TITLE: Preventing Ototoxic Synergy of Prior Noise Trauma during Aminoglycoside Therapy

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Preventing Ototoxic Synergy of Prior Noise Trauma during Aminoglycoside Therapy

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Exposure to loud sounds causes temporary or permanent threshold shifts in auditory perception, with reversible or irreversible cellular damage in the cochlea. Noise trauma, or loud sound exposure, is particularly associated with military environments, especially when sustaining blast injuries. These injuries are frequently treated with aminoglycoside antibiotics that have broad-spectrum bactericidal activity for treating or preventing life-threatening infections. However, aminoglycosides are also toxic to the cochlea, leading to hearing loss and further degradation from pre-injury status. The combination of both prior noise trauma and aminoglycoside treatment can degrade auditory function greater than simple summation of the two insults. We have found that prior sound exposure enhances cochlear uptake of aminoglycosides, providing a mechanistic basis for the observed ototoxic synergy due to noise trauma and subsequent aminoglycoside treatment.

Noise trauma, combat injury, otoprotection, aminoglycoside antibiotic, bacterial infection, ototoxicity, auditory function, hearing loss

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1. INTRODUCTION

Exposure to loud sounds causes temporary or permanent threshold shifts in auditory perception, with reversible or irreversible cellular damage in the cochlea. Noise trauma, or loud sound exposure, is particularly associated with military environments, especially when sustaining blast injuries. These injuries are frequently treated with aminoglycoside antibiotics that have broad-spectrum bactericidal activity for treating or preventing life-threatening infections. However, aminoglycosides are also toxic to the cochlea, leading to hearing loss and further degradation from pre-injury status. The combination of both prior noise trauma and aminoglycoside treatment can degrade auditory function greater than simple summation of the two insults. We have found that prior sound exposure enhances cochlear uptake of aminoglycosides, providing a mechanistic basis for the observed ototoxic synergy due to noise trauma and subsequent aminoglycoside treatment.

In the mammalian inner ear – the cochlea, the auditory sensory cells, particularly outer hair cells (OHCs), are more susceptible to aminoglycoside-induced cytotoxicity than other cochlear cells, particularly at the base of the cochlea most sensitive to higher frequency sound. Once these OHCs are lost, these sensory cells cannot be endogenously regenerated, leading to life-long hearing loss and deafness. Thus, extensive efforts are underway to ameliorate and prevent aminoglycoside-induced hair cell death. Under normal physiological condition, aminoglycosides can rapidly cross the blood-labyrinth barrier (BLB) into the cochlear tissues and fluids and enter OHCs through a number of conduits. The best-characterized conduit is permeation through the mechanoelectrical transduction (MET) channel. The MET channel is mechanically-gated by the extracellular, heterodimeric tip links between two stereocilia. Other mechanisms by which aminoglycosides can enter hair cells include endocytosis, and/or other aminoglycoside cation channels (e.g. TRP channels) expressed by hair cells besides the MET channel, such as TRPV4 on the apical membranes, or TRPA1 on the basolateral membranes, of OHCs.

The ultimate goal of this research is to prevent aminoglycoside-induced cochleotoxicity (as well as vestibulotoxicity and nephrotoxicity) that can severely debilitating the recovery of military personnel, including combatants and associated casualties to pre-injury effectiveness. In this project, we hypothesize that prior noise trauma induces synergistic ototoxicity with systemically-administered aminoglycosides by potentiating cochlear uptake of the drug. We also hypothesize that specific aminoglycoside-permeant cation channels directly facilitate noise trauma-enhanced uptake of aminoglycosides in the cochlea.

2. KEYWORDS

Noise trauma, combat injury, otoprotection, aminoglycoside antibiotic, bacterial infection, ototoxicity, auditory function, hearing loss
3. OVERALL PROJECT SUMMARY

What were the major goals of the project?

Aim 1: Determine the acoustic parameters that induce noise-enhanced aminoglycoside uptake in auditory sensory hair cells.

