AWARD NUMBER:  W81XWH-14-1-0500

TITLE:  Towards a Molecular Understanding of Noise-Induced Hearing Loss

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The purpose of this project is to generate the cell type-specific molecular blueprint of changes in gene expression following different types of noise exposure and their treatments, in the inner ear. To this end, we have (a) Established the hair cell and supporting cell-specific transcriptome of adult mouse inner ears; (b) Established the molecular changes induced by PTS-resulting noise exposure in hair cells, support cells and whole inner ears, 6 and 24 hours after noise exposure; (c) Collected and processed most of the tissue for TTS-resulting noise exposure. In addition, we have two manuscripts in preparation describing findings that arose as sub-projects of the main proposal.
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1. INTRODUCTION

Noise induced hearing loss (NIHL) is a major health concern for the Department of Defense. Noise exposure often is inevitable, and may result in a permanent loss of hearing. Unfortunately, there are no treatments to prevent or reverse NIHL. As a first step towards designing targeted therapeutics, we suggested to generate mouse models which allow cell type-specific translatome analysis in the ear.

These, in turn, will be used to analyze the genes expressed in the hair cells (HC) and supporting cells (SC) of adult mice before and after different types of noise exposure as well as pre-conditioning treatments, which in mice, can ameliorate NIHL. Here we report our progress over the second year of the project, in which (a) HC and SC from adult mice were sequenced and their expression patterns were compared; (b) we established the molecular cell type-specific blueprint following PTS-inducing noise exposure; (c) we developed, tested and compared protocols for sequencing low input translomes (manuscript in preparation); (d) we identified critical differences in the response of male and female mice to noise exposure that are likely to have important implications in future study designs for NIHL (manuscript in preparation).

2. KEYWORDS

Permanent threshold shift, Temporary threshold shift, Noise induced hearing loss, Ribotag, RNA-seq, Hair cell, Supporting cell, SAHA, Heat shock, Sex differences.

3. OVERALL PROJECT SUMMARY

1. Specific Aim 1: To determine the OHC- and SC-specific transcriptional and signaling cascades activated in vivo in response to PTS-inducing noise exposure

   a. Major Task 1: To establish the OHC- and SC-specific translatome of adult mouse inner ears. Progress by subtasks:

   i. Obtain ACURO approval following UMSOM IACUC approval – complete.

   ii. Mouse crosses and tissue harvesting – complete.

   iii. Tissue processing – complete, polysome IP – complete, submission of samples for RiboTag-seq – complete; RiboTag-seq – complete.

   Of note, calibration experiments showed that our standard RNA-seq protocol using the NuGEN Ovation kit resulted in over-representation of introns. Standard RNA-seq protocols
would have required substantially larger amounts of RNA than we could obtained from our inner ear translatomes. We therefore developed modified protocols for RNA-seq using the TruSeq and NEB kits and performed a comparative analysis of these modified protocols as well as the NuGEN and Clontech SMARTer Stranded Total RNA-Seq Kit (designed for <10ng of total RNA). The results of our comparative analysis show that our newly revised protocols for NEB and TruSeq perform very well and generate expression profiles that are similar to each other. Conversely, the NuGEN and Clontech had poor performance metrics (Figures 1 and 2). These data are now being prepared for submission as a manuscript.
Figure 1: Hierarchical clustering of expression levels obtained using the four different kits is shown with a dendrogram. The dendrogram represents Pearson correlation coefficients between pairs of samples. The results show that all input and immunoprecipitated (IP) samples from NEB and TruSeq processed kits clustered together, with a clear distinction between input and IP samples, as expected. The NuGEN samples clustered separately, with a good distinction between input and IP. Finally, the Clontech samples clustered separately and a clear separation between input and IP could not be obtained. IP1-3: ribotag-IP, input1-3: total RNA, SMT: SMARTer, NEB: NEBNext® Ultra™, NuG: NuGEN Ovation® Ultralow System v2, Tru4: TruSeq using 4ng RNA, Tru70: TruSeq using 70ng RNA. Red: SMARTer samples (Clontech), blue: NEB samples, yellow: NuGEN samples, orange and pink: TruSeq.

