitude is deemed as a legitimate non-invasive physiological indicator of the functionality of the afferent AN, including the ribbon synapses. As ABR thresholds in G/F-treated mice were measured, the wave I I/O function was also routinely acquired at 12 kHz with a broad range of sound levels as illustrated in Fig. 3. For lower G/F dosages, including the 200G/50F one shown in Fig. 3A, the 200G/100F dose of Fig. 3B and the 200G/200F one in Fig. 3C, no severe OHC injury was observed based on DPOAEs, and the complete range of wave I amplitudes could be acquired as well. The group average of posttreatment wave I I/O functions, at 3 and 7 days, were never lower than their pretreatment control-function counterparts. These data suggested the ribbon synapses, at the tested frequency location, were resistant to G/F treatments. For the 200G/400F dose, for group averaging purposes, wave I amplitudes were assigned by zeros for sub-threshold ABR waveforms. Only with the contribution of "zero assignments", the average posttreatment wave I I/O curves were lower than the pretreatment curves.

Thus, if the hearing sensitivity at the testing frequency of 12 kHz remained after G/F treatment, ABR wave I amplitude was not reduced. Yet, focusing only at the higher-sound levels, wave I amplitudes actually increased for certain treatment conditions and were more significant at earlier posttreatment time points, for instance, at the 200G/50F dose shown in Fig. 3A (2-way ANOVA, 3-day *vs.* pre, $F(1,40)=11.42$, $p=0.0016$; 7-day *vs.* pre, $F(1,40)=4.126$, $p=0.049$). To further investigate the underlying mechanism of the wave I amplitude increment, wave I I/O functions were regrouped by the severity of OHC injuries, *i.e.* DPOAE deficit, as shown in Figs. 3E-G. Intriguingly, it was the ears with moderate OHC injury, instead of the ears with mild injury, exhibiting increased wave I amplitudes at higher sound levels as demonstrated in Fig. 3G, for example (2-way ANOVA, 3-day *vs.* pre, $F(1,129)=8.985$, $p=0.0033$; 7-day *vs.* pre, $F(1,129)=3.550$, $p=0.0618$).

Results from DPOAE measurements and ABR responses, including both ABR thresholds and wave I amplitudes, collectively, indicated that 400 mg/kg furosemide dosing represents an unfavorable dosage to pursue synaptic damage independent of HC loss.

3.3 Morphological alteration of synaptic ribbon after G/F treatment

Seven days after the one-time G/F treatment, synaptic ribbons that were identified by the anti-CtBP2 labeling exhibited various degrees of morphological change. On the *xy* plane at the cochlear location equivalent to 12 kHz (Muller et al., 2005), the ribbon distribution appeared normal for some low-dose G/F treatments, demonstrated by a vacant zone basally located to the IHC nuclei, without a

reduction in the number of ribbons, *i.e.*, ribbon density, as illustrated by the micrographs of Figs. 4A1-A4. Among them, Figs. 4A2-A4 are the confocal images reconstructed in the *yz* plane from three adjacent IHCs. Similar to our previous findings in healthy control cochleae, the size of ribbons near the nuclei was relatively small, while those located at the IHC basal pole were larger (Edderkaoui et al., 2018). In addition, it is noteworthy that with the mounting technique used, the IHCs from the apex and the middle coils of the cochlea generally orientate in parallel to the cover slide, which allowed a convenient quantifying of the distance of individual ribbons to the nucleus along the *y* axis, *i.e.*, the main axis of the IHC. Thus, a shape of normal distribution along the main axis was typically anticipated, as indicated in Fig. 4A5. Yet, under most G/F treatment conditions, a certain distribution disorder was observed, for example, in Figs. 4B1-B5, manifested by some ribbons being shifted toward the IHC nucleus without a reduction in the number of ribbons. In addition, the "nucleus-ward or upward" shift appeared predominantly toward the pillar side of the IHC as shown on the left side of Figs. 4B2 and 4B4. The axial gradient based on the ribbon size was also disturbed. In the extreme ototoxic scenario, the ribbon numbers did decline after some high-dose G/F treatments, with extensive upward shift of the ribbons as illustrated in Fig. 4C2, and enlarged ribbons in many cases as demonstrated in Fig. 4C1. More examples showing the axial distribution of synaptic ribbons, ranging from normal to extensive upward shift, with and without reduction in ribbon density can be seen in Suppl. Fig. 3.

3.4 OHC damage occurs in between synaptic plasticity and ribbon density reduction

The ribbon numbers per IHC, *i.e.*, ribbon density, was quantified systemically at the 12-kHz cochlear location and number reduction was rare, only occurring in some cases with the highest G/F dosings at 200G/400F (see Fig. 5A) and 400G/200F (see Fig. 5B), although the reduction was not significant in either dosing group due to inter-animal variations. Otherwise, G/F treatment typically resulted in a marginal increase in ribbon density, a hallmark of synaptic plasticity, and the increment was statistically significant for the 200G/200F group (Figs. 5A and B; p=0.006, unpaired t-test with Welch's correction). Thus, combining with the ABR and DPOAE results, the data collectively suggest that the reduction of ribbon density only occurred in the situation with substantial cochlear damage due to G/F treatment, summarized by Fig. 5C. When the ribbon dataset was sorted according to the severity of OHC damage (*i.e.*, DPOAE damage) that was defined earlier, the reduction became significant with severe DPOAE deficit as shown in Fig. 5D (p=0.004, unpaired t-test with Welch's correction).

Cochlear tissue was typically processed with anti-Myo7a antibodies and/or phalloidin in addition to anti-CtBP2 antibodies, in order to address HC integrity at the same cochlear location where the synaptic ribbons were assessed. With our treatment protocols and doses, G/F-induced IHC loss was extremely rare, while OHC loss frequently was extreme at each imaged cochlear location. That is, the OHC loss was often either complete or none, as shown in Fig. 6A. Thus, the cochlear samples at each examined location could be characterized into one of the two groups, "no OHC loss" group *vs.* "OHC loss" group. As partially reported earlier, 7 days after 200G/200F treatment, the overall ribbon density was elevated at examined locations, a sign of synaptic plasticity, including at 12-, 32- and 48-kHz locations as shown in Fig. 6B (2-way ANOVA, $F(1,21)=9.15$, $p=0.0064$). Intriguingly, the G/F-induced increment in ribbon density was exclusively contributed by the samples exhibiting "no OHC loss" as illustrated in Fig. 6C (2-way ANOVA, $F(1,17)=44.75$, $p<0.0001$). Representative cochlear samples at the 12-kHz location from "no OHC loss" group (see Fig. 6E) and "OHC loss" group (see Fig. 6F) showed OHC conditions in accordance with the DPOAE results on the same ear measured immediately prior to tissue collection, shown in Figs. 2E and 2F, respectively. Corresponding values of the ribbon density are depicted in Fig. 6D. Note that without identifiable OHC survival, the ribbon density still fell into the normal range for non-G/F-treated control samples, further confirming that OHC structural damage occurred prior to a ribbon-density reduction.

