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TITLE: Accelerating Translation of Estrogen Signaling as a Treatment for Noise-Induced Hidden Hearing Loss in Both Sexes

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14. ABSTRACT

The objective of the proposed project, focused on noise induced hidden hearing loss (NIHHL), is to accelerate translation of biological repair mechanisms following acoustic trauma into therapies that treat auditory system injury and restore auditory function. We plan to test the efficacy of drugs that modulate the estrogen pathway (using estrogen, DPN and Raloxifene) and the mevalonate pathway (using metformin and statins) to prevent/treat NIHHL.

15. SUBJECT TERMS
Noise-induced hidden hearing loss, estrogen signaling, mevalonate signaling, synapses, single nucleus RNA-sequencing

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1. Introduction

Noise-induced hidden hearing loss (NIHHL) is a major health concern for the Department of Defense. The results of our previously funded CDMRP grant to study the cell type-specific molecular basis of noise-induced hearing loss (MR130240) identified critical sex differences in the response to noise involving the estrogen receptor 2 (ESR2), and identified the mevalonate pathway, which is targeted by the widely used FDA-approved drugs statins and metformin, as upregulated in the response to noise. Therefore, the objective of the proposed project is to 1) determine if activation of ESR2-mediated signaling can prevent/treat noise induced-hidden hearing loss (NIHHL) in intact female mice by testing estradiol (E2), DPN (an ESR2-specific agonist) and raloxifene, an FDA-approved selective estrogen receptor modulator (SERM) that functions in neural tissues as an ESR-2 agonist; 2) to determine if ESR2-agonists, statins, and metformin— alone or in combination— can prevent/treat NIHHL in intact males and females; and 3) uncover the transcriptional underpinnings of the otoprotective effects of these treatments.

2. Keywords

Noise-induced hidden hearing loss, estrogen signaling, mevalonate signaling, synaptopathy, single nucleus RNA-sequencing.

3. Accomplishments

Major goals of the project and their accomplishments:

As outlined in the proposal, we used a temporary threshold shift (TTS) inducing noise paradigm (8-16 kHz at 97dB SPL for 2 hours) and evaluated (a) ABR threshold shifts (**Figure 1**); (b) ABR wave-I amplitude as a proxy to evaluate cochlear synaptopathy (**Figure 2**); (c) Distortion Product Otoacoustic Emissions (DPOAE) threshold shifts as a proxy for outer hair cell function (**Figure 3**); and (d) histological analysis of the procured cochlear tissue for outer hair cell and inner hair cell synapse counts (**Figure 4**).

Specific Aim 1: To determine if ESR2-mediated signaling can be used to prevent/treat NIHHL in intact female mice.

Major Task 1: To establish the use of DPN and E2 as a treatment for NIHHL in female mice. Progress by subtasks:

Subtask 1: Obtain ACURO approval following UMSOM IACUC approval –these approvals were obtained on August 4th, 2021.

Subtask 2: Functional testing of DPN and E2 as therapeutics for NIHHL.

Subtask 2a: First group of 18 females: B6CBAF1/J female mice: n=6 placebo, n=6 E2, n=6 DPN – **Outcome for this subtask was submitted in year 1 progress report**

Subtask 2b: Second group of 18 females: B6CBAF1/J female mice: n=6 placebo, n=6 E2, n=6 DPN – **Outcome for this subtask was submitted in year 1 progress report**

Subtask 2c: Third group of 18 females: B6CBAF1/J female mice: n=6 placebo, n=6 E2, n=6 DPN – **Outcome for this subtask was submitted in year 1 progress report**

Subtask 3: Histological experiments and analysis – **Complete** / **Outcome for this subtask was submitted in year 1 progress report. Additionally, see Figure 5 in the Changes/Problem section.**

Major Task 2: To establish the use of Raloxifene as a treatment for NIHHL in female mice:

Subtask 1: Obtain ACURO approval following UMSOM IACUC approval – **Complete**, **these approvals were obtained on August 4th, 2021, and August 1st, 2022**

Subtask 2: Functional testing of Raloxifene as therapeutic for NIHHL – Group of 36 B6CBAF1/J female mice: n=18 raloxifene, n=18 placebo. (n=18 placebo was approved via an amendment to the original protocol, more details provided in the Changes/Problems section on page 10).

