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14. ABSTRACT

Hearing loss and balance disorders are widespread among OIF/OEF veterans as a result of blast-induced damage, yet relatively little is known about the underlying mechanisms of injury. In this project, we are characterizing the effects of blast exposure on auditory and vestibular organs in the inner ear of rodents as well as developing strategies for mitigating or reversing vestibular injury that originates from damage to mechanosensory hair cells. Using a compression driven shock tube, we seek to determine whether exposure to a single blast, repeated blasts, or blunt head trauma or blast in combination with blunt head trauma causes deficits in vestibular function that are matched by damage to the vestibular organs within the mouse inner ear; to determine whether functional and morphological changes within the auditory organs of the mouse inner ear differ after exposure to single blast, repeated blasts, blunt head trauma or blast in combination with blunt head trauma; to determine the cell-type-specific changes in gene expression that occur within auditory supporting cells and hair cells after repeated blast exposure, and to determine whether overexpression of Atoh1, inhibition of Notch signaling, or a combination of the two can induce meaningful hair cell regeneration and/or functional recovery in mouse vestibular organs that have been damaged by exposure to different blast profiles.

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1. Introduction

The devastating consequences of acute blast-induced auditory and vestibular disorders (e.g. hearing loss, tinnitus, and imbalance) often continue and worsen with age and the etiology is largely undefined. A comprehensive understanding of the deleterious effects of blast waves to the structure of the inner ear, and molecular components affected by injury, is essential for the development of the most appropriate therapies for hearing and balance deficits resulting from blast exposure. We hypothesize that loss or damage of hair cells and their connecting neurons is the primary reason for sensorineural auditory and vestibular deficits. Research on inner ear development indicates that overexpression of hair cell-specific transcription factors may convert supporting cells into hair cells in adult organs. We utilize an air-driven shock tube simulation of blast to pre-clinically evaluate blast-induced auditory and vestibular injuries in mice and characterize structural, physiological and molecular changes in the inner ear and brain, and determine whether gene therapy using hair cell transcription factors can be used to induce functional recovery in blast-damaged inner ears.

2. Keywords: Blast, hearing loss, balance disorders, inner ear, cochlea, vestibular, gene expression, RNA sequencing, auditory brainstem response, distortion product otoacoustic emission

3. Accomplishments

- **What were the major goals for the project?**

The major objectives for the project were: (1) to determine whether exposure to a single blast, repeated blasts, or blast in combination with blunt head trauma causes deficits in vestibular function that are matched by damage to the vestibular organs within the mouse inner ear; (2) to determine whether functional and morphological changes within the auditory organs of the mouse inner ear differ after exposure to single blast, repeated blasts, blunt head trauma or blast in combination with blunt head trauma; (3) to determine the cell-type-specific changes in gene expression that occur within auditory supporting cells and hair cells after repeated blast exposure; (4) to determine whether overexpression of hair cell-specific transcription factors in transfected supporting cells can induce meaningful hair cell regeneration and/or functional recovery in mouse vestibular organs that have been damaged by exposure to different blast profiles.

- **Bulleted list of key research accomplishment emanating from this research**

  - We have defined mouse models of blast shockwave- and combination injury-induced auditory and vestibular deficits using an advance blast simulator.
  - We have completed auditory functional assessments longitudinally following blast and combined blast with weight drop injuries for six months.
  - We have completed vestibular functional assessments following exposures to blast and combined blast with weight drop injuries.
  - We improved our tissue preparation methods, resulting in better quality images, more reliable cell counts, and data analysis.

- We continued evaluation of the possibility that the inner hair cells have sustained damage to the neural synapses within spiral ganglion neurons which would impede the transmission of sound to the brain.
- We verified the damage to the tympanic membrane and inner ear from 1 day through 28 days post blast exposures.
- We have performed successfully hair cell and supporting cell sorting, RNA extraction, library preparation and RNA sequencing.
- We discovered the most differential gene expression (DEG) in the cochleae at acute (1 day) and chronic (28 days) phases after blast exposures.
- We have successfully generated the complex transgenic animals required for specific aim 4 and confirmed that we are able to genetically mark supporting cells using tamoxifen.
- We have determined the appropriate dose of the aminoglycoside antibiotic, neomycin, needed to selectively kill the majority of hair cells in the utricle.
- We have confirmed that supporting cells can be transfected with our viral vector containing hair cell-specific transcription factors, and shown that we can keep utricule cultures alive and in good conditions after multiple treatments (tamoxifen, neomycin, virus) for up to 8 days.

o **What was accomplished under these goals?**

During the study period, we have determined the effects of various intensities of a single blast, repeated blasts, and blast in combination with blunt head trauma on auditory and vestibular functional deficits, as well as on morphological changes within the auditory and vestibular organs of the mouse inner ear. We also performed single cell RNA sequencing (scRNA-seq) analyses using transgenic mice to determine the cell-type-specific changes in gene expression that occur within auditory supporting cells and hair cells after repeated blast exposures.

I. Animals and injury models

Animal protocol 16-PN-02 was approved by WRAIR/NMRC IACUC and ACURO. Protocol 1385-16 was approved by NIH IACUC. Mice strains of CBA, B6.129S-Atoh1<tm4.1Hzo> and FVB/N-Tg(GFAPGFP)14Mes were provided by the Jackson Laboritory.

A. **Blast shockwave induced injury.** The blast overpressure was generated by rupture of a PVC fabric membrane positioned in an Advanced Blast Simulator (ABS); a 0.5 ft long compression chamber is separated by this membrane from a 21 ft long transition/expansion test section (Fig 1). The compression chamber is rapidly pressurized with air causing the membrane to rupture at a pressure that is dependent upon its thickness, yielding a supersonic shockwave that impacts the experimental subject. The ABS can reproducibly provide a range of blast flow conditions, including the peak static pressure of 16 psi with a 4 msec positive phase duration illustrated in Fig 2.

Fig 1. Advanced Blast Simulator (ABS)

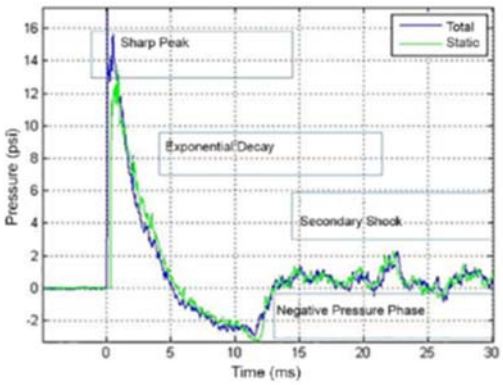


Fig 2. Pressure recording from tip/side gauge

B. Combined blast and weight-drop injury. An airblast exposed mouse (described above) was treated immediately afterwards by a weight-drop to the head as originally described by Marmarou (Marmarou et al. 1994). The injury device consists of a 1 m long Plexiglas tube with a 10 mm inner diameter clamped in a vertical orientation. Isoflurane-anesthetized mice were secured to the foam bed and the tube was positioned directly over the mouse's head. The injury was produced by dropping cylindrical weights of 80 g from a predetermined height of 100 cm. Rebound impact by the weights was prevented by sliding the foam bed and mouse away from the tube immediately after impact.