Figure 2. Enrichment analysis. In sequencing of translatome samples from RiboTag mice, the ability to measure enrichment of cell type-specific samples is critical for the analysis of the sequenced material. This analysis shows that TruSeq and NEB processed samples detect
the best enrichment and are most suited for sequencing of translatome from RiboTag mice, while the Clontech processed samples have a limited dynamic range for enrichment. Specifically, the plot shows the enrichment of transcripts with ratio of IP/Input >2. X axis represents transcripts; Y axis represents the log2 value of enrichment (IP/Input). The features with enrichment or depletion number greater than 2 were used in this plot and sorted based on the value of TruSeq with 70ng of RNA. Dotted lines: ribotag-IP. Solid lines: input samples.

iv. Data analysis – we have completed the alignment and enrichment analysis for the OHC and SC-specific translatome. We identified 410 genes with a significantly higher expression in outer hair cells (OHC) and 282 genes with a significantly higher expression in supporting cells (SC). We have further analyzed these genes informatically for functional groups and pathways, and are in the process of selecting genes for validation.

v. Validation experiments – polysome IP to be used for RT-qPCR – complete. Tissue harvesting for immunohistochemistry and in situ hybridization – complete. Large scale validation method was changed from the planned use of Real Time RT-PCR to NanoString Technology. The latter, allows for validation of a larger number of genes, using less starting material and with greater accuracy and significantly less time investment. However, all genes from all parts of the project need to be validated at the same time to reduce cost. Therefore, we are waiting for the middle of year III before finalizing the gene list (this part was planned in the submitted proposal for months 31-36).

b. **Major Task 2**: To determine the OHC- and SC-specific transcriptional and signaling cascades activated in response to PTS-inducing noise injury. Progress by subtasks:
   i. Mouse crosses, noise exposure, tissue harvesting, histological analysis, ABR and DPOAE measurements. Complete.
   ii. Tissue processing – complete.
   iii. Data analysis – data analysis of the PTS-dataset from an informatics perspective is complete. This analysis identified the major cell type-specific pathways that are activated and inhibited in response to PTS-inducing noise (as described in
progress report year II quarter III). We are now in the process of careful reading, analysis and synthesis of the analysis results to identify key molecules for validation and intervention. In addition, the data will be re-analyzed when the dataset of the TTS-inducing hearing loss will be complete. This will allow us to differentiate between genes that change and have a ‘harmful’ effect from genes that change but are ‘protective’; without being able to make this distinction, the interpretation of any change in gene expression as a result of noise exposure is somewhat speculative.

iv. Validation experiments – polysome IP to be used for RT-qPCR – complete. Tissue harvesting for immunohistochemistry and in situ hybridization - complete. Validation experiments to be performed with NanoString Technology as described above (this part was planned in the submitted proposal for months 31-36).

II. Specific Aim 2: To determine the OHC- and SC-specific signaling cascades activated in vivo in response to otoprotective interventions.

a. This Aim was designed to define the cell type-specific molecular blueprint of interventions that may ameliorate NIHL. Specifically, we planned to characterize TTS-inducing noise exposure, heat shock and restraint stress.

b. TTS-inducing noise exposure: crosses, calibration, validation cytocochleograms, noise exposure, tissue harvesting, polysome IP and RNA extraction was completed both for the OHC and SC crosses. Unfortunately, four (out of 24) of the RNA samples did not meet the necessary quality criteria for sequencing, and additional tissue is being collected. We believe that this was the result of a malfunction of the -80 freezer that contained those samples. We anticipate having all of the necessary tissue and RNA for sequencing by February 2017. Data analysis and validation will continue in the third year of the grant.