Subtask 2a: First group of 18 B6CBAF1/J female mice: n=9 placebo, n=9 Raloxifene. **Completed in October 2022**

Subtask 2b: Second group of 18 B6CBAF1/J female mice: n=9 placebo, n=9 Raloxifene. **Completed in February 2023**

Figure 1 compares ABR thresholds and threshold shifts between placebo and raloxifene treated groups. In **Figure 1 A**, ABR threshold shifts were smaller in raloxifene treated group when measured 6-weeks after noise exposure. Interestingly, at 6-weeks after noise exposure (subplot on the right), the difference in ABR threshold shifts between placebo and raloxifene treated groups were statistically significant at all measured frequencies.

Figure 1

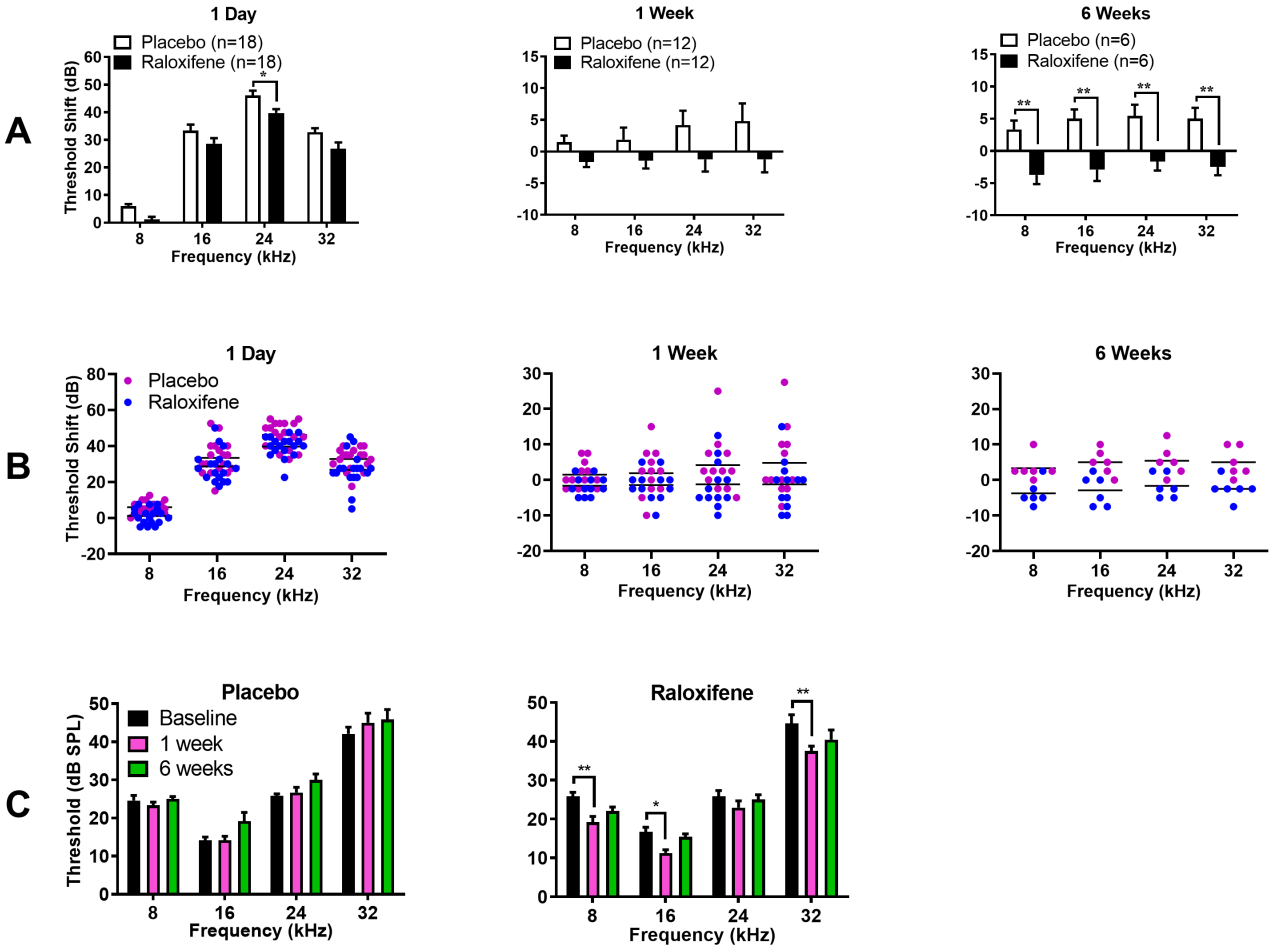

Figure 1 – Raloxifene-treated mice display significantly smaller threshold shifts at all measured frequencies compared to placebo group at 6-weeks post noise exposure. **(A)** ABR threshold shifts following a TTS-inducing noise exposure (97 dB SPL, 8-16 kHz, 2h) at 1 day, 1 week and 6 weeks post noise exposure. **(B)** Individual threshold shifts for placebo and raloxifene treated groups at 1-day, 1-week, and 6-weeks post noise exposures are shown in these three subplots. **(C)** Compares ABR thresholds at baseline, 1-week, and 6-weeks post noise exposure in placebo and raloxifene-treated groups. (ABR threshold data analyzed using 2-way ANOVA followed by the Bonferroni post-hoc test for multiple comparisons; ABR thresholds/threshold shifts in (A, C): mean ± SEM; *p<0.05; **p<0.01; ***p<0.001.)

Figure 2 shows that for placebo group (left column), there exists a significant reduction in wave-1 amplitude at 16 and 24 kHz at both 1-week and 6-weeks post noise exposure.

For Raloxifene treated mice (right column), there is no significant reduction in wave-1 amplitude at 6-weeks post noise at any measured frequency, however for wave-1 amplitude measured 1-week after noise exposure, there exists a small reduction at 8 and 16 kHz.