Eight week old male mice were randomly assigned to experimental groups including single blast exposure (BE1), tightly coupled double (BE2) or triple (BE3) blast exposures, blast combined with weight-drop (BW), noise control (NC) and sham control (SC). Animals were secured in the ABS in a prone position facing the oncoming shockwave, immediately after anesthesia by exposure to 4% isoflurane gas in an induction chamber for 6 min (O2 flow rate 1.5L/min). BE2 and BE3 experimental subjects received the second and/or third blast shockwaves with a 2 min interval, during which time additional isoflurane anesthesia was delivered. Varied blast overpressure intensities (4, 8, 12 or 16 psi) were applied. BW subjects received 16 psi blast exposure plus blunt impact by dropping an 80g weight from 100 cm through a cylindrical column on to the head. NC animals were placed outside of ABS after anesthesia. SC animals were handled in the same fashion, including being subjected to isoflurane anesthesia, but without exposure to blast shockwaves.

II. Effects of blast on auditory system

A. **Hearing loss following blast exposure.** Recordings of distortion product otoacoustic emissions (DPOAE) and auditory brainstem response (ABR) were used to assess auditory function. Mice were anesthetized by intraperitoneal injection of a mixture of Ketamine and Dexdomitor (60 mg/kg and 0.4 mg/kg, respectively). Baseline ABR and DPOAE were recorded at 3 - 5 days before blast treatment. A time-course of blast effects on auditory function was assessed at 1, 7, 14 and 28 days, then each month up to 6 months after blast exposure.

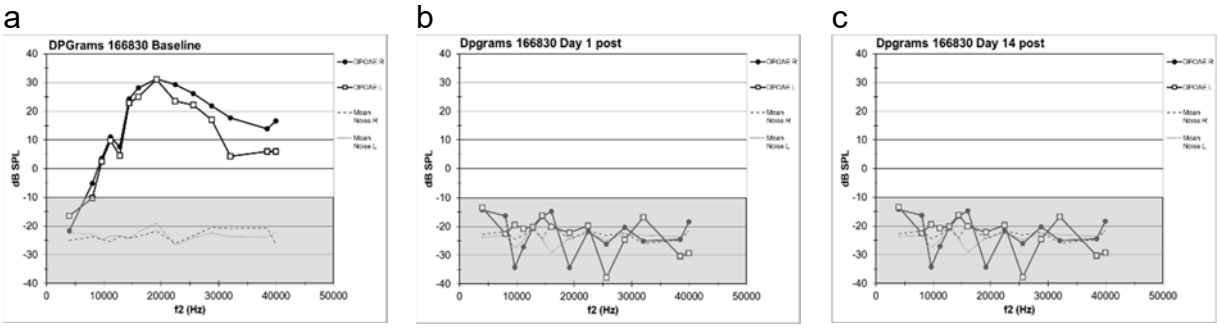


Fig 3. Loss of DPOAE after blast exposure indicated structural injury of inner ear. (a) baseline, (b) 1 day post-injury, (c) 14 days post-injury

DPOAE response to 16 psi blast exposure (Fig 3) showed immediate loss of DP signals that persisted for 14 days. At 3 months post injury, 53% of BE1 mice and 60% of BW mice recovered, whereas no recovery was observed in BE3 mice. At 6 months post exposure, 65% (11) of mice had a DPOAE when tested (Fig. 4). Out of the 11 mice that showed some level of recovery, 7 regained some signal only in one ear and 4 regained DPOAE signal in both ears. The DPOAE signal typically recovered around low-mid frequencies (9 – 16 kHz). Following blast exposure, damage to the inner ear is evident through this loss of a DPOAE. Mice showing restoration of some signal when tested usually showed stronger signals at the low-mid frequency ranges which greatly faded when when tested at higher frequencies. Complete recovery of the DPOAE signal was not seen following exposure to a 16 psi blast. Mice were also exposed to lower pressure single blasts [12psi (n=5), 8psi (n=5), and 4psi (n=4)] which were tested up to two months post-injury. Compared to the 16 psi exposures, these mice showed much greater recovery and responsiveness in DPOAE testing. Throughout these experiments, noise control animals showed no significant difference in responses from those that were recorded in sham animals. Overall, these results reveal intensity-dependent blast damage to the inner ear.

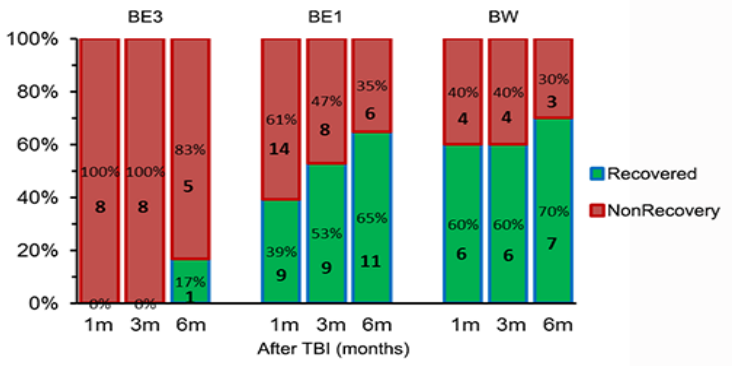


Fig 4. DPOAE changes at 1, 3 and 6 months post blast exposure

ABR results showed significant intensity-dependent reductions in wave amplitudes (Fig 5a) after blast exposure accompanied by elevations in thresholds. At 28 days post blast exposure, ABR thresholds in BE1 mice were significantly elevated in comparison to those of the NC mice. Compared to BE1, ABR responses to 8 KHz and 40 KHz sound stimuli were also significantly altered in the EB3 mice (*$p<0.05$, ***$p<0.0005$, Fig 5b).

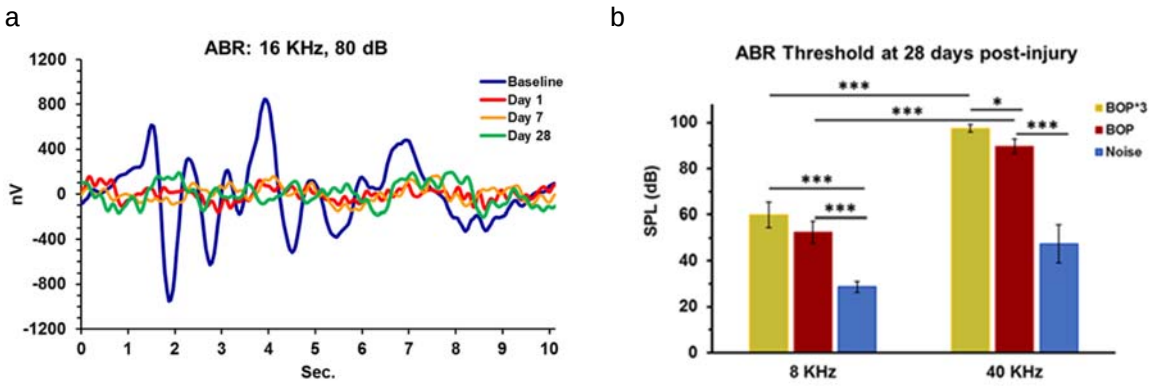


Fig 5. Blast-induced ABR changes. (a) Representative picture of ABR amplitude changes to 16 kHz sound stimulus, (b) ABR threshold changes at 28 days post injury

We also determined a time-course of the effects of various intensities of blast overpressure on ABR threshold changes. As showed in Fig 6, the timing of auditory functional testing ranged from preinjury baseline through 1, 7, 14 and 28 days, as well as through 2, 3, 4, 5 and 6 month after blast exposure. In the acute phase, blast exposure at 16 psi impacted the whole hearing spectrum. Data showed a complete hearing loss at frequencies of 8, 16, 32 and 40 KHz (threshold > 90 dB) at 1 day after exposure to blast overpressure which persisted over several months. Mice receiving 3 repeated blast exposures lost hearing to high frequency sound (32k and 40 kHz) for over six months, and 50% showed partial recovery to low frequency sound (8 kHz) during this timeframe.