3.5 Gentamicin/furosemide-induced ribbons unlikely functional

The marginal increase in ribbon density indicated a small number of synaptic ribbons were newly formed after the titrated G/F treatment. The physiological significance of this morphological alteration is unclear. According to the observation that the G/F-induced elevation of the ABR wave I I/O function was slightly more overt by 3 days posttreatment compared to 7 days as indicated in Fig. 3, we speculated that the G/F-enhanced ribbon density also occurred at earlier time points following treatment. Thus, we focused on a subset of *C57BL/6* mice that were administered with 400G/200F doses, and had their cochlear tissue harvested 2 days posttreatment, to better understand the mechanism of the newly generated, G/F-induced synaptic ribbons. The selected group of mice exhibited normal ABR thresholds 2 days after treatment (data not shown), but elevated wave-I amplitudes across the entire range of the tested sound levels as illustrated in Fig. 7A (2-way ANOVA, $F(1, 151)=6.827$, $p=0.0099$). In addition, the ribbon density appeared increased at multiple locations throughout the cochlea as indicated in Fig. 7B. However, when we interrogated the correlation

between the ABR wave-I amplitude and the ribbon density from individual cochleae at the 12-kHz location, the correlation coefficient was low (see Fig. 7C, R^2=0.14). This outcome was further confirmed from the larger, 7-day post treatment dataset shown in Fig. 7D. Here, except for the extremely low ribbon numbers (*i.e.*, two incidences) that corresponded to the unidentifiable wave I, the G/F-enhanced ribbon density did not result in increased wave-I amplitudes with a coefficient index near 0. Consequently, the lack of correlation between the electrophysiologic measures and the morphological quantification indicated that the newly G/F-induced ribbons did not directly contribute to the elevated wave-I amplitude, and the synaptic plasticity based on morphological observations was not functional in the present study.

4. Discussion

Similar to acoustic overstimulation and aging, it has long been considered that the primary ototoxic target of aminoglycosides including gentamicin is the sensory hair cells in the mammalian cochlea, while the damage to the SGNs is secondary (Forge and Schacht, 2000; Hirose and Sato, 2011). This dogma has recently been challenged (Liberman and Kujawa, 2017; Ruan et al., 2014), particularly with the recent research interest in HHL, a unique form of auditory neuropathy (Starr and Rance, 2015), in which the temporal processing capacity is drastically compromised, while sensitivity to pure tones remains intact.

In the present study, we used DPOAE methods to measure OHC integrity along with morphological counter-labeling agents including phalloidin and anti-Myo7a to identify sensory HCs. DPOAE measures are fast and reliable and, more importantly, a non-invasive approach to evaluate OHC function, and allowing multiple assessment time points upon G/F treatment. Thus, we could readily observe the progress of G/F-induced cochleotoxicity. Together with immunofluorescence techniques, the present study concluded that the G/F treatment did alter the distribution of synaptic ribbons in the IHC, but more likely in a neuroplasticity fashion instead of in an HHL manner, prior to damage to the OHCs.

4.1 Gentamicin/furosemide treatment as an effective ototoxicity model in mice

A single dose of gentamicin combined with 400 mg/kg of furosemide is widely used as an effective experimental strategy to ablate OHCs in the inner ear (Kraft et al., 2013; Ruan et al., 2014; Schmitz et

al., 2014). In the present study, DPOAE responses confirmed that G/F treatment resulted in HC damage, with equivalent damage severity bilaterally (Forge and Schacht, 2000). Based on our observations, unilateral severe ototoxic damage is extremely rare with only one observed case. This drug-induced cochlear damage has been used as a disease model to study corrective approaches such as cochlear implantation and HC regeneration (Kraft et al., 2013). Since we were keen on producing damage specific to the synaptic ribbon without HC loss, a variety of titrated dose combinations between gentamicin and furosemide were used (see Suppl. Fig. 1). The overall results indicated that the 400 mg/kg dosage was on the high side of dose selection that frequently caused OHC damage. Between the conditions of 200G/400F and 400G/200F, the latter was gentler in terms of stressing OHCs.

From both experimental animal studies and human temporal bone histology, furosemide and other loop diuretics exert reversible damage or edema to the cochlear lateral wall and the stria vascularis within it (Ding et al., 2016; Rybak, 1993; Santos and Nadol, 2017). The edema is typically manifested by the enlargement of the extracellular spaces from the marginal cell tight junctions to the basal cell layer with broadening the stria vascularis. Diuretics alone do not directly damage HCs in the cochlea and the vestibular system, but, rather, cause a secondary effect from changes in the stria vascularis. Studies in mice suggested that furosemide targets the renin-angiotensin aldosterone system in the stria vascularis and disrupts the blood-cochlear barrier (Ding et al., 2016; Rybak, 1993), which allows ototoxins such as gentamicin to flush into the endolymphatic space many times more than the regular volume, and to quickly enter HCs from there (Li and Steyger, 2011). The effectiveness of furosemide on the stria vascularis likely forms a gradient from the cochlear apex to the base (Hirose and Sato, 2011), while the cochleotoxicity of gentamicin forms a gradient from base to apex. The combined ototoxic efficacy of G/F treatment appears more towards the cochlear base, similar to findings previously reported with kanamycin/furosemide treatment (Hirose and Sato, 2011). Yet, an exception did occur and a few individual cases showed apical-only G/F ototoxicity (DPOAE data not shown). In addition, G/F-induced IHC loss was rare in the present study, given the examination time points were no more than 2 weeks after treatment. Thus, this treatment protocol produces a good model for research efforts on cochlear electrode implantation and regeneration of OHCs.

4.2 Using wave-I amplitude to estimate ribbon density was not practical after gentamicin/furosemide treatment

Firstly, as shown in Fig. 3, we did observe a marginal G/F-enhanced wave-I amplitude from group data, and the increment appeared more evident from the ears exhibiting moderate DPOAE deficit as illustrated in Fig. 3G. It is established that a correlation exists between the wave-I ABR amplitude and ribbon density in age-related and/or noise-induced HHL (D et al., 2020). However, when we pooled data from ears with both ABR wave-I measurement and ribbon quantification at the 12-kHz location, this correlation did not exist for individual G/F-treated ears as seen in Figs. 7C and D as new synaptic ribbons formed. In addition, based on our observations, the prevalence of G/F-enhanced ribbon density was greater than that of G/F-increased wave-I amplitudes. One reason, of course, could be due to the lack of stability of ABR measurements. Or alternatively, the newly formed ribbons were not functional in our protocol and the increased wave-I amplitudes were supported by the augmentation of other functional modalities within the cochlea. For instance, the loss of sensory input from damaged HCs at higher-frequency locations can modify the tuning of succeeding SGNs, resulting in an increased population of SGNs responding to sounds at adjacent lower frequencies, and, consequently, a greater wave-I amplitude. This acute auditory effect is termed the "edge effect" (Snyder et al., 2000). The fact that ears with moderate DPOAE deficit exhibit greater G/F-enhanced wave-I amplitudes at the 12-kHz location supports this explanation, given that the 12-kHz location in these cases were closer to the OHC injury "edge" as indicated in Figs. 3G and 2C. This acute effect could fade away to a certain extent as the succeeding SGNs gradually lost their function due to the loss of intended input from the corresponding sensory epithelium. Concordantly, the fact that 7-day wave-I amplitude elevations were slightly less than that of the 3-day elevations did support the hypothesis as corroborated in Fig. 3.