Figure 2

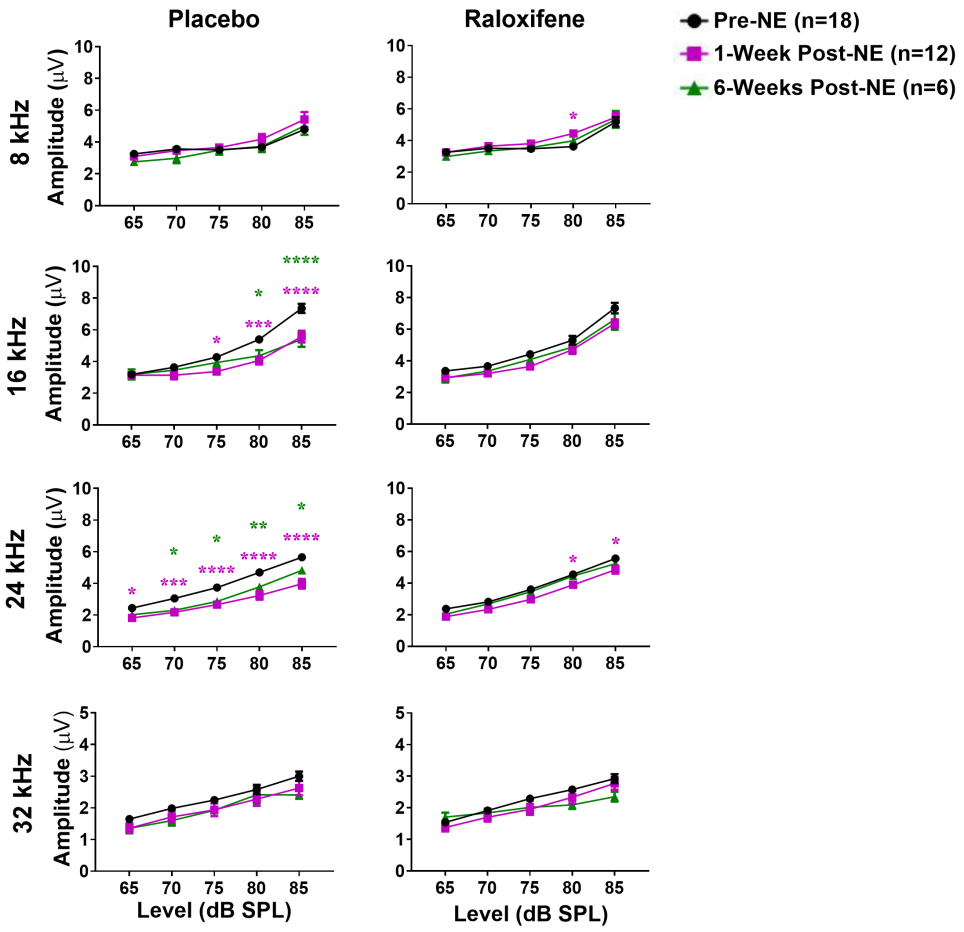

Figure 2 – Raloxifene protects against reduction in wave-1 amplitude after noise exposure (NE= noise exposure; ABR wave-1 data analyzed using 2-way ANOVA followed by the Bonferroni post-hoc test for multiple comparisons; ABR wave-1 amplitudes: mean ± SEM; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.)

Figures 3 A and **3 B** show similar DPOAE threshold shifts and DPOAE thresholds for placebo and Raloxifene-treated mice when measured at the three time points after noise exposure.

Figure 3

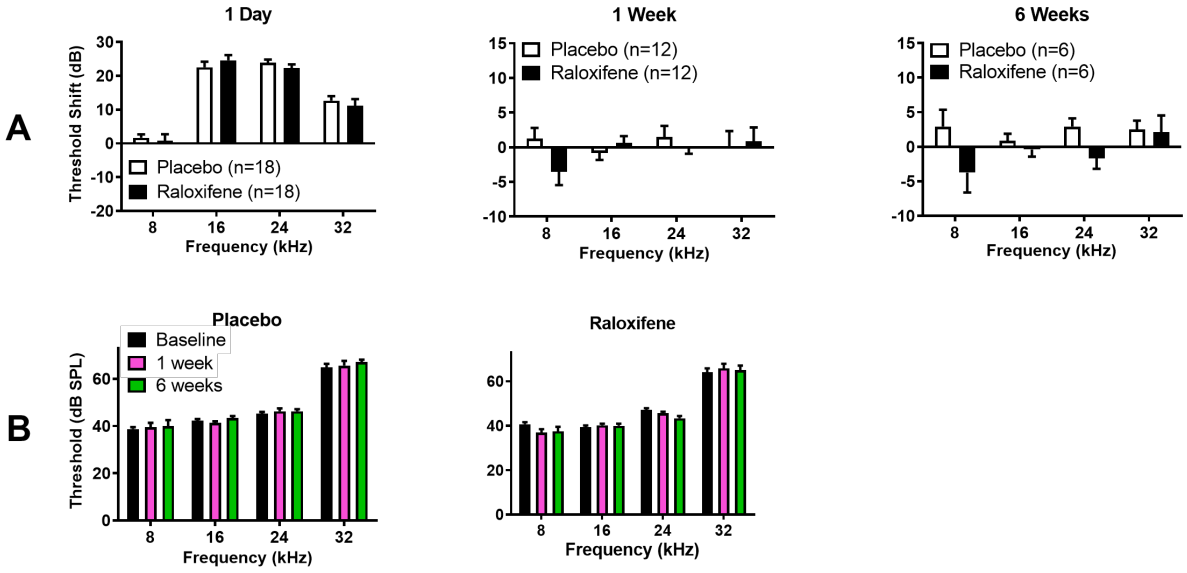

Figure 3 – Raloxifene treatment does not reduce DPOAE threshold shift following a TTS-inducing noise-exposure. **(A)** DPOAE threshold shifts following a TTS-inducing noise exposure at 1 day, 1 week and 6 weeks post noise exposure. **(B)** DPOAE thresholds at baseline, and 1 week and 6 weeks post TTS-inducing noise exposure.

Subtask 3: Histological experiments and analysis

All tissue for cohort 1 has been processed and analyzed. 90% of the tissue for cohort 2 has been processed and 60% has been analyzed. Cochlear ducts were immunostained with antibodies directed against a pre-synaptic marker (CTBP2) and a post-synaptic marker (GLUR2). Synapses at inner hair cells (IHC) are considered active if CTBP2 and GLUR2 co-localize. **Figure 4** shows the number of active synapses per IHC at 1-day, 1-week, and 6-weeks after noise with or without Raloxifene treatment (all tissue analyzed is included in the figures). Our results show that our noise paradigm leads to a loss of synapses at 24 kHz and 32 kHz. Additionally, a partial recovery of synapses is detected at 24 kHz and 32 kHz at the 6-week time point. However, there is no significant difference between the vehicle and the raloxifene treated animals.