c. Heat shock: we modified the originally proposed heat shock protocol (which was based on historically published heat shock experiments in the literature) to a ‘modern’ approach using an Infra-red heating pad connected with a feedback controlled heating system (Kent Scientific, CT) set to raise body temperature to 42°C. Mouse and pad temperatures in this setup are monitored constantly using separate probes. Control
mice undergo the same treatment but with the temperature set at 37°C. We tested the efficacy of heat shock as a protective intervention for NIHL on 10 week old BL6CB AF1/J male mice that were exposed to 102.5 dB SPL octave band noise for 2 hours. This testing/calibration was crucial as the strain of our mice was not previously tested for the efficacy of heat shock to protect from NIHL. In addition, we found in year I of our work that some of the published data regarding pre-conditioning treatments, produces results that are not always consistent. We found a statically significant and robust protective effect at 16-24 kHz at all time points tested (24h, 8 and 21 days post exposure) as shown in Figure 3. In addition, we found, as expected, induction of canonical heat shock-protein mRNAs (data not shown).

![Figure 3](image.png)

**Figure 3. Protective effect of heat shock from NIHL.** The graphs show ABR threshold shifts at 24 hours, 8 days and 21 days after exposure to PTS-inducing noise. Mice were either pre-treated with a 15 min 42°C heat shock (red bars) or maintained at 37°C (blue bars). The data show a strong protective effect that is statistically significant at 16 and 24 kHz.

d. **Restraint stress:** In our progress report from last year, we reported that restraint stress and steroids resulted in a significantly lesser than expected protective effect. This year, further discussion with other researchers in the field (Lisa Cunningham, PhD, Charles Liberman, PhD) revealed that the protective effect of restraint stress can be
inconsistent. We therefore decided to not molecularly characterize this third intervention.

e. **SAHA**: In search for a third intervention for molecular characterization, we pursued the testing of SAHA – a histone deacetylase inhibitor, which was published to have a protective effect in the ear from noise, aminoglycoside and cisplatin ototoxicity. Of note, all of the work that has been published for SAHA in mice focused on males only, while current NIH guidelines as well the requested work for this proposal require the use of an equal number of male and female mice. The results of our calibration experiments were surprising. We found that our SAHA treated mice, when analyzed as a group, did not benefit from a protective effect from NIHL (**Figure 5**). However, when male and female mice were analyzed separately, male mice were found to benefit from the treatment, as previously published (**Figure 6**). To determine the reason for the apparent lost efficacy in females we compared the response to PTS-inducing noise exposure between male and female mice (**Figure 7**). We found that male mice have significantly greater threshold shifts, in comparison to female mice as a result of noise exposure (**Figure 7**). To complement our hearing threshold studies, we performed cytocochleograms and synapse counts from the mice included in the study (**Figure 8**, representative image).

Our data show that in the frequencies affected with a measured threshold shift (16 and 24 kHz) there is only minimal (less than 1%) loss of hair cells, and the loss of synapses is limited primarily to the higher frequencies (24 kHz and above). In addition, SAHA did not have a statically significant protective effect on loss of synapses. *Taken together, these data suggest that the effect of SAHA is through changes in hair cell function rather than survival*, and that the differences between the hearing loss in males and female mice are likely secondary to the severity of the injury of the hair cells (although other causes such as changes in endocochlear potential could be considered and future experiments will include DPOAE measurements to determine OHC function). Finally, should SAHA be chosen to be studied molecularly, it would be best studied using male mice only.
Figure 4. Selection of SAHA treatment for prevention of NIHL. Graphs showing threshold shift at 24 hours, 8 days and 15 days after noise exposure (101 dB SPL, 2 hours, octave band) in mice treated with different regimen of SAHA (or DMSO as a vehicle). These preliminary data suggest that injection of SAHA 3 days before and 2 hours after noise exposure is the only regimen resulting in a decrease in threshold shifts (framed in red). n = 4, error bars are SEM.