Animals exposed to 4 or 8 psi shockwaves displayed a significant increase in ABR threshold to low frequency (8 kHz) stimuli which persisted for a few days, and an increase in ABR threshold to high frequency (40 kHz) which persisted for 14 days. These findings indicated that blast exposure impaired the signal generation and transduction from inner ear through brainstem.

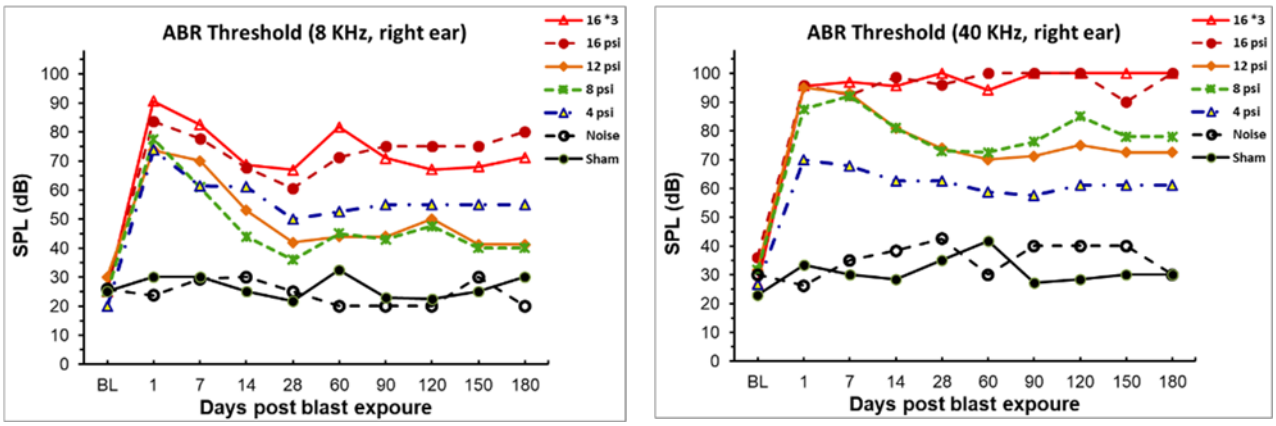


Fig 6. ABRs response to 8 kHz and 40 kHz following various pressure blast exposure.

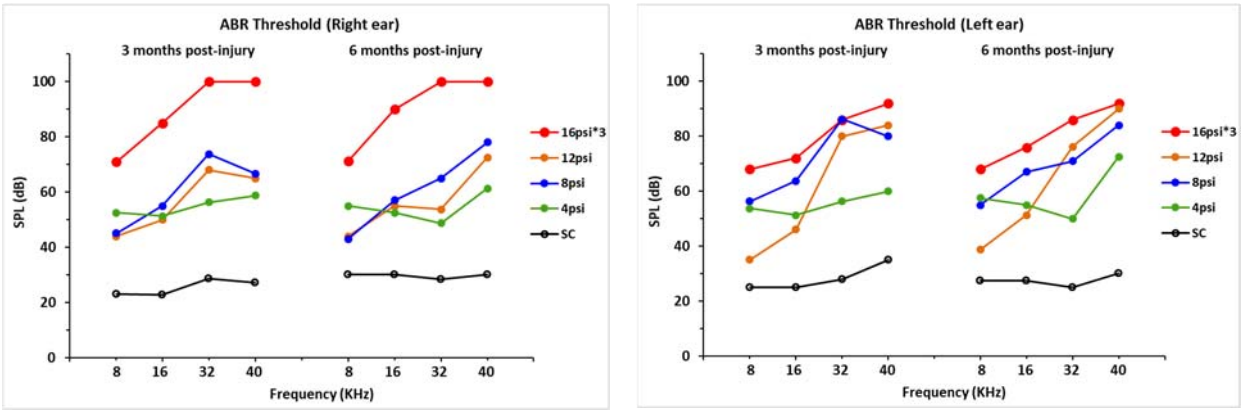


Fig 7. ABR threshold changes at 3 and 6 months after balst exposure.

ABR data analysis revealed that wave I latency changed significantly that was compared to the baseline measurements across experimental groups (Fig 8).

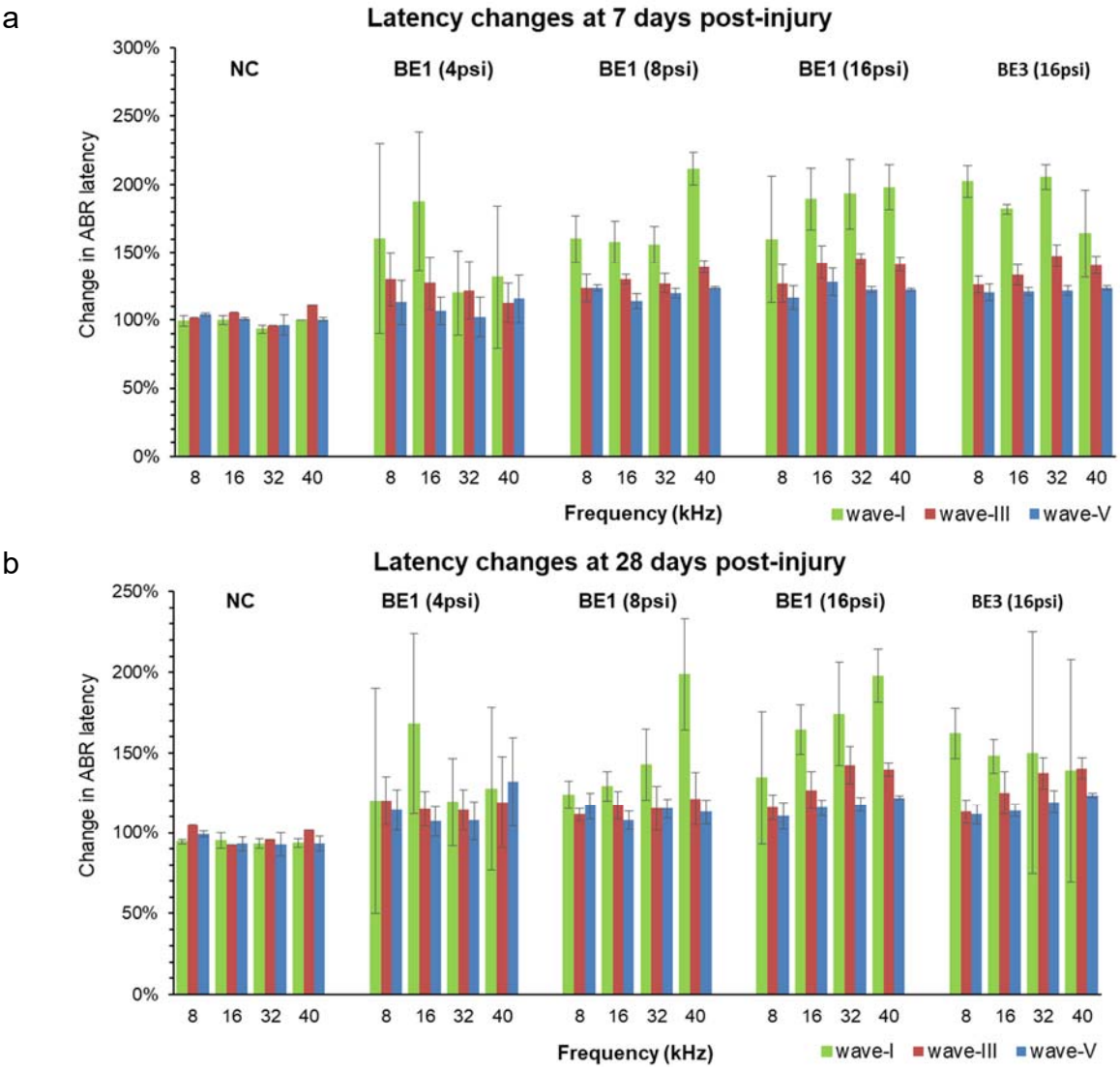


Fig 8. Increase in the latencies of ABR waves I, III and V at 7 days (a) and 28 days (b) after blast exposure

Auditory functional changes following the combined blast with weight drop injury (BW). Longitudinal assessment of ABRs were presented in Fig 9.