Figure 4

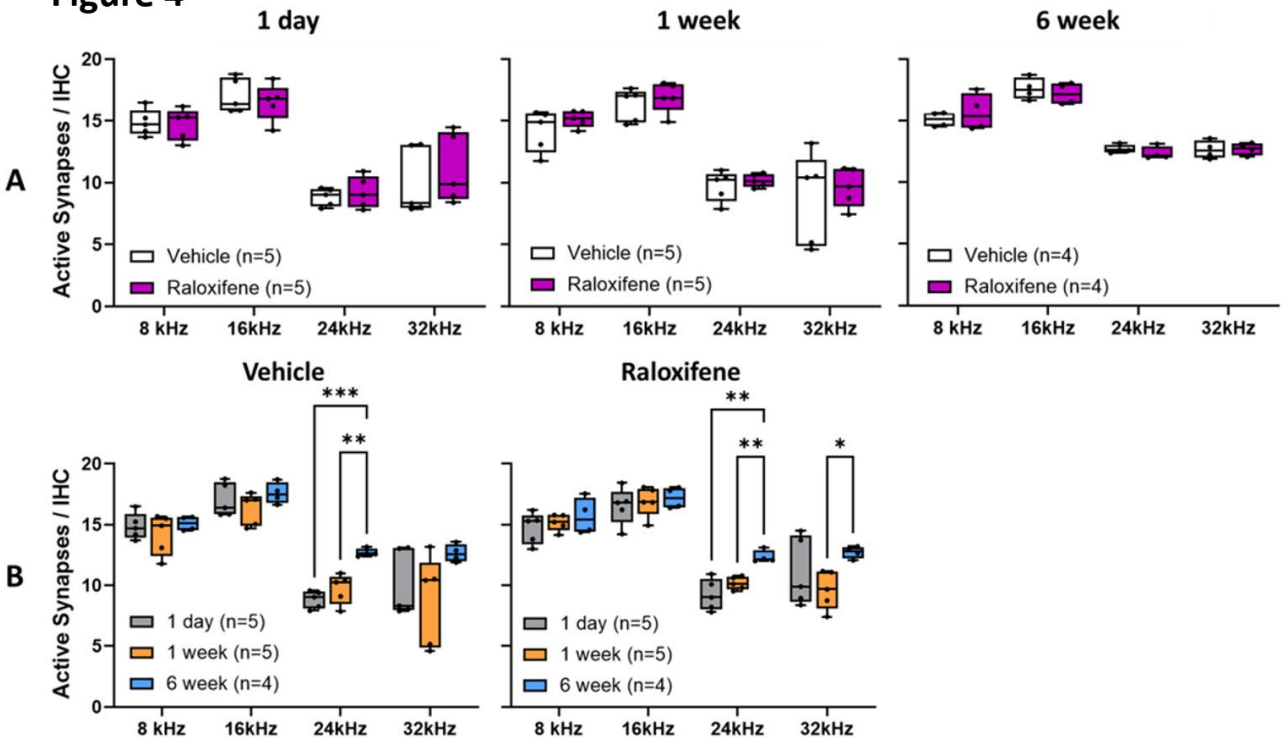

Figure 4 - Active synapses per IHC following noise exposure in mice treated with vehicle or raloxifene. **(A)** Data comparing Vehicle and Raloxifene at the 3 time points show a loss of synapses at 24 kHz and 32 kHz. However, no significant difference is detected between treatments at all time points analyzed. **(B)** Data comparing the time points for each treatment reveal that by 6-week post noise, there is a recovery of synapses at 24 kHz and 32 kHz for all treatments. Error bar – SEM; *p-value<0.05; **p-value<0.01; ***p-value<0.001.