Figure 5. No difference in threshold shifts between SAHA treated and untreated mice when male and female mice are combined. A box plot graph showing the combined threshold shifts of all animals in response to SAHA versus vehicle at 15 days. No statistically significant differences were measured.
Figure 6. SAHA induction of statistically significant protection from noise trauma at 24 kHz in male mice only. Males and females respond differently to treatment with SAHA; Males have a statistically significant reduction in threshold shift at 24 kHz, while females show similar shifts in both the treatment and control groups (p value = 0.0481).

Figure 7. Control male and female mice have statistically significant different responses to noise exposure. Male mice have significantly worse hearing thresholds 8 days following noise exposure, in comparison to female mice (* p < 0.02, *** p < 0.0001).
Figure 8. Representative images of CtBP2 immunostaining in a non-exposed control mouse (top panel) and in a DMSO injected mouse exposed to 101dB SPL for 2h (bottom panel) in the 32kHz region of the organ of Corti. A decrease in the number of ribbons associated with inner hair cells is seen in noise-exposed mice as compared to control mice. Scale bar is 10um (16.2/IHC in control vs 10.2/IHC in exposed animals).

III. **Specific Aim 3**: To evaluate the OHC and SC-specific signaling and transcriptional cascades affected *in vivo* in mice that underwent prophylactic otoprotection and were subsequently exposed to PTS-inducing noise.

The work described in Aim 2, which was beyond the scope of the grant, set the foundation to be able to appropriately perform this aim. This aim will be performed in year 3 of the proposal.

IV. **Actual or anticipated problems or delays and plan to resolve them**

- An initial delay occurred due to late ACURO approval. As animal experiments started later than originally planned, we hired the post doc for the project only in January of 2015 and plan to continue working on the project 3-9 months following its official completion (year I).
- As a result of the variability in the efficacy of pre-conditioning treatments, we added a calibration and cross-comparison step (year I&II).
- Mice are generated slightly slower than originally planned. To adapt to this change and allow for completion of the project, we decreased the man-power dedicated to the project by 33% to allow for funds to support it for a few months after the end of year III.

- Four out of 24 RNA samples did not meet quality criteria in year II of the project. This delays the sequencing as all samples from an experiment are sequenced together to avoid a batch effect.

V. **Change in approach and reason for change**

- Pre-conditioning treatments were compared and based on efficacy and we decided to not perform a detailed molecular characterization of the response to restraint stress/steroids. We will perform the molecular response to TTS-inducing noise exposure and Heat Shock, as originally planned.

- Testing of SAHA – due to the limited efficacy of restraint stress/steroids, we searched for another intervention that is likely to protect from NIHL through large transcriptional changes. This year we performed calibration experiments and testing of SAHA. We are still deciding whether we would like to pursue a molecular analysis of this intervention.

- Focus on differences in the response to noise between male and female mice. Our calibration experiments for SAHA led us to find that there are profound differences in the response to noise exposure between male and female mice. These were described in the progress report above. We need to decide if to analyze males and females separately, specifically for the Heat Shock and SAHA experiments. Taking this approach will result in a significant increase in cost. Alternatively, sequencing could be done using male mice and validation on both males and females, separately.

- Change in sequencing approach – the original project called for sequencing using the NuGEN kit. Detailed analysis identified multiple deficiencies when taking this approach. We therefore performed a comprehensive analysis and now perform our sequencing using the NEB kit. The analysis will be published as a manuscript to be submitted before the end of 2016.

VI. **Changes that have a significant impact on expenditure**

- Last year we implemented also sequencing of the whole ears and not just HC and SC from each sample and explained the rationale for it. While this results in a much better
understanding of the molecular changes and their origin in the sequenced samples, it does increase the cost. We will probably therefore forgo either sequencing a third intervention or forgo some of the sequencing of the mixed noise+treatment and limit the sequencing to treatment and noise, separately.

- This year, the calibration experiments and identification of significant differences in the response to noise between male and female mice resulted in an increase in animal costs and reagents for histology, confocal analysis and personnel time. The findings, however, are of great significance, both to our project as well as to the ear research community that is focused on studying NIHL. The project, nevertheless, is in good financial health.