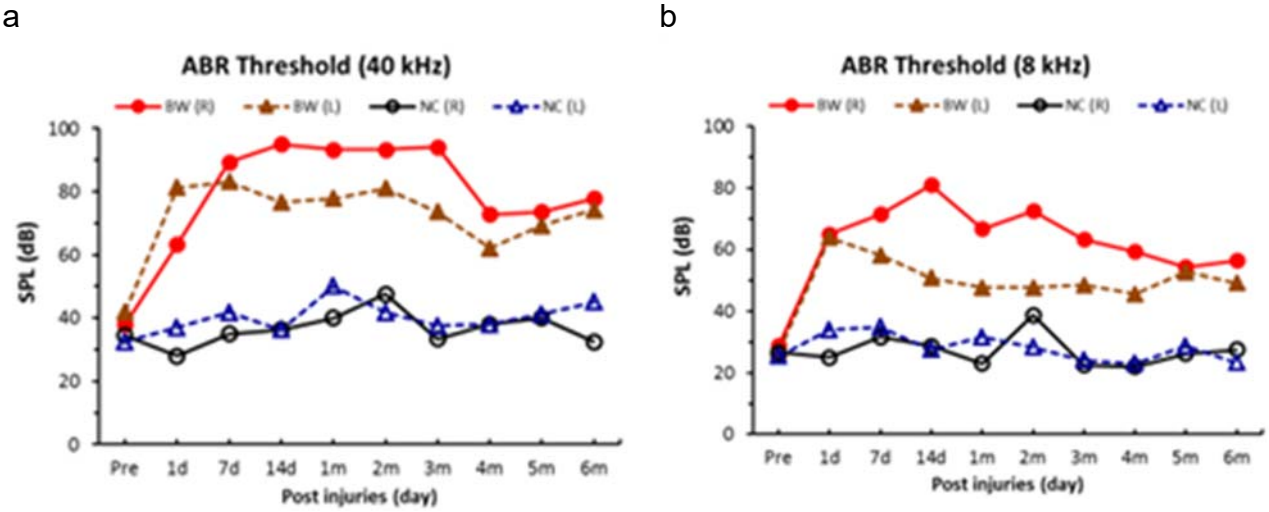


Fig. 9. BW-induced ABR threshold changes to 40 kHz (a) and 8 kHz (b) frequency stimuli

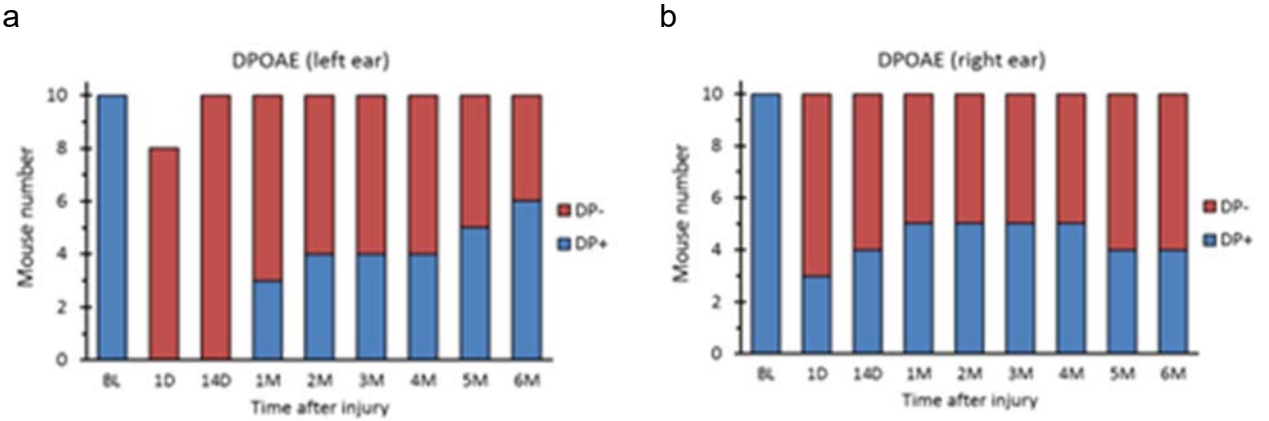


Fig. 10. BW-induced DPOAE changes of left ear (a) right ear (b)

At 6 months after blast and weight drop combination injuries (BW), around 50% of animals (n=10) showed complete loss of high frequency (40 kHz) hearing. The brainstem response to 8 KHz acoustic stimuli showed a limited degree of recovery, but compared to its baseline, ABR threshold remained significantly elevated (Fig 9). Loss of DPOAE was observed in all mice for 14 days after insult. Approximately half of these mice continued to show DPOAE disruptions up to 6 months (Fig 10).

Compared to the BW group, increased ABR thresholds were significantly higher in the 3 repetitive blast exposures (B*3) group after 6 months post-injuries, although there was no distinguishable difference in functional recovery at 3 months post-injuries (Fig 11).

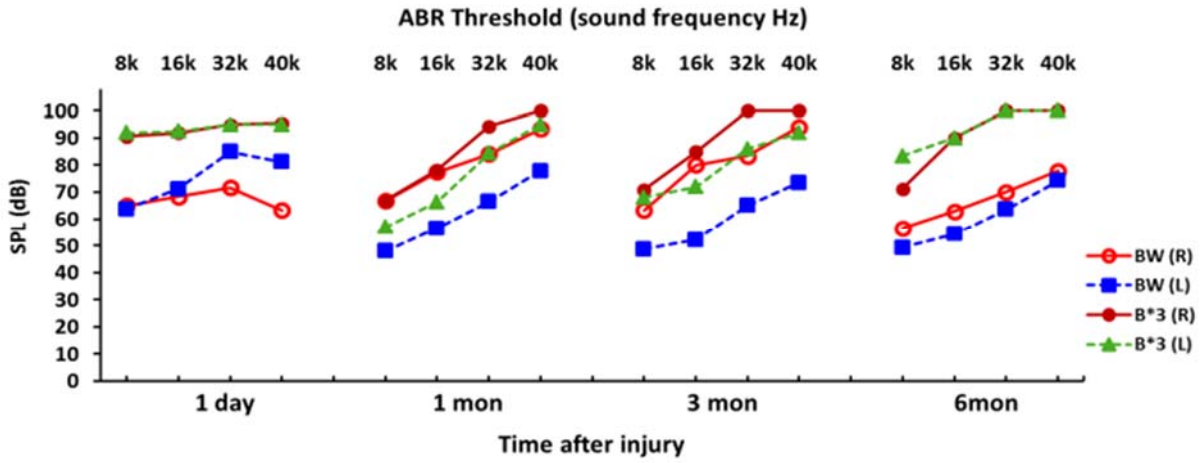


Fig.11. Comparison of ABR threshold change between WB and 3-repetitive blasts

B. **Middle ear structural changes following blast injuries**

The integrity of tympanic membrane (TM) was evaluated using a Sony MCC-500 HD camera attached to a Zeiss Opmi Pico S100 microscope. The TMs of blast-exposed mice showed signs of damage after blast exposure at all time points examined. Tympanic injury was particularly pronounced one and three days after blast, then often showed signs of healing with scarring at later time points. Control TMs were intact and did not show scarring or blood at any time points, while those of blast exposed mice were often perforated with hemorrhage and scarring evident at 2 weeks post injury (Fig 12).