Specific Aim 2: statins and metformin, alone or in combination, with/without ESR2-agonists, as a treatment for NIHHL in mice of both sexes

Major Task 3: Testing pravastatin and metformin alone

Subtask 1: Obtain IACUC and ACURO approval: **These approvals were obtained on August 4th, 2021**

Subtasks 2-3: **Will begin in September 2023**

Major Task 4-5: **Planned for year 3 and 4 (All experiments are delayed by 6-8 months as a result of the team relocation to the NIH. This relocation was discussed and approved by the DOD, operations continue as a sub-award from the University of Maryland, and the team plans to file a 1 year no-cost extension)**

Specific Aim 3: A mechanistic understanding of the otoprotective effects of ESR2-agonists, statins, and metformin signaling on the inner ear hair cells and auditory nerve fibers.

Major Task 6: Tissue collection for scRNA-seq and validation

Subtask 1: Obtain ACURO approval following UMSOM IACUC approval – **Completed**, these approvals were obtained on August 4th, 2021 and January 19th, 2022. **We also obtained**

approval by ACUC of NIH and is pending approval by ACURO. No work reported in this report was performed at NIH.

Subtask 2: Tissue collection from Fbxo2VHC; CBA/CaJ

For this subtask, we optimized the nuclear extraction from spiral ganglion tissue and the organ of Corti of adult Fbxo2$^{VHC/+}$ mice which will be used for Major Task 6. This optimization created a slight delay in tissue collection but was necessary to guarantee successful outcome of specific aim 3.

Subtask 2a – Collecting tissue for placebo (DPN control) at baseline and 24h post noise exposure, Mice of each sex: n=12 sham, n=12 noise – **48% complete - Collected tissue from noise exposed mice: Female n=11 and Male n=12**

Subtask 2b – Collecting tissue for DPN at baseline and 24h post noise exposure, Mice of each sex: n=12 baseline sham, n=12 noise – **37.5% Complete - Collected tissue from noise exposed mice: Female n=10 and Male n=8**

Subtasks 2c-e – **Collecting tissue for these Subtasks will begin in September 2023.**

Major Tasks 7 to 9: **Planned for year 3 and 4 (All experiments are delayed by 6-8 months as a result of the team relocation to the NIH. This relocation was discussed and approved by the DOD, operations continue as a sub-award from the University of Maryland, and the team plans to file a 1 year no-cost extension)**

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

Carissa Byrd was taught by Dr. Beatrice Milon how to perform fine dissection of cochlea.

Our abstract showing our findings in this project has been accepted for presentation at the Society for Neuroscience 2023 meeting in Washington, DC.

How were the results disseminated to communities of interest?

Our team presented our results at Association for Research in Otolaryngology (ARO) midwinter meeting in February 2023 in Orlando, Florida, National Institute for Deafness and Other Communication Disorder (NIDCD) DIR Retreat event in May 2023 in Bethesda, Maryland, and Modeling Hearing and Balance Disorders in Mice in September 2022 in Bar Harbor, Maine.

Our team will present our findings at the Society for Neuroscience (SfN) meeting on November 15th, 2023, in Washington, DC.

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

Our main goals for the next reporting period will be:

Specific Aim 2, Major Task 3, Subtasks 1-3. To determine if Metformin and Pravastatin can independently protect against NIHHL in intact female and male mice.

Specific Aim 3, Major Task 6, Subtasks 1 and 2. Collecting tissue from sham (non-noise exposed) and noise exposed subjects from the Fbxo2VHC;C57BL/6 mice treated with a placebo, DPN, pravastatin and metformin.

4. Impact

Nothing to report.

5. Changes/Problems

Changes in approach and reasons for change

Changes already approved by ACUC at NIH and pending approval by ACURO

We submitted an amendment to ACUC from NIH requesting 24 additional Fbxo2VHC;C57BL/6J to confirm that our noise exposure also results in a TTS in this strain. The amendment was approved by ACUC on August 10th, 2023, and was submitted to ACURO on August 16th, 2023.

Problems and opportunities

When we were completing Specific Aim 1, Major task 1, Subtask 2b, we unintentionally noise exposed 18 B6CBAF1/J Female mice to a lower intensity noise level (93 dB SPL instead of the target 97 dB SPL), due to a faulty calibration microphone. Interestingly, 1-week after noise exposure, 93 dB SPL noise led to significant higher number (or recovery) of synapses in both E2 and DPN treated subjects (**Figure 1B**, right subplot), at the same time no recovery in number of synapses occurred after 97 dB SPL noise exposure (**Figure 1A**, right subplot). The physiological data from both exposure intensities resulted in similar conclusions, that E2 and DPN treatment reduce threshold shifts following a TTS-inducing noise exposure.