4.3 Pillar-side upward shift of synaptic ribbons with G/F treatment

After G/F treatment in *C57BL/6* mice, pillar-side upward shift of synaptic ribbon is a prevalent morphological event, as illustrated in Fig. 8. The marginally increased number of ribbons appear not adequate to be solely responsible for their upward shift. That is, the shifted ribbons are unlikely the newly generated synaptic ribbons, but, rather, are the relocated ribbons from the basal pole of IHCs. Specimens processed with both CtPB2 and GluR2 antibodies confirmed the expected pairing between the presynaptic ribbon and postsynaptic AMPA receptor (Figs. 4A4 and 4B4, and Suppl. Fig. 4), which suggests that the ribbons are mature, instead of freshly assembled. However, if the overall ribbon density increased significantly with G/F treatment, it is reasonable to hypothesize that some new ribbons are recruited to the cellular membrane not far from the nucleus. Ribbons that are located on the

pillar side are considered to connect with ANFs with low-threshold and high-spontaneous discharge rates. These fibers also present a narrow dynamic range of discharge rate, meaning less of an increment in the overall discharge rate as the sound level of the acoustic stimulus increased. This could partially explain the marginal, and mostly non-significant increase of the ABR wave-I amplitudes shown in Fig. 3, as well as the lack of a correlation between the wave I amplitudes and ribbon density.

4.4 Why was not there HHL-type of reduction of ribbon density?

Ribbon synaptic transmission between IHCs and SGNs is excitatory and glutamatergic, and the ribbon synapse is susceptible to excitotoxicity, presumably due to excessive glutamate release from IHCs. Intracochlear perfusion of glutamate agonists *in vivo* results in the degeneration of SGN synaptic terminals on IHCs (Pujol et al., 1996; Pujol et al., 1985; Ruel et al., 2007). To date, excitotoxicity is the leading underlying mechanism of the HHL-type synaptopathy due to acoustic overexposure (Kujawa and Liberman, 2009, 2015). Intriguingly, excitotoxicity has also been proposed as one of the toxic mechanisms impeding auditory function with aminoglycoside treatment (Duan et al., 2000; Sedo-Cabezon et al., 2014). Given that excitotoxicity primarily targets the synaptic terminals of ANFs, *i.e.*, ribbon synapses, it is legitimate to speculate that aminoglycosides could cause synaptopathy, and more specifically, an HHL-type of synaptopathy with carefully titrated doses of aminoglycosides (Jiang et al., 2017; Liberman and Kujawa, 2017). In the present study, with combined G/F treatment, synaptic ribbons were often reorganized, and ribbon density was rarely reduced, at least, not prior to overt damage at the OHC level. This observation is contrary to a model of aminoglycoside-induced excitotoxicity. Since the cochlear damage by a furosemide-induced ototoxicity is rather rapid (Taylor et al., 2008), we postulate that a chronic situation is necessary for the aminoglycoside to be effectively excitotoxic at the synaptic terminals of the ANF, thus, multiday aminoglycoside treatment might be necessary to encourage an adequate excitotoxicity that results in an HHL-type synaptopathy. Further investigations are thus warranted to confirm such a notion.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figure legends

Figure 1: Dose response of G/F treatment as determined by ABR thresholds. A: Average ABR thresholds with 200G/0F treatment measured before, 3-day and 7-day posttreatment. **B**: 200G/50F. **C**: 200G/100F. **D**: 200G/400F. N=2-5 mice per group, both ears tested. Error bar=SEM. **E**: Selected ABR thresholds from individual ears measured at 3-day posttreatment, and **F**: at 7-day posttreatment.

Figure 2: DPOAE revealed OHC damage from G/F treatment in individual mice. In the fixed dose of gentamicin groups, the introduction of furosemide at varied doses caused a DPOAE deficit in the cochlea at various degrees of severity, from **A**: no damage, to **B**: mild damage, **C**: moderate damage, and **D**: severe damage 7-day posttreatment. Considerable inter-animal variation existed with the same treatment regimen. For instance, both with 200G/200F treatment, **E**: no DPOAE deficit were observed in mouse #388 7-day posttreatment, while **F**: severe functional damage was observed in mouse #387. Sound levels of the primary tones were equal at L_1=L_2=55 dB SPL, f_2/f_1=1.25. NF=noise floor.

Figure 3: Input/output functions (I/Os) of wave-I amplitude from ABR to 12-kHz tones. A: Average ABR wave I amplitude I/O functions with 200G/50F treatment measured before, 3-day and 7-day posttreatment. **B**: 200G/100F. **C**: 200G/200F. **D**: 200G/400F. **E**: Average I/O functions from cochleae with no DPOAE deficit, **F**: mild DPOAE decrements, and **G**: moderate DPOAE losses. N=2-4 mice per group, both ears tested. Error bar=SEM.

Figure 4: Alteration of synaptic ribbons after G/F treatment. A: In the apical and middle cochlear locations free of OHC damage, low-dose G/F treatment might not induce any visible change in CtBP2-labeled synaptic ribbons (red puncta), showing a characteristic vacant zone depicted by brackets and dotted lines basal to the IHC nucleus in the *xy* plane (**A1**). Error bar=20 μm. Reconstructed images in the *yz* plane from three adjacent IHCs exhibited the distribution of ribbons along the pillar-modiolar axis (**A2**), with identification of the cytoplasm of the IHC by anti-Myo7a immunolabeling (**A3**, blue), and paired postsynaptic AMPA receptors by anti-GluR2 immunolabeling (**A4**, green). A semi-normal distribution of the ribbon along the main cell axis was often observed (**A5**). **B1-4**: Seven days after G/F treatment, at cochlear locations free of OHC damage, a pillar-side upward shift of synaptic ribbons was often seen without a reduction in ribbon number. **B5**: ribbon distribution along the main cell axis was skewed to the nucleus. **C1**: With severe cochlear damage induced by G/F treatment, the ribbon density could be drastically reduced and many individual ribbons enlarged (arrowhead), and again, often exhibiting an upward shift (**C2**).

Figure 5: Group analysis of synaptic ribbon density after G/F treatment. A: Ribbon density with a fixed dose of gentamicin and varied dose of furosemide. N is identified in each column. **B**: Ribbon density with fixed doses of furosemide and varied doses of gentamicin. **C**: Illustration of G/F treatment dosages that modified ribbon density. **D**: Ribbon density is categorized based on the severity of DPOAE deficit. ** p< 0.01, 2-tailed Welch's t test. Error bar=SD.