This is completed by the end of Year one at OHSU.

Aim 2: Determine if prior noise trauma modifies intra-cochlear trafficking of aminoglycosides.

Aim 2a: Use cochlear perfusion techniques to determine the contribution of endolymph or perilymph trafficking of aminoglycosides to hair cells with prior noise exposure. GTTR will be administrated either systemically or by scala tympani infusion to the animal.

This is completed at OHSU.

Aim 3: Determine if aminoglycoside-permeant channels on the hair cell apical membrane contribute to aminoglycoside uptake by cochlear hair cells.

Aim 3a: Determine if prior noise trauma enhances drug uptake in hair cells, by using mouse models with MET apparatus defects, including Pcdh15<sup>3J/3J</sup> (Ames waltzer) mice, Myo7a<sup>3J/3J</sup> (Shaker 1) mice; and TrpV4<sup>−/−</sup> and P2X<sub>2</sub><sup>−/−</sup> mice with channelopathies, compared to heterozygous littermates.

This is completed approaching to the end of Year one at Loma Linda.

Aim 4: Determine if TRP channels on the basolateral membrane of cochlear hair cells also contribute to aminoglycoside uptake.

What was accomplished under these goals?

1) Major activities

Due to lab relocation, the project was interrupted in 2015, and resumed on June 1<sup>st</sup>, 2016, and re-budgeted. This is the first annual report after the relocation to VA Loma Linda Healthcare System, in Loma Linda, California.

After resuming the project, we re-established mouse cohorts, acquired and calibrated instruments for auditory and physiological measurement in rodents. We also completed searching, hiring and training process of lab personals. Experiments using electrophysiology and immunohistochemistry have been carried to produce data for the proposed studies in this project.
2) **Specific objectives**

a) We initiated mouse cohorts including *TrpV1* mice (#3770) and wildtype C57BL/6 mice (#0664) in the animal facility at Loma Linda. Breeding pairs were purchased from Jackson Laboratory (Bar Harbor, ME).

b) We acquired and setup lab equipment, including noise exposure instrument and auditory brainstem response (ABR) measurement apparatus. One of the ABR system is primarily driven by a TDT-RZ6 system, purchased from Tucker-Davis Technology (Alachua, FL) through the PI’s startup fund. In addition, both the noise chamber and the sound delivery component of the ABR system has been calibrated using a ¼” B&K microphone, to ensure the accuracy of sound levels that are presented to experimental animals.

c) We have accumulated adequate number of *TrpV1* mice at Loma Linda, and characterized the mutant mice by ABR testing. Newly acquired experimental data are compared to those from PI’s previous lab at Oregon Health & Science University (OHSU).

d) We also developed genotyping protocols to identify mutant mice and heterozygous mice in the *TrpV1* cohort. We could either use the traditional PCR method or a quantitative PCR system to achieve the genotyping purpose within the lab.

4. **KEY RESEARCH ACCOMPLISHMENTS**

a) After successfully genotyping the *TrpV1* mice, we measured baseline ABR thresholds in young adults. The newly acquired data (Fig. 1A) is noticeably similar to the data (Fig. 1B) obtained from the PI’s previous lab, offering us confidence in the new *TrpV1* colony and the new electrophysiological system.

b) Mice with BL/6 background manifest an age-related hearing loss. With ABR recordings, we have been longitudinally following the hearing sensitivity of a group of *TrpV1* mice, including mutant mice and heterozygous mice. In addition, our previous observation before the lab relocation suggested expedited hearing deterioration in mutant *TrpV1* mice. This approach provides us fundamental knowledge to achieve the goals in Specific Aims 3 and 4, given that the TRPV1 channel is a candidate channel for aminoglycoside uptake by sensory hair cells.