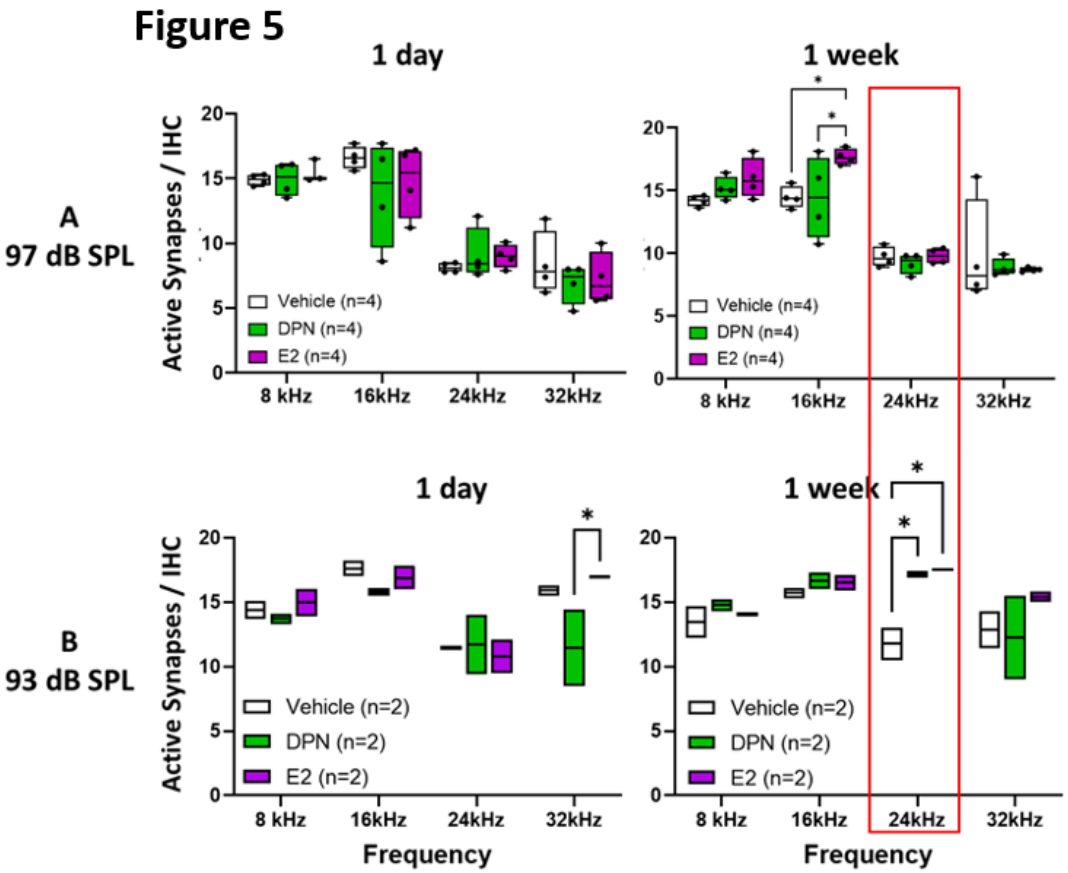

Figure 5 - Active synapses per IHC following noise exposure in mice treated with vehicle, DPN, or E2. Data separated by treatment, **(A)** The 97 dB SPL noise exposure resulted in no recovery of IHC synapses between the 1-day and 1-week post noise exposure time points. No difference in loss of synapses was observed between treated and placebo subjects. **(B)** 1-day after the 93 dB SPL noise exposure, a similar loss of synapses was observed for all groups. However, a recovery in number of synapses was observed within treated groups at 24 kHz. This difference between treated and placebo groups was statistically significant. Error bar – SEM; *p-value<0.05; **p-value< 0.01; ***p-value<0.001.

Based on these interesting results, we will consider submitting an amendment to request additional animals to increase the number of subjects (currently n=2) and repeat the exposure at 93 dB SPL to confirm the results.

Expected changes

As explained earlier, we expect a longer time line and will request a no-cost extension.

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

None

Changes that had a significant impact on expenditures

Nothing to report.

Significant changes in use or care of human subjects

Not applicable.