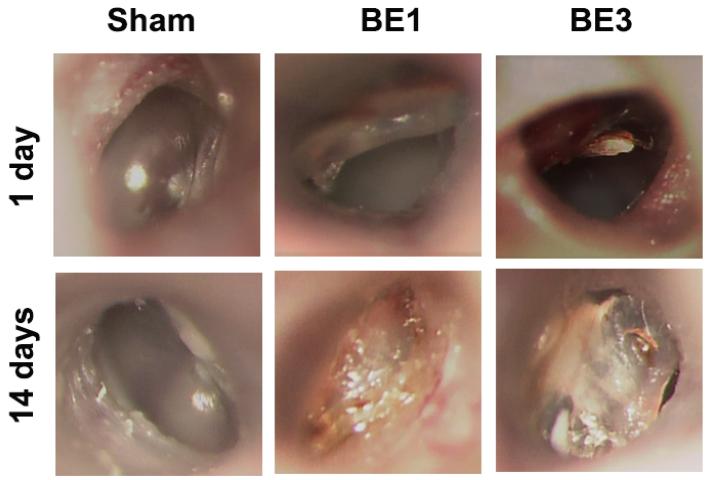


Fig 12. Tympanic membranes of mouse damaged at 1 day and healed at 14 days after blast exposure.

C. **Inner ear morphological changes following blast injuries**

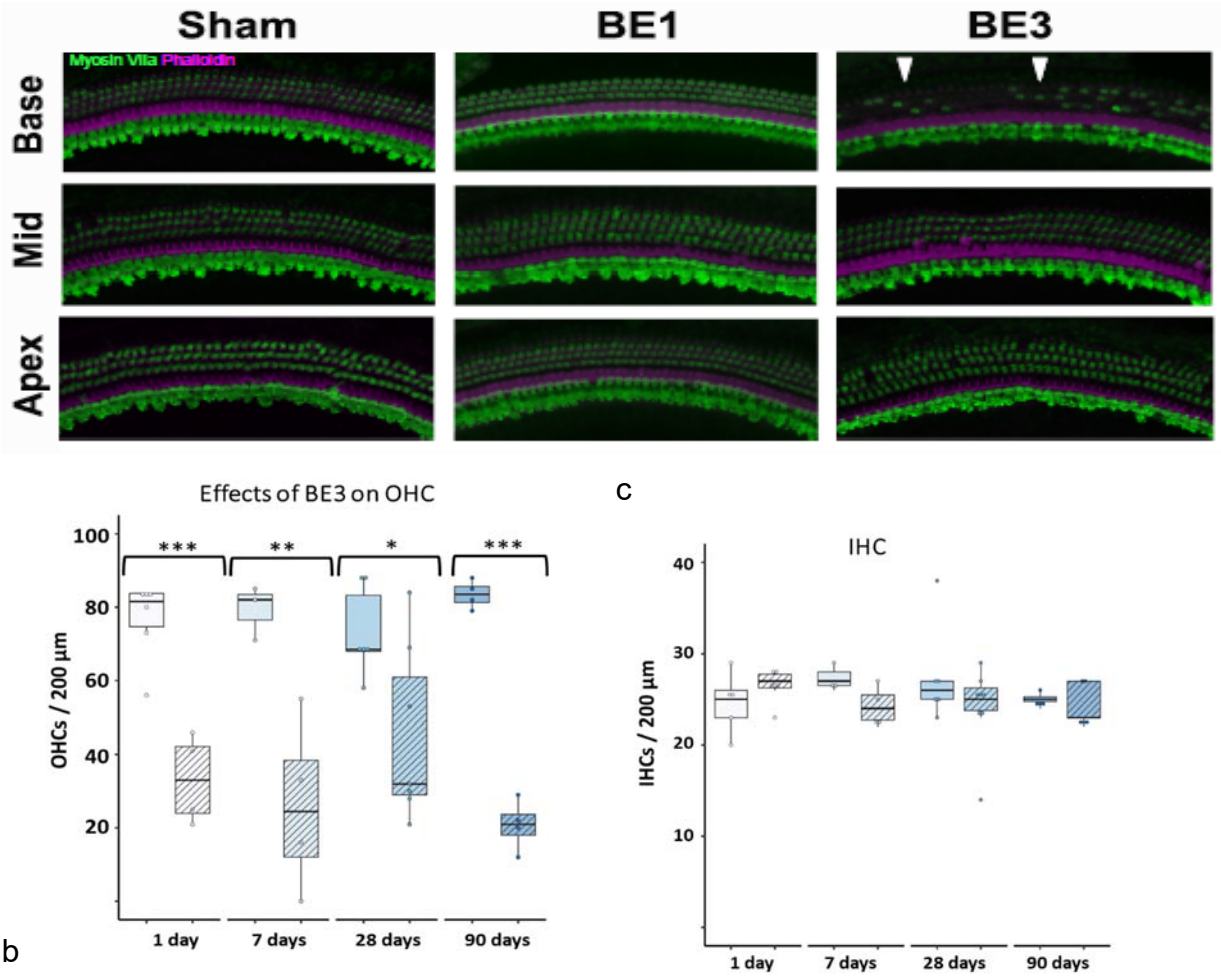
Under deep anesthesia, mice received transcardial perfusion with 4% PFA. A specimen including middle and inner ears was dissected from temporal bone, washed with cold PBS, and immersed in 0.12 M EDTA in 0.1 M PB (pH 7.0) for 7 days while rocking on a platform. With whole-mount sections, cochleae and utricles were dissected from decalcified inner ear for immunostaining.

Impact of cochlear hair cells **(HC)** following blast exposure was evaluated by immunohistochemistry. Briefly, the whole-mount sections were then incubated with the primary antibodies against HCs (MyosinVIIa, Pou4f3) and supporting cells (Sox2), phalloidin, and labeled with AlexaFluor-conjugated secondary antibodies, then mounted in SlowFade, and imaged on a Zeiss LSM 710 microscope. Quantification of

cochlear HC counts was made across a distance of 200 µm. Analysis was completed using ZEN Black (ZEISS Microscopy).

Compared to SC at 1 day, 7 days, 1 and 3 months post-injury, outer hair cells (OHC) in the basal turn of the cochlea of BE3 mice were significantly reduced in number (Fig 13). However, inner hair cells (IHCs) appeared to survive in the apical, middle, and basal turns. The number of IHCs did not differ among the different experimental groups.

A



b

Fig 13. Immunohistrochemistry of cochlear tissue (a) showed OHCs loss (b) and no changes in IHCs (c)

Synaptic integrity was assessed by counting the number of synapses per inner hair cell (C). Dissected cochlear tissue was immunostained with primary antibodies for synaptic markers (Ctpb2 and GluR2) and outer hair cells (Prestin), DAPI, and AlexaFluor-conjugated secondary antibodies. Synaptic counts of Ctpb2 puncta were averaged across 15 inner hair cells (IHCs).