Figure 6: Synaptic ribbon density after 200G/200F treatment. A: The OHC loss at each cochlear location where a confocal image acquired, was often complete or none. **B**: Average ribbon density from multiple cochlear locations 7 days posttreatment. Error bar=SD. **C**: Ribbon density grouped by whether OHCs were largely survived at the same cochlear location. **D**: Ribbon densities from

individual cochleae at the 12-kHz location. Red: showing OHC loss. Green: without OHC loss. **E**: A representative confocal image showing intact OHCs and an elevated number of synaptic ribbons. Scale bar=10 µm. **F**: A representative confocal image showing a complete loss of OHCs. Phalloidin labeling (green) identified scar formation at the sites of missing OHCs.

Figure 7: The lack of correlation between the wave-I ABR amplitudes and ribbon density. A: Average ABR I/O-amplitude functions for 400G/200F treatment measured before (N=8) and 2 days posttreatment (N=6). Error bar=SEM. **B**: Average ribbon density from multiple cochlear locations 2-day posttreatment (N=5-11). Error bar=SD. **C**: Scatter plot indicates the lack of correlation between wave-I ABR amplitude and ribbon density from individual cochleae for the 400G/200F treatment at 2 days posttreatment. Gray zone indicates the ribbon density from control ears, ±1 SD. **D**: Scatter plot shows poor correlation coefficient between the wave I ABR amplitude and the ribbon density, from individual cochleae with intact OHCs, treated by 200 mg/kg gentamicin and varied furosemide at 7-day posttreatment.

Figure 8: Illustration demonstrates the pillar-side upward shift of the synaptic ribbons after G/F treatment. Red particles depict individual synaptic ribbons. p=pillar side; m=modiolar side.

Supplementary Materials

Supplemental Figure 1: G/F dose combinations applied to *C57BL/6* in the present study.

Supplemental Figure 2: Average ABR thresholds to the G/F treatment of 0G/200F (**A**), 50G/200F (**B**), 100G/200F (**C**), and 200G/200F (**D**).

Supplemental Figure 3: Ribbon distribution along the main IHC axis in the normal formation (**A**), or presenting an upward shift without a reduction of ribbon density (**B**), or with a drastic reduction (**C**).

Supplemental Figure 4: Postsynaptic AMPA receptors were identified by anti-GluR2 immunolabeling. The pre- and post-synaptic pairing were typically maintained after G/F treatment when OHCs were survived at the same examined region, either with (**B**) or without upward shift of the synaptic ribbons (**A**). However, unpairing did occur after the OHC loss (**C**), detected by phalloidin labeling (blue). Error bar=20 µm.