VII. Significant changes in biohazards

An amendment was submitted and approved to include SAHA as pre-conditioning treatments.

4. KEY RESEARCH ACCOMPLISHMENTS

* Identification of the adult OHC and SC-specific translatome. This will allow for the identification of new deafness genes and additional cell type-specific markers in the adult mouse inner ear.

* Generated the cell type-specific molecular blueprint of PTS-inducing noise exposure. This will allow for design of targeted therapeutics to prevent and treat NIHL.

* Identification of critical differences in the response to noise between male and female mice. This will result in a change in experiment design both for us but likely for other researchers funded to study NIHL.

* Development of new protocols for sequencing low input translatome. This will allow more researchers in the field to apply the RiboTag mice to study cell type-specific changes using significantly less mice for each biological replicate in their experiments.

5. CONCLUSION

The results of the cell type-specific molecular changes as a result of PTS-inducing noise exposure will set a foundation for rationale design of drugs to treat NIHL. This is critically important for the military, hearing loss and tinnitus are a principal cause for disability in the DoD and to date there are no efficient preventative or curative treatments. Maximal benefit from our molecular
data is pending the TTS-inducing noise exposure dataset, as the latter will allow to distinguish protective changes in gene expression from those that represent a detrimental effect on hearing. In addition, our results demonstrate the feasibility of using cell type-specific molecular changes as a readout in future studies to protect from NIHL. For example, in future studies partial ’rescue’ of hearing loss could be explained in a molecular way, thereby identifying pathways that remain to be counteracted for augmented protection (for example, if of 700 genes changes to be counteracted, 300 are corrected, the common inhibitors of the remaining 400 can be searched). The improved sequencing protocols we designed, calibrated and tested decrease the number of animals needed thereby increasing feasibility and decreasing the cost of future studies using adult inner ears for cell type-specific translatome analysis. Our identified differences in the physiologic response to noise between male and female mice are important, not only for the experimental design of future studies, but as both men and women now serves in the army in positions where they suffer from NIHL, and we should therefore design treatments that will be efficacious in both sexes.

Plans for year III:
- Completion of the TTS-inducing noise trauma and a molecular description of the cell type-specific changes that occur as a result of this noise exposure.
- Detailed comparative analysis of the cell type-specific molecular changes that occur as a result of PTS-inducing noise exposure and TTS-inducing noise exposure.
- A detailed molecular description of the cell type-specific events as a result of heat shock as this treatment has been validated in our hands to be protective of NIHL.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS
   a. Manuscripts and abstracts
      Two manuscripts are currently being prepared for publication.
      Secondary outcomes (publications acknowledging DoD support as the PI and laboratory were supported by the DoD during this time):
      RFX transcription factors are essential for hearing in mice.
b. Presentations

Three abstracts submitted to the ARO of 2017 for presentations.

7. INVENTIONS, PATENTS AND LICENCES

Nothing to report

8. REPORTABLE OUTCOMES

Nothing to report

9. OTHER ACHIEVEMENTS

- New protocols for sequencing low input samples
- The current funding is for work that will set a foundation for studies that will follow a strong rationale design to treat NIHL. We believe that our data, even thus far, set the foundation for designing applications that will focus on testing therapeutics that are based on the results of this project.
- We applied for an equipment grant from the Army and Navy to increase the throughput of our histologic analysis of inner ear tissue for the study of NIHL.