*Figure 1. Baseline ABR thresholds in young adult *TrpV1* mice from Loma Linda (A) and OHSU (B). Error bars are SEM.*
c) We documented ABR thresholds at 6 time points, including 5, 9, 13 (Fig. 2A), 17 (Fig. 2B), 21 and 29 weeks of age, in both male (open symbols) and female (filled symbols) mice. A sub-population of monitored mice started exhibiting age-related hearing loss at higher frequencies (24 and 32 kHz) from week 13 (Fig. 2A). The hearing deterioration was more evident at week 17 (Fig. 2B). A legitimate question is whether these mice with expedited hearing loss are more vulnerable to noise trauma.

Figure 2. ABR thresholds from individual TrpV1 mice at 13 (A) and 17 weeks of age (B).

d) We thoroughly analyzed the longitudinal ABR data in measuring the hearing sensitivity of *TrpV1* knockout mice. It led to the conclusion that up to 29 weeks of age, the hearing deterioration in *TrpV1* knockout mice and that in littermate control mice, are comparable. Evident high frequency hearing loss can be observed from both groups. Previously, our ABR data collected at OHSU suggested an expedited age-related hearing loss in *TrpV1* knockout mice, which occurs as early as 11 weeks of age in the knockout. We suspect certain environment factor is responsible for the observational deviance between two different research sites.

e) Further data analysis on the raw ABR data revealed a subtle, but statistically significant delay in ABR wave-I latency in *TrpV1* knockout mice (Fig. 3A&B, 9- &17-week old, respectively, 2-way ANOVA, p<0.0001 for both). The physiological relevance of this observation is to be determined. Wave-I amplitudes were comparable between groups (Fig. 3C, 9 week, 2-way ANOVA, p=0.51). The extended delay in wave-I latency in *TrpV1* knockout mice was not limited at 12 kHz. An across frequency analysis, at the sound level about 18 dB above the group-average response threshold, showed significant delay in knockout mice by a 2-way ANOVA test (Fig. 4; p=0.016), although the post-hoc Bonferroni comparison did not reveal

Figure 3. ABR wave-I latencies and amplitudes measured to tone pips at 12 kHz in adult TrpV1 mice at 9 (A, C) and 17 (B) weeks of age. Error bars are SEM.
significant difference at individual frequencies (p>0.05). The electrophysiological source of ABR wave-I is the spiral ganglion along the auditory pathway. The delay in wave-I latency warrants an investigation in the synaptic morphology in the organ of Corti. Part of data has been reported and discussed at the 40th ARO Annual MidWinter Meeting in Baltimore, MD.

f) We initiated immunofluorescent experiments in which the ribbon synapse of the inner hair cell afferent inneravation is evaluated using cochlear micro-dissection and confocal imaging techniques. Our preliminary result in aged mice (~28 weeks of age) suggested that *TrpV1* deficiency appeared to reduce the number of ribbon synapses while enlarged the size of individual ribbons (Fig. 5B), suggesting an incomplete compensatory mechanism. We will study more animals with various ages to confirm this observation.

![Figure 4. ABR wave-I latencies measured to tone pips at the sound level about 18 dB above the group-average response threshold, and across frequencies. Error bars are SEM.](image)

![Figure 5. Ribbon synapses from control mice (A) and TrpV1 knockout mice (B). Arrows depict exemplified ribbon synapses that were immunolabeled by anti-CtBP2 IgG. Note that the antibody also labeled the nucleus of inner hair cells. Scale bar is 10 µm.](image)
5. CONCLUSION

Candidate aminoglycoside channels (e.g. TRPV1) and their regulating components in the inner ear, control hearing sensitivity in a characteristic fashion. In addition, our data suggest the neurotransmission between the inner hair cell and the spiral ganglion neuron is another major ototoxic target. It is already known that noise exposure does modify this neural transmission adversely. How prior noise exposure and aminoglycoside-induced ototoxicity interplay at this pivotal functional region is open question in auditory research, and needless to say, very relevant to personals in military settings.