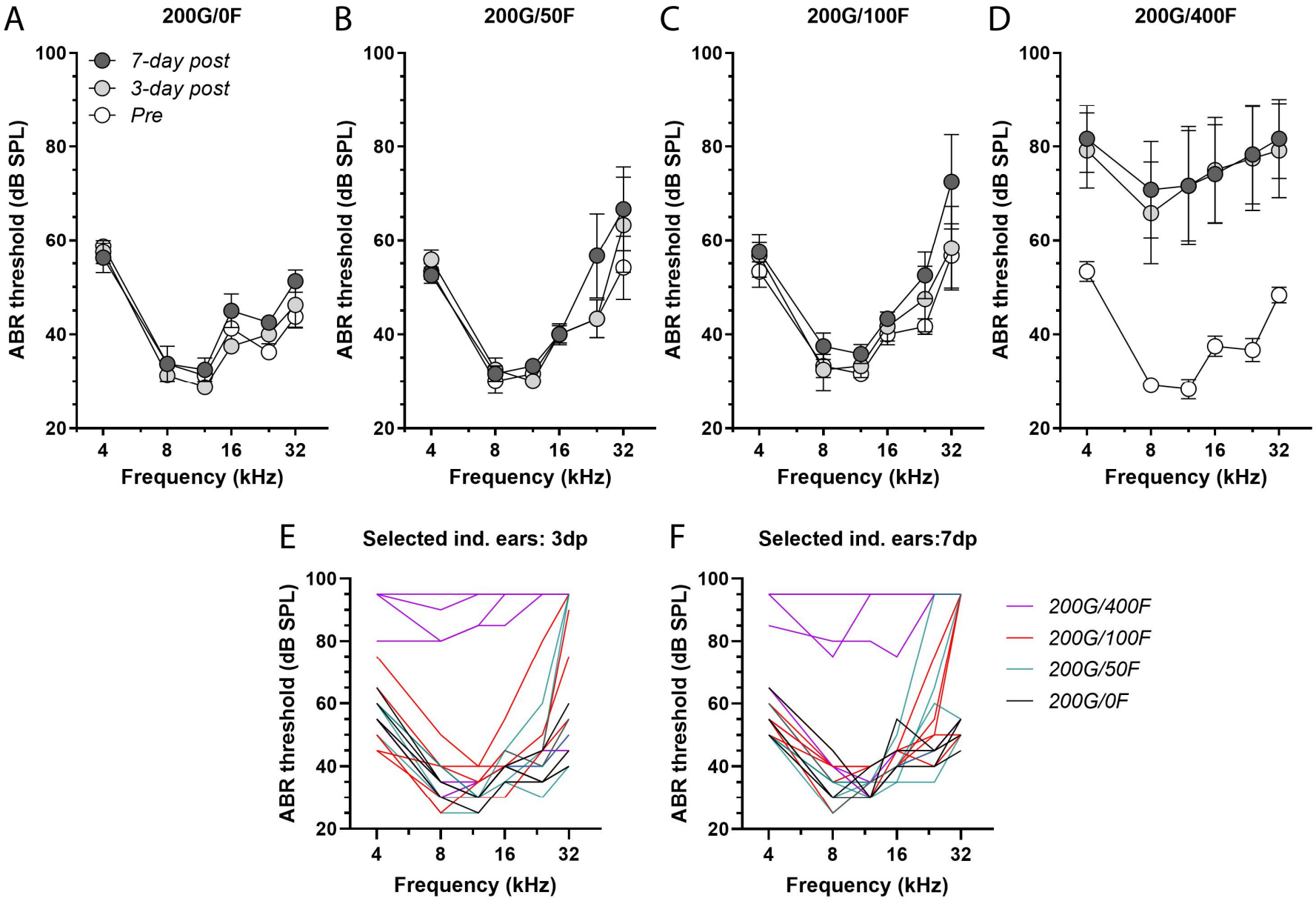

Figure 1

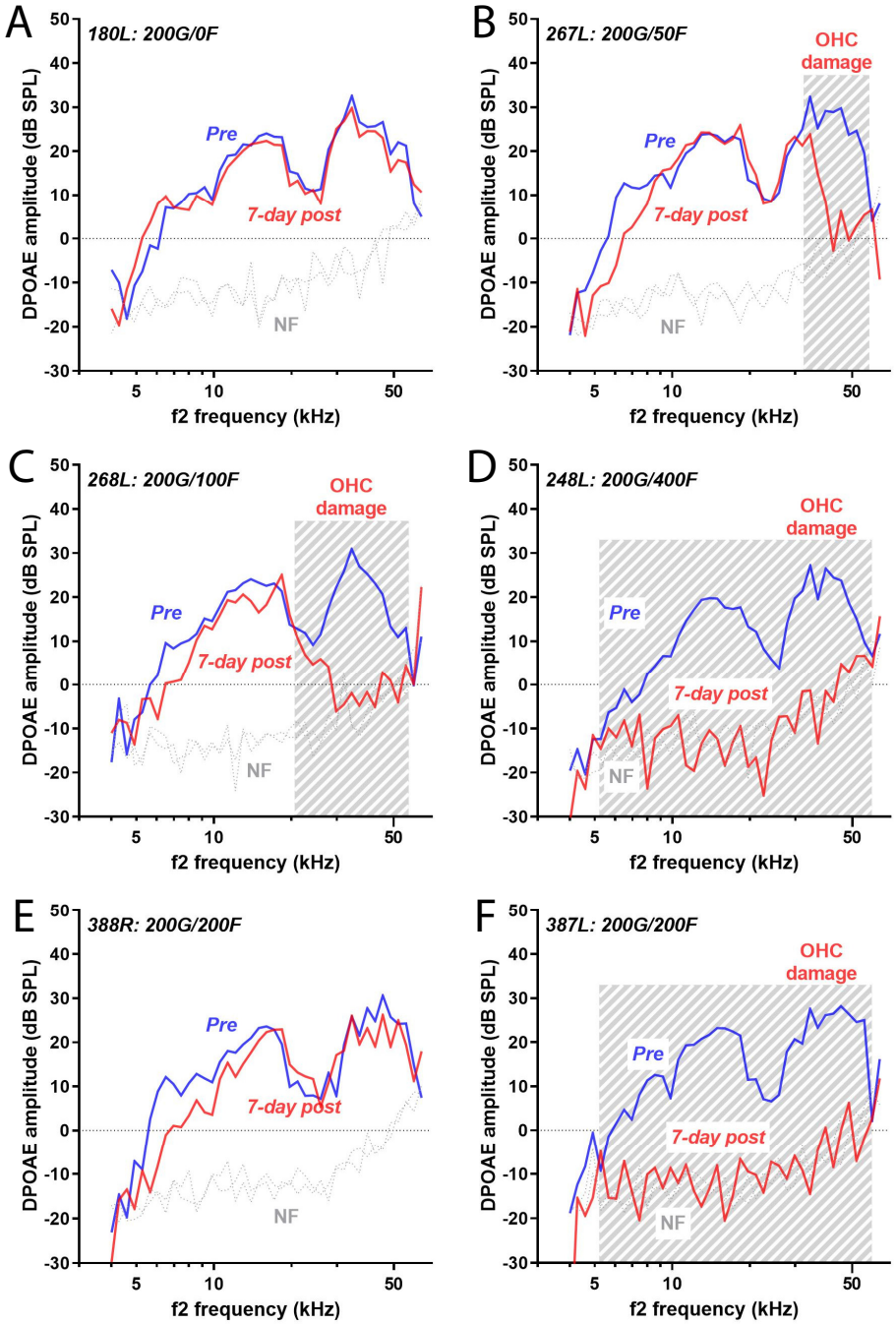
A
180L: 200G/0F
B
267L: 200G/50F
OHC damage
C
268L: 200G/100F
OHC damage
D
248L: 200G/400F
OHC damage
E
388R: 200G/200F
F
387L: 200G/200F
OHC damage
Pre
7-day post
NF
DPOAE amplitude (dB SPL)
f2 frequency (kHz)
50
40
30
20
10
0
-10
-20
-30
5
10
50

Figure 2

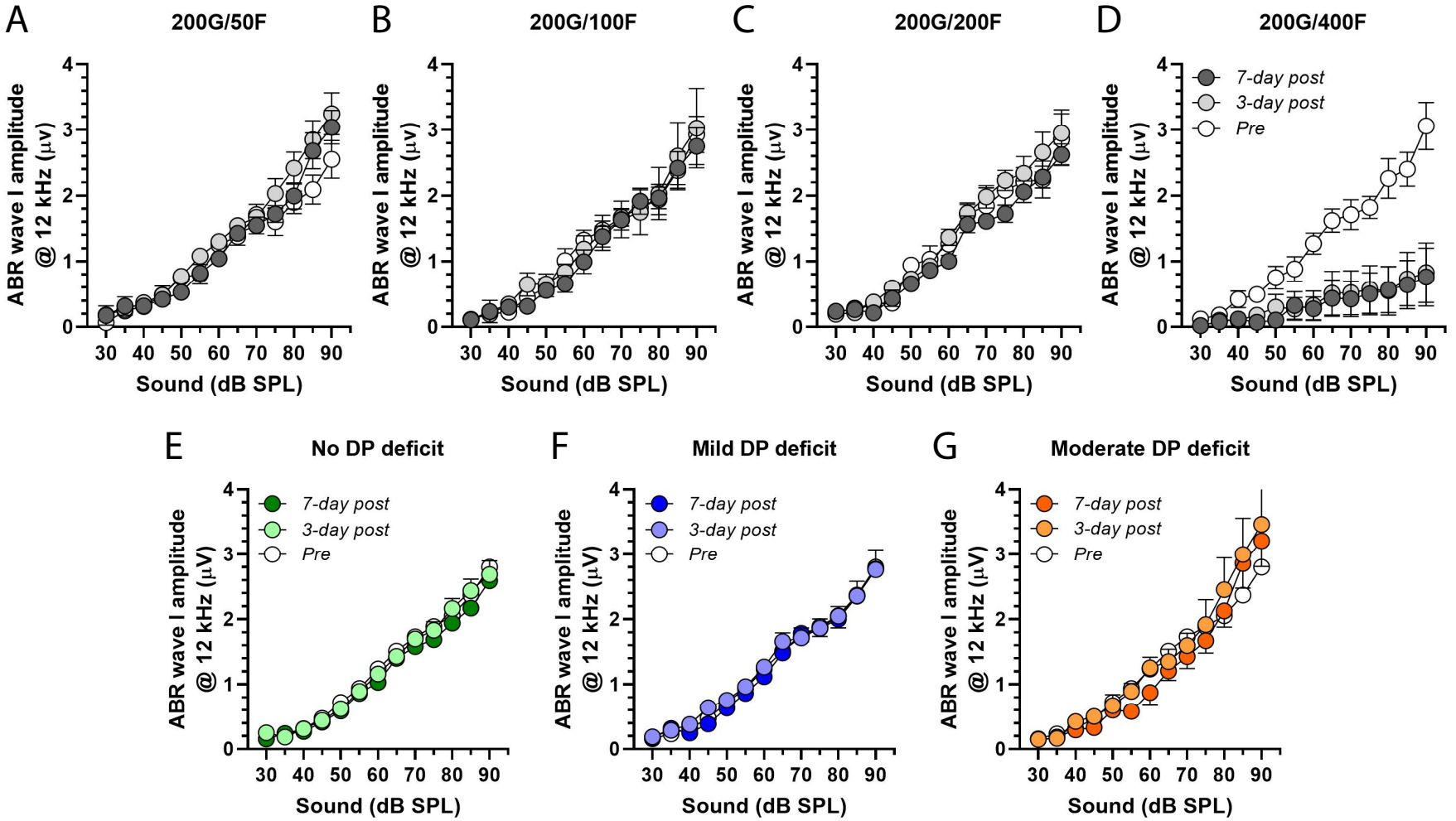
A
200G/50F
B
200G/100F
C
200G/200F
D
200G/400F
7-day post
3-day post
Pre
ABR wave I amplitude @ 12 kHz (μv)
Sound (dB SPL)
E
No DP deficit
F
Mild DP deficit
G
Moderate DP deficit