A trend of synapse loss in the cochlear mid and apical turns was observed that may contribute to post-blast hearing loss in the mid and low frequencies (Fig 14). Some synaptic data are still being quantified, so the difference between blast and control samples may become more pronounced after all counts have been made.

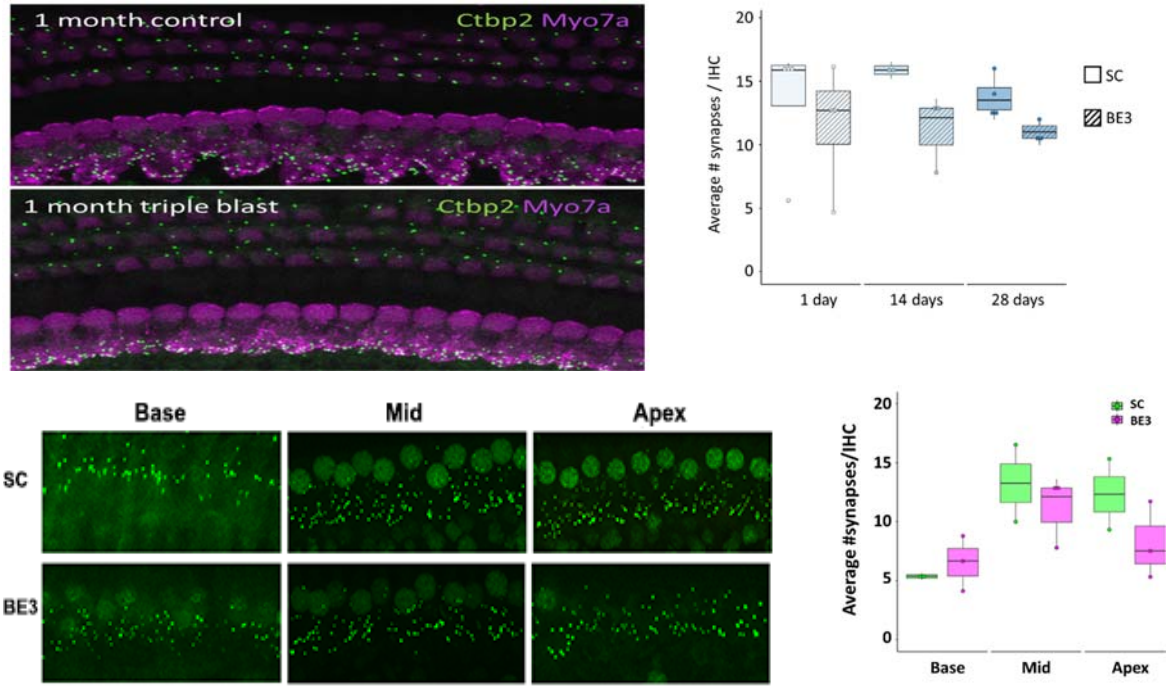


Fig 14. Inner hair cell synapses changes after blast exposures

Evaluation of neurons in spiral ganglia following blast exposure was performed using immunohistochemistry. Plastic embedded sections (4 μm) of inner ear were cut by a Leica microtome and an acidophilic stain was applied (0.05% thionin). Images of the organ of Corti and spiral ganglion pockets were photographed (Fig 15) by a confocal microscope. Spiral ganglion neurons was quantified using Velocity software. Data showed that the density of neurons in the spiral ganglia at 28 days after blast exposure (BE3, 24.4 ±1.43) was decreased in comparison to that of sham controls (SC, 37.46 ±6.65). This observation indicates that the blast shockwave may damage the neural synapses among spiral ganglion neurons which would impede the transmission of sound to the brain.

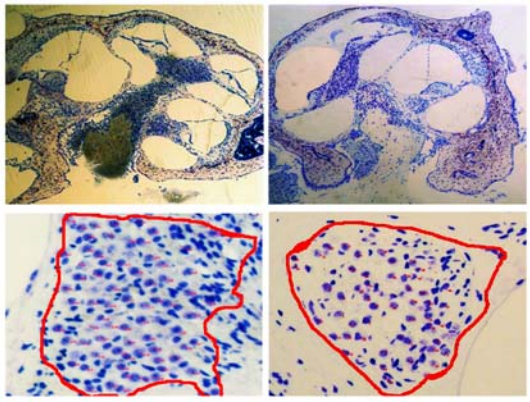


Fig 15. Representative histology of neurona in spiral ganglion.

III. Effects of blast injury on vestibular system

A. Changes in righting reflex time (RRT) following blast exposure The righting reflex is a reflex that corrects the orientation of the body when it is taken out of its normal upright position. It is initiated by the vestibular system, which is composed of inner ear organs forming the "labyrinth", which consists of the semicircular canals, the otoliths, and the cochlea. Sensory information from the vestibular system allows the head to move back into position when disturbed as the rest of the body follows. In our study, RRT can be influenced by conditions of brain and vestibular organs.

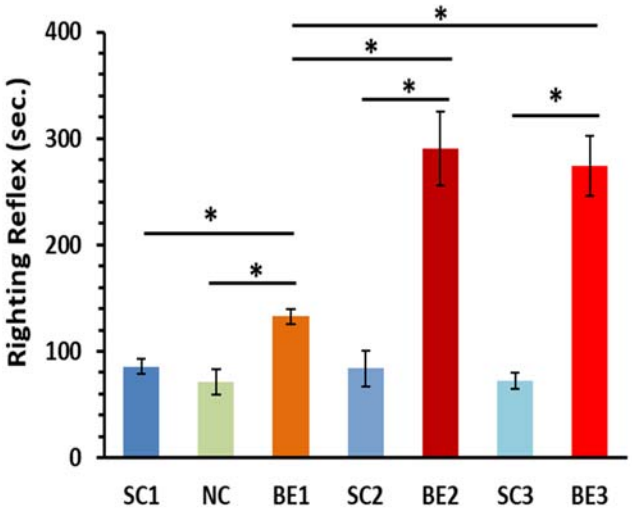


Fig 16. Righting reflex time among experimental groups.

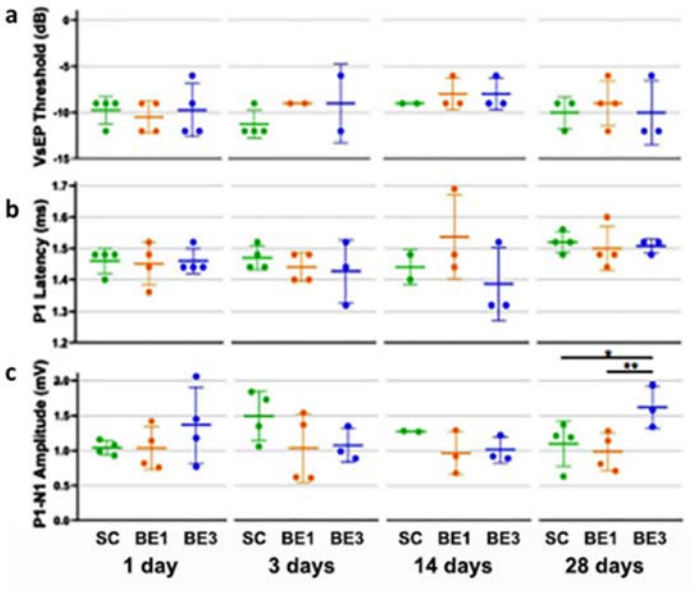


Fig 17. VsEP thresholds (a) and P1 latencies (b) and P1-N1 amplitudes (c) changes after blast exposure.