10. REFERENCES

11. APPENDICES

1. TRAINING OR FELLOWSHIP AWARDS:

Training

a. the PI, Ronna Hertzano, participated in a RNA-seq course performed at the NIH. This lead to the identification of new analysis platforms and specifically, familiarity with the Cytoscape environment now used in the laboratory (year I).

b. Sunayana Mitra, PhD and Zachary Margulies, were mentored by the Co-I Didier Depireux on how perform and analyze ABR and DPOAE (year I & II).

c. Yoko Ogawa, PhD, a developmental biologist originally trained in the field of zebrafish, who was hired for the project, was trained by Beatrice Milon, PhD, to perform inner ear dissections, cytocochleograms and immunohistochemistry (year I).

d. Sunayana Mitra, PhD – participated in a pre-conditioning conference: “Preconditioning in Biology and Medicine Mechanisms and Translational Research” April 19-20, 2016. Dr. Mitra carries out the pre-conditioning experiments for the project (year II).
e. Beatrice Milon, PhD – learned to perform cytocochleograms, and synapse counts. Has been training the other members of the team to perform these techniques.

f. Lab meetings – since obtaining funding from the DoD the entire Hertzano laboratory engages in in-depth study of current literature and techniques to study NIHL and has been increasing their knowledge and experience through laboratory meetings and journal clubs. The team has also trained two additional laboratories in the department (laboratories of Drs. Ahmed and Riazuddin) who now focus some of their work on NIHL.

2. Professional Development

All members working on the DoD project participate in laboratory meetings, the translational Auditory and Vestibular research day, the Association for Research in Otolaryngology mid-winter meeting (Beatrice Milon, PhD – last year, all team two years ago, and all members of the team have submitted abstracts for this upcoming meeting).

3. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

Individuals who work on the project

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<th>Ronna Hertzano</th>
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<td>NIH R01, DC013817; Action on Hearing Loss. G65_Bowl; NIH R01, DC003544; Hearing Health Foundation – HRP support for gEAR.</td>
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<td>Oversight of the noise exposure protocols, ABR and DPOAE setup and measurements; Discussion and analysis of Male/Female data.</td>
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<td>MII, Translational Research in Hearing Foundation, Capita foundation, NIH/NIDCR</td>
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4. Changes in the other support of the PI/ other key personnel:

   a. The PI and Beatrice Milon, PhD are now supported also by a NIH grant. This does not conflict with the current project. The PI changed effort from 20% to 15% (year I).

   b. Ran Elkon (Co-I) changed position from a research fellow in the Netherlands Cancer Institute to a Principal Investigator at the Sackler School of Medicine, Tel Aviv University (year I).

   c. Yang Song, PhD, has been working on the project 20% effort (informatics support) but the PI has not been asked to pay for her salary (supported by the institute).
Towards a molecular understanding of noise induced hearing loss

Log number: MR130240

PI: Ronna Hertzano  Org: University of Maryland School of Medicine  Award Amount: $1,500,000

5. QUAD CHART

**Study/Product Aim(s)**
- Identification of the principal cells affected by noise trauma – hair cells versus supporting cells.
- Identification, characterization and validation of the cell type-specific translational changes secondary to noise induced hearing loss.
- Identification of the cell type-specific signaling cascades activated during successful treatment of noise induced hearing loss in mice.
- Define at least two key signaling cascades that could be targeted to develop new therapeutic interventions to prevent and treat noise induced hearing loss.

**Approach**
We established animal models that allow cell type-specific RNA extraction from adult mouse inner ears. We will apply these mice to determine the cell type-specific translational changes at different time points following noise exposure with and without treatment, compared with the baseline translatomes prior to exposure. Identified key regulatory pathways will be validated using real-time RT-PCR.

**Goals/Milestones**
- **CY14-15** Goal – OHC- and SC-specific translatomes at baseline:
  - Identification and characterization of the OHC and SC-specific translatomes
- **CY15-16** Goal – Defining the inner ear translatome in response to noise trauma:
  - Identification of the key signaling cascades initiated by noise trauma
  - Identification of differences in the response of male and female mice to noise
- **CY16-17** Goal – In vivo detection of cell type-specific changes in response to treatment:
  - Identification and characterization of the signaling pathways induced by otoprotective interventions from noise-induced hearing loss
  - Comparison of the cell type-specific molecular changes in response to FKB- and TT90-induced noise exposure

**Budget Expenditure to Date**
- Projected Expenditure: $1,000,000
- Actual Expenditure: $1,019,241