Figure 3

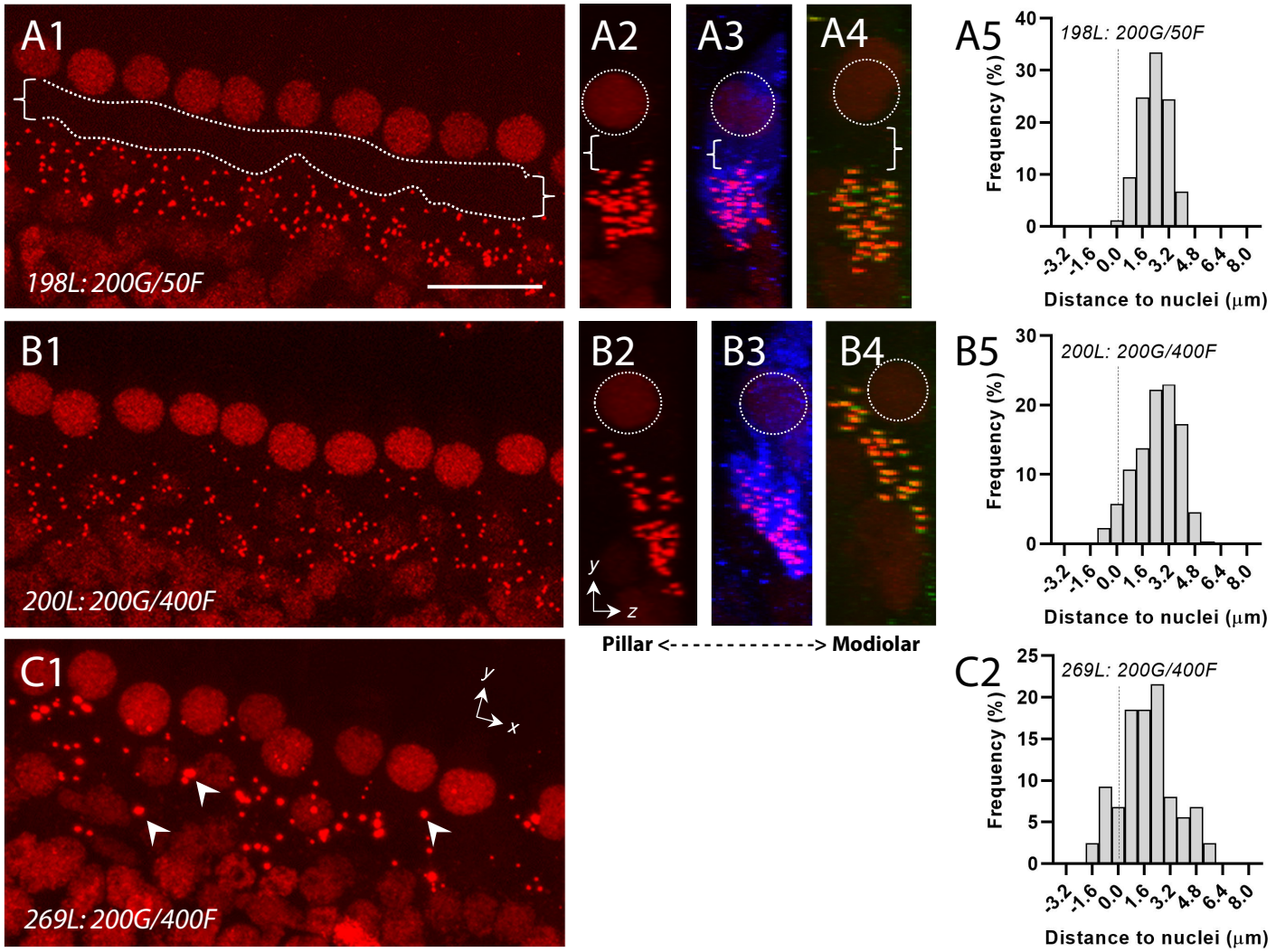
A1
198L: 200G/50F
A2
A3
A4
A5
198L: 200G/50F
Frequency (%)
Distance to nuclei (μm)
B1
200L: 200G/400F
B2
B3
B4
B5
200L: 200G/400F
Pillar <----------> Modiolar
C1
269L: 200G/400F
C2
269L: 200G/400F