B. Effects of blast on vestibular functions Vestibular sensory evoked potentials (VsEP) were assessed. Mice were anesthetized by intraperitoneal injection of a mixture of Ketamine and Dexdomitor (60 mg/kg and 0.4 mg/kg, respectively). VsEPs were recorded using stimuli in 3 dB steps from -18 to +6 dB re: 1.0 g/ms and at least two waveforms were obtained at each stimulus level to ensure repeatability of the response. Threshold was defined as the stimulus intensity midway between the lowest stimulus level that produces a visible response waveform and the next stimulus level that produces no visible response waveform. Data (Fig 17) showed that BE3 mice had a significantly larger P1-N1 wave amplitude than SC and BE1 mice at one month post blast exposure. But VsEP thresholds (top panel) and P1 latencies (middle panel) were not significantly different in single or triple-blasted mice compared to control mice at any time point tested.

C. **Morphological evaluation** The utricles of mice were dissected and performed immunohistochemistry after euthanasia. Image analysis was completed using ZEN

Black (ZEISS Microscopy). Quantification of HC counts were averaged across 5 different regions with a total area of 2500 μm². Data (Fig 18) showed no significant difference in an average number of hair cells per unit area between SC and BE3 at any time point examined. The result indicated blast shockwave didn't cause significant loss of hair cells in the utricle.

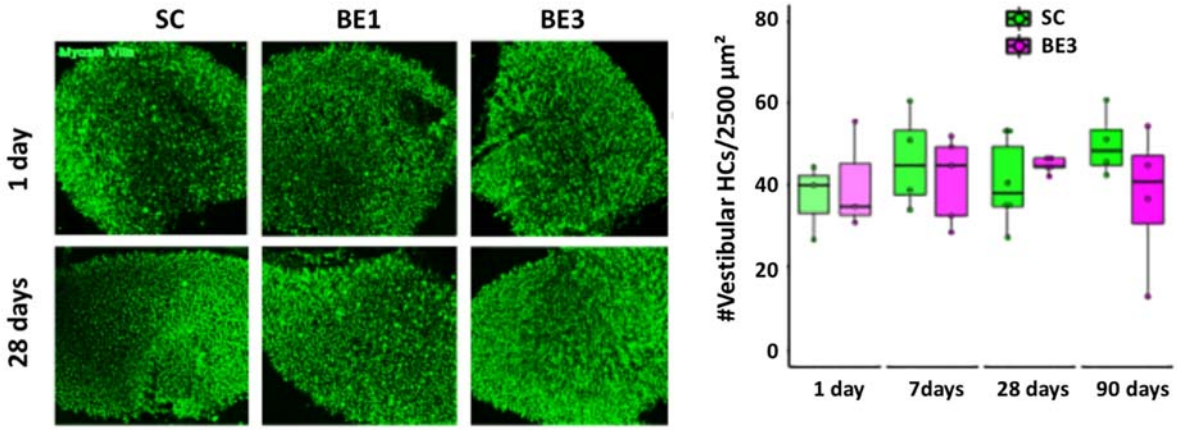


Fig 18. Vestibular HC counting showed no significant difference between control and 3-repeated blast expoures.

IV. Differential expressed genes in cochlea after blast exposure

We used two transgenic mice to determine the changes in gene expression that occur within auditory supporting cells and hair cells after repeated blast exposure. B6.129S-Atoh1<tm4.1Hzo>/J mouse expresses GFP in hair cells under control of the Atoh1 enhancer. FVB/N-Tg(GFAPGFP)14Mes/J mouse expresses GFP in supporting cells under control of the GFAP promoter. Mice from these transgenic lines were subjected to blast or control conditions, then euthanized 1 day or 1 month post-exposure.

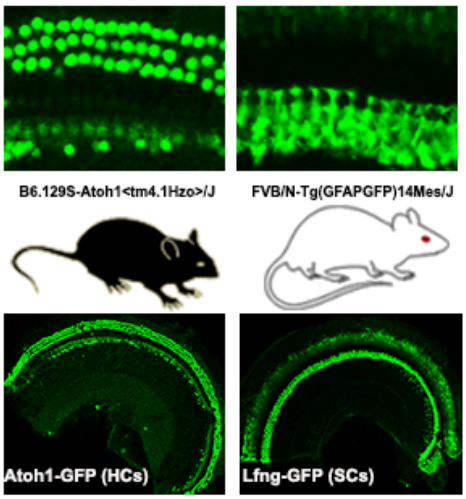


Fig 19. Cochleae from Atoh1 and GFAPGFP transgenic mice.

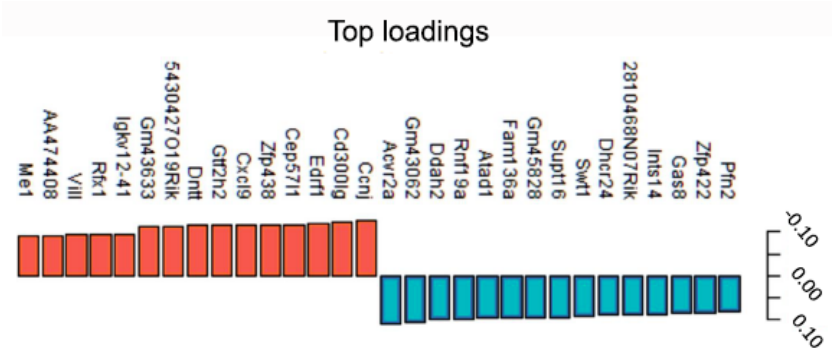


Fig 20. TOP 15 upregulatted and downregulated differentially expressed genes in BE3 vs. SC.

Cochleae (Fig 19) were dissected immediately after euthanasia which were pooled from 2-4 mice and dissociated before fluorescence-activated cell sorting (FACS). This resulted in selective populations of either hair cells or supporting cells. RNA was extracted using the RNeasy Micro Kit (QIAGEN). Libraries were produced using the Nextera protocol and sequenced on an Illumina NextSeq500 using paired-end reads. Transcriptomic analyses were performed to evaluate changes in gene expression (Fig 20).

Gene ontology (GO) analysis was performed using DAVID 6.8 and showed that differentially expressed genes (DEG, Fig 21) in cochlear HCs at 1 day post blast exposure were associated with cytoskeleton, DNA-termplated transcription and hormone response (Fig 22).

ID	Description	Differentially Expression of Genes
GO:0005856	cytoskeleton	PFN2, CEP57L1, RNF19A, GAS8, DHCR24
GO:0006351	transcription, DNA-templated	ZFP422, SUPT16, EDRF1, RFX1, SWT1, GTF2H2
GO:0009725	response to hormone	ME1, DHCR24
GO:0005634	nucleus	ZFP422, CCNJ, DNTT, SUPT16, EDRF1, ZFP438, RFX1, SWT1, GTF2H2, ATAD1, DHCR24
GO:0005813	centrosome	CEP57L1, RNF19A, DDAH2
GO:0007368	determination of left/right symmetry	ACVR2A, GAS8
GO:0005737	cytoplasm	ME1, ACVR2A, PFN2, DNTT, CEP57L1, RNF19A, ZFP438, FAM136A, DDAH2, GAS8, DHCR24

Fig 21. Gene ontology enrichment analysis.