Aminoglycoside antibiotics, like gentamicin and tobramycin, are clinically-essential antibiotics for treating life-threatening Gram-negative bacterial infections. They are being pervasively used, seeing wide application in outpost clinics to national army/veterans hospital. In civilian population, they are particularly used in premature babies, and for patients with cystic fibrosis, or Gram-positive infections like tuberculosis and protozoal infections. Despite their wide use, broad-spectrum, bactericidal efficacy and low cost, clinical dosing with aminoglycosides is limited by the risk of acute nephrotoxicity and life-long ototoxicity, with significant ramifications for quality of life. This research is to search countermeasures to prevent aminoglycoside-induced cochleotoxicity (as well as vestibulotoxicity and nephrotoxicity) that can severely debilitating the recovery of military personnel, as well as civilians received aminoglycoside therapy with a history of (or likely ongoing) acoustic insult.

6. PUBLICATIONS, ABSTRACTS AND PRESENTATIONS

Peer reviewed publication


Conference abstracts, papers and podium presentations


“Ototoxicity under bacterial infection and inflammation”, 2nd Hearing Science Symposium: from Hair Cells to Cortex, Shandong ENT Infirmary, Shandong University, Jinan, China, Oct 2016.

7. INVENTIONS, PATENTS AND LICENSES

Nothing to report.

8. REPORTABLE OUTCOMES

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

This research project provided opportunities for people with interest and motivation in biomedicine research, including college students and international physicians. For instance, Brandon Yeoh, a college student from Brown University, has involved in this project as a volunteer during summer time. Liana Sargsyan, a European trained physician who is specialized in otology has joined the research group since the beginning of 2017, providing the lab with hands-on experience in cochlear dissection, and image acquisition etc. This experience will certainly provide positive impact on their career development.

What individuals have worked on the project?

Name: Hongzhe Li, PhD
Project Role: PI
Nearest person month worked: 6.0
Contribution to Project: Dr. Li has performed work in experimental design, tissue harvest and processing, confocal imaging, image acquisition and quantification, data analysis, documents, reports and manuscript preparation.

Name: Alisa Hetrick, BSc
Project Role: Research Technician
Nearest person month worked: 6.0
Contribution to Project: Ms. Hetrick has performed work in ABR recordings and managed mouse cohort with genotyping procedures. She also assisted in acquiring lab equipment and consumables, and protocol development.
Name: Liana Sargsyan, MSc
Project Role: Research Assistant
Nearest person month worked: 5.0
Contribution to Project: Ms. Sargsyan has performed work in cochlear microdissection, confocal microscopy and part of data analysis.

Name: Brandon Yeoh
Project Role: Research Volunteer
Nearest person month worked: 1.0
Contribution to Project: Mr. Yeoh has assisted on ABR recording and data analysis.

**How were the results disseminated to communities of interest?**

Part of the content in this report has been published at the 5th Joint Meeting of Acoustical Society of America & Acoustical Society of Japan, Honolulu HI, and at 2017 Midwinter meeting of Association for Research in Otolaryngology, Baltimore MD, as attached in the Appendix.

**9. OTHER ACHIEVEMENTS**

a) Other general lab activities included personal recruitment and lab orientation, lab safety and compliance training, equipment acquisition and setup, and protocol selection or development etc. For instance, in compliance with DoD Instruction and US Army Regulation, we submitted institute-approved IACUC protocol to ACURO, with a complete form of Animal Use Appendix for Research Involving Animals. The animal protocol was approved by the USAMRMC ACURO on March 8th, 2017.

b) Research technician has been trained on mouse genotyping techniques, and we are now proficient on identifying mutant mice and heterozygous mice in the *TrpV1* cohort.

**10. REFERENCES**

Not applicable.