Figure 4

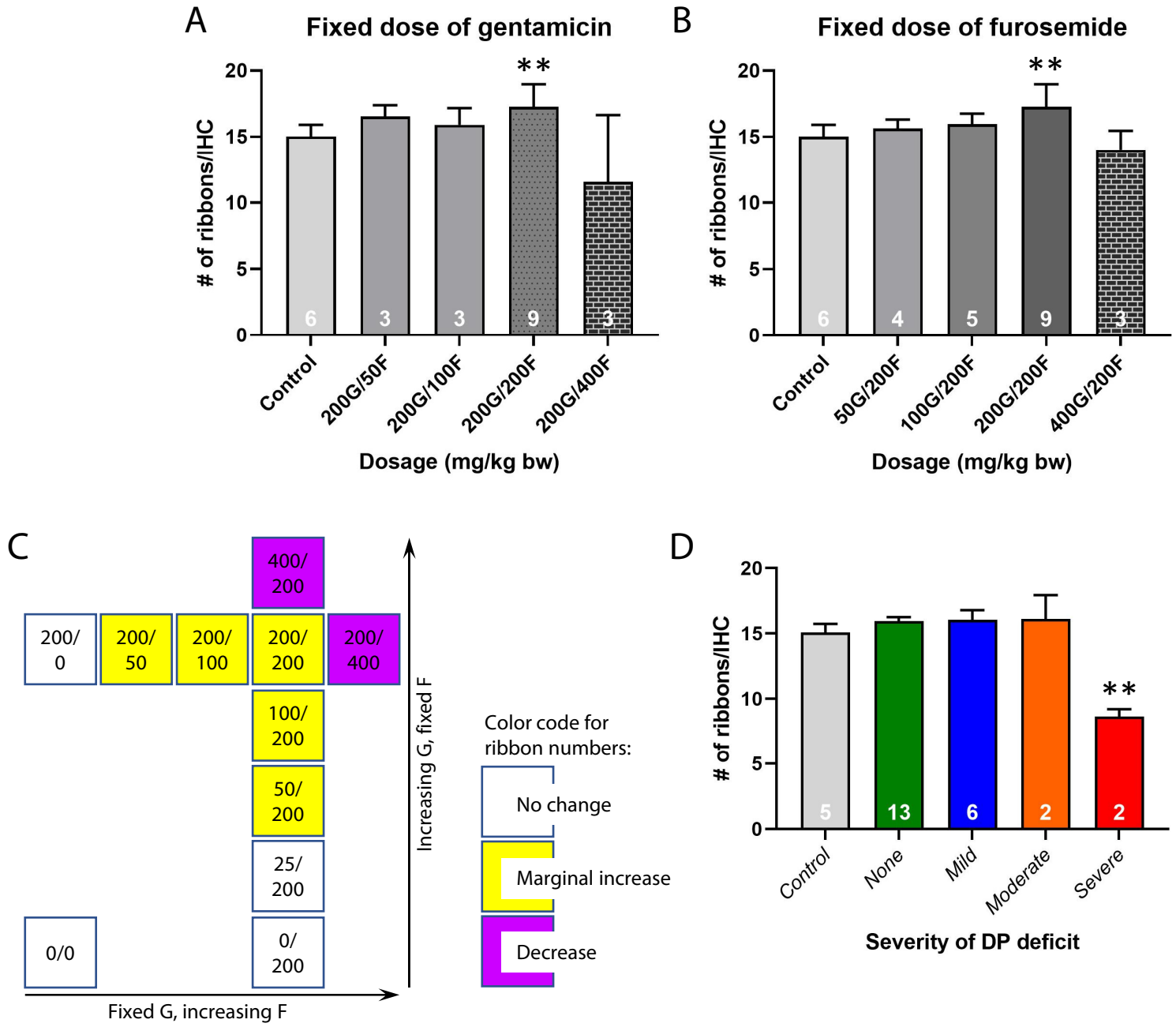
A
Fixed dose of gentamicin
# of ribbons/IHC
20
15
10
5
0
**
6
3
3
9
3
Control
200G/50F
200G/100F
200G/200F
200G/400F
Dosage (mg/kg bw)
B
Fixed dose of furosemide
# of ribbons/IHC
20
15
10
5
0
**
6
4
5
9
3
Control
50G/200F
100G/200F
200G/200F
400G/200F
Dosage (mg/kg bw)
C
400/ 200
200/ 0
200/ 50
200/ 100
200/ 200
200/ 400
100/ 200
50/ 200
25/ 200
0/0
0/ 200
Increasing G, fixed F
Fixed G, increasing F
Color code for ribbon numbers:
No change
Marginal increase
Decrease
D
# of ribbons/IHC
20
15
10
5
0
**
5
13
6
2
2
Control
None
Mild
Moderate
Severe
Severity of DP deficit

Figure 5

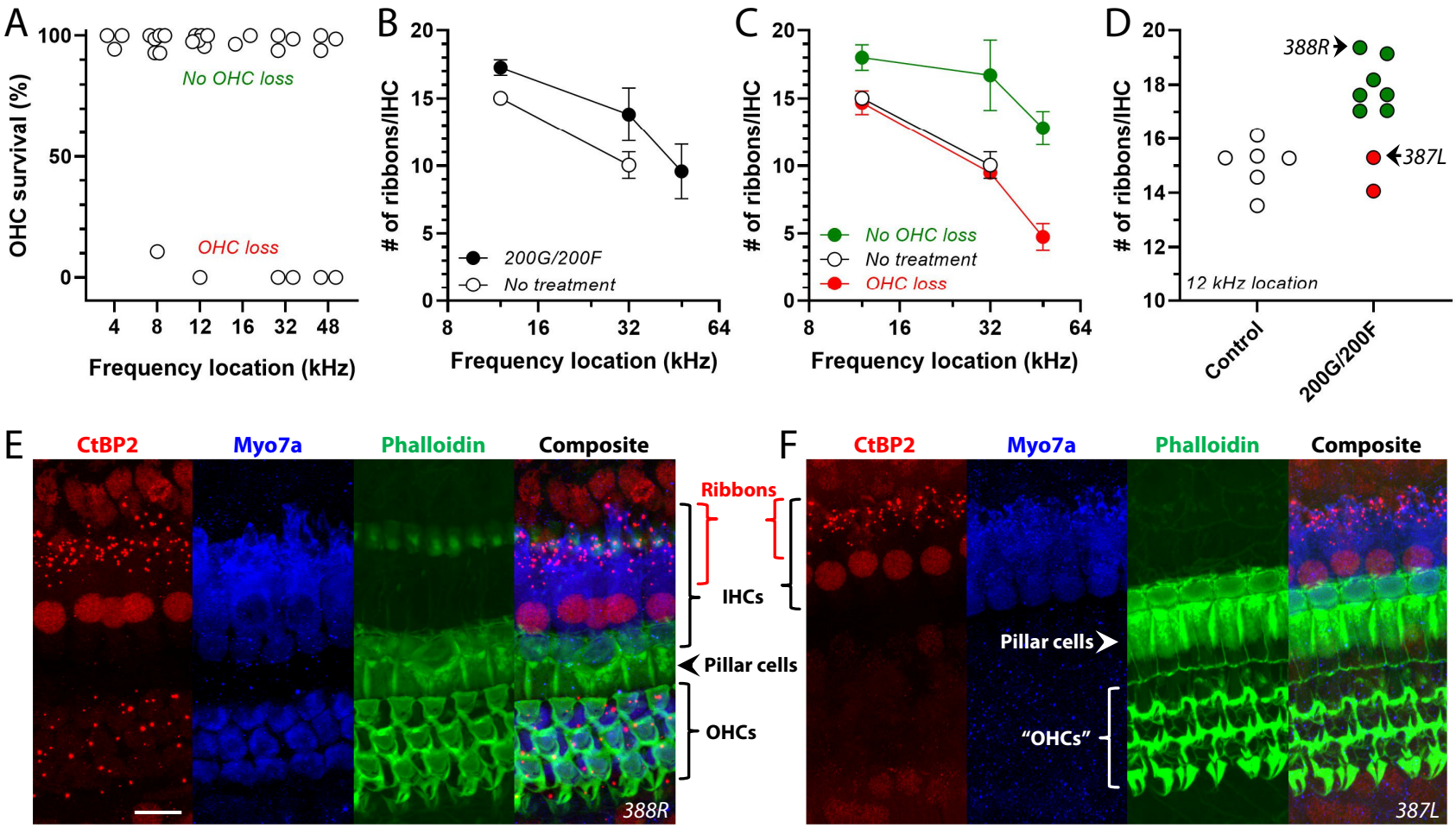
A
OHC survival (%)
100
50
0
No OHC loss
OHC loss
4
8
12
16
32
48
Frequency location (kHz)
B
# of ribbons/IHC
20
15
10
5
0
200G/200F
No treatment
8
16
32
64
Frequency location (kHz)
C
# of ribbons/IHC
No OHC loss
No treatment
OHC loss
Frequency location (kHz)
D
# of ribbons/IHC
388R
387L
12 kHz location
Control
200G/200F
E
CtBP2
Myo7a
Phalloidin
Composite
Ribbons
IHCs
Pillar cells
OHCs
388R
F
CtBP2
Myo7a
Phalloidin
Composite
Pillar cells
"OHCs"
387L