Significant changes in use or care of vertebrate animals.

One amendment to the animal protocol was submitted and pending approval by ACURO (listed above).

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. Products

Publications, conference papers, and presentations

Publication

Kennedy, C. L.*, Shuster, B.*, Amanipour, R., Milon, B., Patel, P., Elkon, R., Hertzano, R. (2023). “Metformin Protects Against Noise-Induced Hearing Loss in Male Mice.” Otology & Neurotology. Accepted.

Presentations at conferences:

Amanipour R, Shuster B, Milon B, Hertzano R. Estrogen Receptor 2 Agonists Ameliorate Noise-Induced Hidden Hearing Loss in Gonadally Intact Female Mice. Association for Research in Otolaryngology, 46th Annual MidWinter Meeting, February 11-15, 2023

Shuster B, Kennedy C, Amanipour R, Milon B, Patel P, Elkon R, Hertzano R. Metformin Protects Male but Not Female Mice Against Noise-Induced Hearing Loss. Association for Research in Otolaryngology, 46th Annual MidWinter Meeting, February 11-15, 2023

Kennedy C, Shuster B, Amanipour R, Milon B, Patel P, Elkon R, Hertzano R. Metformin Protects Male but Not Female Mice Against Noise-Induced Hearing Loss. American Otological Society, 156th Annual Meeting, May 6-7, 2023

Our team presented the outcomes of our project at the NIDCD Division of Intramural Research (DIR) at NIH campus in Bethesda, MD. May 10-11, 2023.

Secondary outcomes: publications acknowledging DoD support as the PI and laboratory were supported by the DoD during this time

Reavis KM, Bisgaard N, Canlon B, Dubno JR, Frisina RD, Hertzano R, Humes LE, Mick P, Phillips NA, Pichora-Fuller MK, Shuster B, Singh G. Sex-Linked Biology and Gender-Related Research Is Essential to Advancing Hearing Health. Ear Hear. 2023 Jan-Feb 01;44(1):10-27. doi: 10.1097/AUD.0000000000001291. Epub 2022 Nov 17. PMID: 36384870; PMCID: PMC10234332.

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

7. Participants & Other Collaborating Organizations

What individuals have worked on the project?

Name:	*Jessica Mong*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	*0000-0002-6992-6288*
Nearest person month worked:	*1.8*
Contribution to Project:	*Intellectual contributions; data interpretations; experimental design*
Funding Support:	*NIH, R01, HL129138; NIH, T32, NS063391*

Name:	*Ronna Hertzano*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	*0000-0002-8093-6567*
Nearest person month worked:	*1.86*
Contribution to Project:	*Overall responsibility for the proposal and all aspects of the research program including: hiring and training personnel, ensuring quality of data, interpretation of data, oversight of methods, administrative responsibility and reporting to the DoD.*
Funding Support:	*Hearing Health Foundation; Binational Scientific Foundation Israeli American; Intramural Program at the National Institute on Deafness and Other Communication Disorders DC000094-01*

Name:	*Reza Amanipour*
Project Role:	*Postdoctoral Fellow*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*12*
Contribution to Project:	*Animal care, noise exposures, auditory brainstem responses, distortion product otoacoustic emissions, tissue collections, data analysis*
Funding Support:	

Name:	*Beatrice Milon*
Project Role:	*Staff Scientist*
Researcher Identifier (e.g. ORCID ID):	*0000-0001-9208-9134*
Nearest person month worked:	*4*
Contribution to Project:	*Animal care, animal protocols/amendments, immunostaining, synapse counts, cytocochleograms, data analysis*
Funding Support:	

Name:	*Benjamin Shuster*
Project Role:	*Biologist*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*3*
Contribution to Project:	*Animal surgeries, animal protocols/amendments, tissue collections, histology, data analysis*
Funding Support:	

Name:	*Carissa Byrd*
Project Role:	*Student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*4*
Contribution to Project:	*Assists with histology*
Funding Support:	

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

The PI is also supported by a Binational Scientific Foundation Israeli American grant and the Intramural Program at the National Institute on Deafness and Other Communication Disorders DC000094-01. This does not conflict with the current project.

What other organizations were involved as partners?

8. Special Reporting Requirements

COLLABORATIVE AWARDS

QUAD CHARTS

9. Appendices