**11. APPENDICES**

Poster presentation at 2017 Midwinter meeting of Association for Research in Otolaryngology, Baltimore MD.
**Introduction**

In mammals, TRPV1 is widely expressed in the nervous system and some non-neuronal tissues1,2, such as the kidney3. In the inner ear, TRPV1 is present in the hair cells and supporting cells of the organ of Corti, and spiral ganglion cells34, and regulates cochlear sensitivity5. TRPV1 is also present in sensory cells of the vestibular and organs and vestibular ganglion cells in. In aged rats, TRPV1 expression in spiral ganglion neurons is heightened, but this is attenuated in the basal cochlear region by acoustic trauma that occurred earlier in life6. This modulated TRPV1 expression in spiral ganglion neurons is thought to be related to neuropathic events that lead to tinnitus and hyperacusis7. Similar to aging and acoustic trauma, treatment with antibiotic aminoglycosides such as kanamycin in mice can also modulate spiral ganglion TRPV1 expression8.

We are not aware of any previous work that reported apparent hearing loss in adult mice, TRPV1 mutant mice also show no pain response, display increased longevity, reduced metabolism, and reduced aging. Interestingly, the homologue TRPV4, which is also present in the cochlea, including hair cells, spiral ganglia and stria vascularis, is implicated in age-related hearing loss9. Here, we assessed the hearing sensitivity of TRPV1 mutant mice as they aged, and vulnerability to noise exposure.

Transient receptor potential (TRP) channels exhibit high capacity/conductance and low selectivity of cations, with high calcium permeability. They are candidate transporters for the cationic aminoglycosides, such as gentamicin, to enter sensory hair cells, and promote cellular toxicity10. Since the gating mechanisms of TRP channels are associated with cellular stress, apoptosis, necrosis, and neurodegeneration, and cytoplasmic uptake of aminoglycosides, these channels, including TRPV1, may represent a functional target that links acoustic trauma and enhanced aminoglycoside trafficking and uptake. If acoustic overexposure modulates the expression of TRPV1 and/or its cationic permeability, this will represent a novel mechanism between acoustic trauma and enhanced aminoglycoside toxicity. In the long-run, we will test whether the loss of TRPV1 function also reduces cellular uptake of gentamicin, and that TRPV1 is a viable pharmacological target to prevent ototoxicity (and nephrotoxicity) during gentamicin therapy, with or without prior acoustic trauma.

**Methods and Materials**

Mice. TRPV1 mutant mice (TRPV1-/-, Tabuchi et al., 2004; J Assoc Res Otolaryngol 7:109-119) were crossed with C57BL/6 (JAX stock 00064) to generate #1 offspring, and then backcrossed with the initial TRPV1 mutant mice to generate experimental animals. Animals were housed in a specific pathogen-free room, with ambient temperature and light cycles.

Acoustic overexposure. Adult noise was exposed to wideband noise (WBN; 96 dB SPL) for 8 hours/day for 3 consecutive days, resulting in a total of 18 hours of noise exposure. We previously reported temporary threshold shifts with this protocol in C57/BL/6 mice11.

ABR. Auditory brainstem responses to clicks and tones with a broad frequency range, from 0.2 to 48 kHz, were measured (i) at multiple age points, (ii) during the development of temporary threshold shift, and (iii) at three different time points after acoustic overexposure, including 1 day, 3 weeks, and 6 weeks post exposure.

Immunofluorescence. Mice were fixed by transcardiac perfusion of 4% paraformaldehyde (PFA) in PBS, excised and immersion fixed overnight with 4% PFA plus 0.5% Triton X-100. The cochlear preparation was then cleared in ascending concentrations of ethanol and xylene, followed by xylene. All tissues were embedded in paraffin and sectioned at 10 mm. Sections were then stained with IHC and counterstained with propidium iodide. Immunostaining was performed using primary antibodies against TRPV1 and/or TRPV4 and secondary antibodies against Alexa 488 or Alexa 568. Sections were mounted on slides and coverslipped.