Figure 6

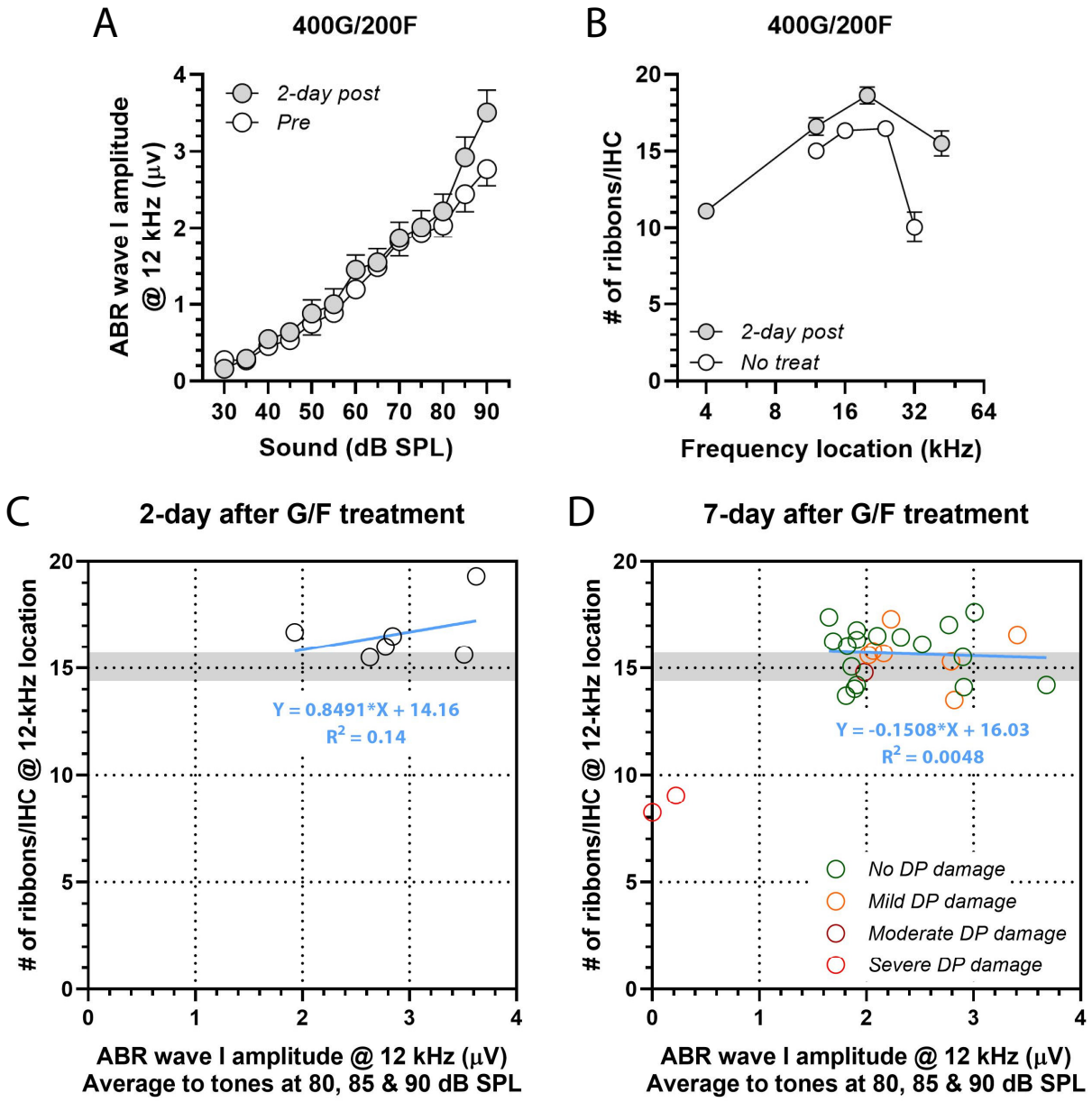
A
400G/200F
ABR wave I amplitude @ 12 kHz (μv)
2-day post
Pre
Sound (dB SPL)
B
400G/200F
# of ribbons/IHC
2-day post
No treat
Frequency location (kHz)
C
2-day after G/F treatment
# of ribbons/IHC @ 12-kHz location
Y = 0.8491*X + 14.16
R2 = 0.14
ABR wave I amplitude @ 12 kHz (μV)
Average to tones at 80, 85 & 90 dB SPL
D
7-day after G/F treatment
# of ribbons/IHC @ 12-kHz location
Y = -0.1508*X + 16.03
R2 = 0.0048
No DP damage
Mild DP damage
Moderate DP damage
Severe DP damage
ABR wave I amplitude @ 12 kHz (μV)
Average to tones at 80, 85 & 90 dB SPL

Figure 7

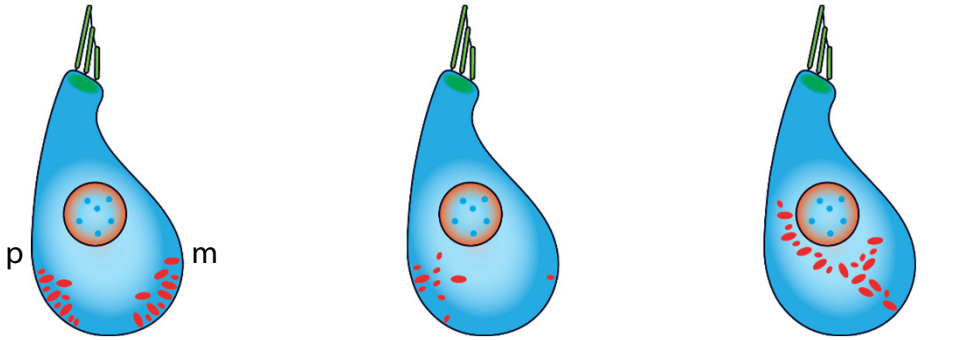
p
m
IHC
no treatment
HHL-type of synaptopathy
after noise exposure
G/F-induced pillar-side
upward shift

Figure 8