**Results**

**Age-related hearing loss in TRPV1 mutant mice**

(A) At 7 weeks of age, ABR thresholds in TRPV1 mutant mice (n=5) were comparable to their littermate heterozygous control (n=8) at most tested frequencies, except at 8 kHz (*p<0.05, t-test). (B) At 11 weeks of age, ABR thresholds in TRPV1 mutant mice were comparable to their littermate control at lower frequencies, from 4 kHz to 16 kHz (*p<0.05), while thresholds were significantly elevated ~30 dB at higher frequencies, from 24 kHz to 48 kHz (*p<0.05). (C) Hearing sensitivity is further diminished in 18-week-old TRPV1 mutant mice. No data were not available from littermate control, ABR thresholds were estimated from mutants compared to age-matched C57/BL/6 mice. ABR thresholds were generally worse in mutants versus the thresholds in controls at 16 kHz and above (*p<0.05).

TRPV expression in the cochlea and in the kidney

(A1-A2) In the stria vascularis, TRPV1 was localized at the luminal surface of marginal cells (mc, red in A2) at the level of the arterioles/tight junctions (green in A2) between adjacent marginal cells. (A3) No primary antibody control. (B) TRPV1 expression was most prominent at the reticular lamina of pilar cells (arrow), in the inner hair cell body (IC) and outer hair cell membranes (OM). (C) No primary antibody control. (C1-C2) In the kidney, TRPV1 was immunolocalized at the apical membranes of proximal tubules and distal tubules. (C2) No primary antibody control. Scale bar in C3 applies to all panels.

The latency and peak-to-peak amplitude of Wave I of ABR were further analyzed in TRPV1 mutant mice, in attempt to assess the auditory sensory integrity. The input/output functions were extracted from 9 kHz to 12 kHz noise tones. Two-way ANOVA indicated for Wave I latency in TRPV1 mutant mice at all ages, including 9 weeks (A, p<0.0001, n=6) and 17 weeks (B, p<0.0001, n=12). In contrast, Wave I amplitudes were comparable between two groups of mice, for instance at 9 weeks (C, p=0.46, 2-way ANOVA).

**Input/output functions of ABR**

(A-C) Wave I latency and peak-to-peak amplitude of ABR. (A) Wave I latency in TRPV1 mutant mice at all ages, including 9 weeks (A, p<0.001, n=6) and 17 weeks (B, p<0.0001, n=12). (C) Wave I amplitudes were comparable between two groups of mice, for instance at 9 weeks (C, p=0.46, 2-way ANOVA).

**Increased vulnerability to acoustic overexposure**

After noise exposure (WBN, 96 dB SPL, 18 hours total) at 11 weeks of age, ABR to click stimuli revealed comparable hearing recovery in TRPV1 and their littermate controls. However, subtle change was found with tonal stimuli. ABR thresholds to tone pips at 4, 8, 16 and 32 kHz were identified in TRPV1 mutant mice (n=4) after noise exposure (gray area in each panel). Thresholds were consistently higher in mutant mice at 16 and 32 kHz, resulting in poor hearing recovery from acoustic overexposure. The ABR threshold at 6 kHz after noise exposure, at 16 kHz in mutants was significantly higher than the threshold in littermate controls with noise exposure (*p=0.02, t-test), and higher than mutants without noise exposure (*p=0.04). Error bars are ±SEM.

**Discussion and Conclusions**

• TRPV1 is not required for normal development of cochlear function, as suggested by normal hearing sensitivity in juvenile TRPV1 mutant mice.
• Genetic disruption of TRPV1 promted late-onset (i.e. age-related) hearing loss compared to age-matched mice.
• TRPV1 may delay an adaptation component contributing the temporary threshold shift.
• TRPV1 mutant mice exhibited marginally increased susceptibility to acoustic overexposure.
• Similar to TRPV1, at 2 months of age, there was no significant difference in ABR thresholds between TRPV1 mutant mice and wild type mice, but thresholds elevated in TRPV1 mutant mice at 8 months of age. This suggests TRP channels are involved in progressive hearing loss.
• Similar to TRPV1, both TRPV4 and TRPV6 mutant mice are more vulnerable to acoustic trauma, resulting in poor recovery on auditory sensitivity12,13,14.

**References**


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**Vulnerability to Acoustic Trauma in TRPV1 Mice**
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