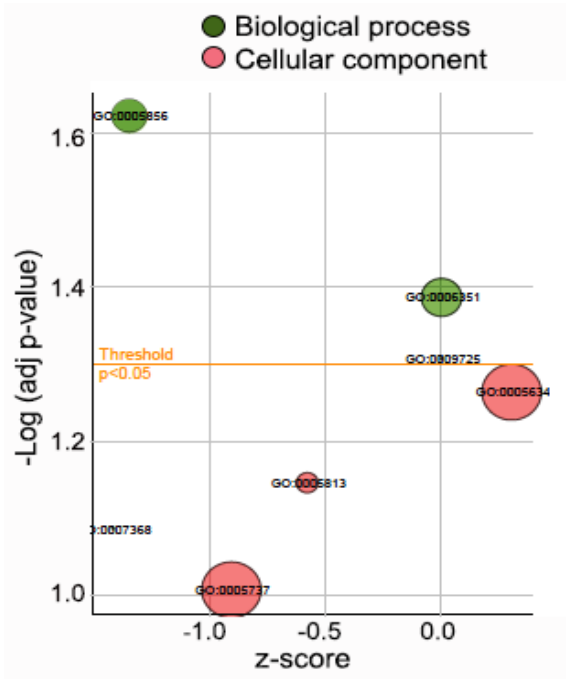


Fig 22. Gene ontology enrichment analysis.

The bioinformatic data were highly variable within a given time point and condition. This high variation was likely due to both biological and experimental factors, in that adult cochlear sensory cells are known to be extremely fragile and prone to rapid degradation. Further, inherent variability between cochlear sensory cells, such as between cells located in the apex (low frequency region) versus the base (high frequency region), likely introduced additional variation in the data. One subset of our dataset, which came from the sensory cells one day post-blast exposure, was identified as the most likely to yield meaningful results. The results also need to be validated by further advanced research technology, such as using single molecule fluorescence in situ hybridization.

V. Induce Hair cell (HC) regeneration by transfecting supporting cells (SC) with transcription factors

Manipulation of non-sensory supporting cells to become hair cells is a major focus of hearing research, because the sensory HCs of the inner ear cannot be regenerated or replaced in adult mammals. However the hearing organ, the cochlea, cannot be cultured beyond early postnatal ages. Therefore, we performed regeneration experiments using mature utricles, which can be maintained in culture for several weeks.

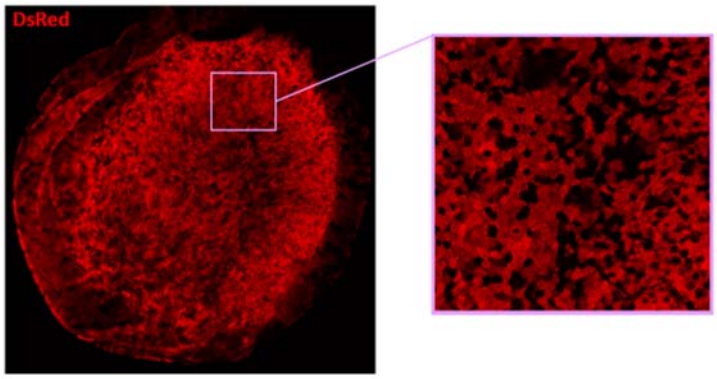


Fig 23 Labeling SCs by adding 4OHT

We generated Plp1-Cre$^{+/-}$/R26R-tdTomato$^{+/-}$ mice which allowed labeling of pre-existing SCs with tdTomato in red fluorescence. As shown in Fig 23, the SCs were labeled after adding 4-hydroxy-tamoxifen (4OHT) in the primary utricle culture. We determined an optimal dose of Neomycin for killing of HCs. A concentration of 3 mM Neomycin for 24 hour and culture up to 7 days is sufficient to kill hair cells *in vitro* without destroying the SCs(Fig 24). Then, we transfected SCs with a virus containing HC-specific transcription factors and an additional label (Fig 25). Once the transfected supporting cells (double labeled in both red and green) would start to express the transcription factors, we would expect them to become more like hair cells. Characterization of the transfected supporting cells for hair cell-like morphology, or signs of expression of hair cell-associated markers, needs to be evaluated.

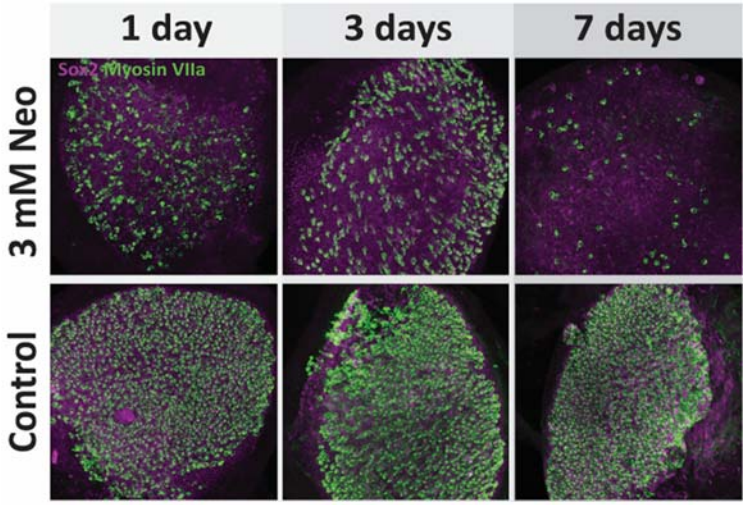


Fig 24. Progressive HC death following 24 hrs Neomycin treatment

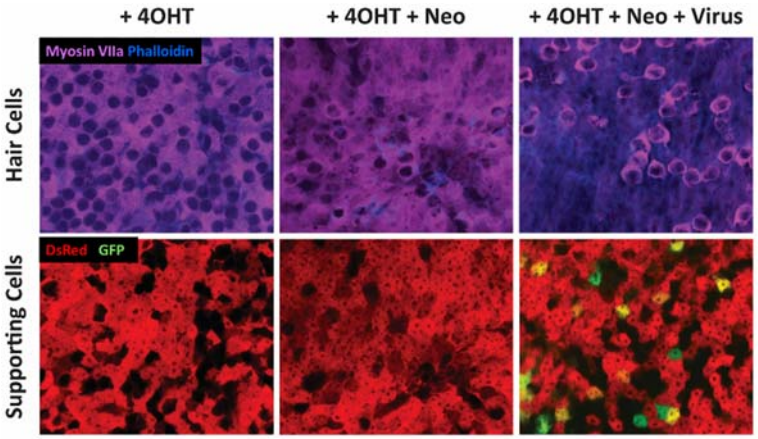


Fig 25. Progressive HC death following 24 hrs Neomycin treatment

The results indicate that we can successfully perform all of the critical steps of the planned work—inducing Cre recombinase to genetically label supporting cells, killing sensory cells with neomycin, then transfecting the supporting cells with a control version of our viral construct—over the course of 8 days in culture. The use of neomycin to kill hair cells is necessary because our proposed method, which utilized a transgenic mouse line, proved to generate results that were to variable for our experiments. In contrast the neomycin treatment produces much more reliable levels of hair cell death.

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

  Technical skill development includes:

  - dissection and whole-mount immunostaining of the adult inner ear
  - ABR, DPOAE and VsEP testing
  - plastic sectioning and staining of plastic-embedded samples on a microtome
  - dissociation of live tissue for FACS sorting
  - RNA extraction
  - culturing of adult mouse utricles
  - viral transfection
  - inducing recombination in transgenic mice
  - genotyping, breeding, and management of mouse colonies
  - image and data analysis

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

  The PIs, Research associates, Postdoctoral fellows, and Postbaccalaureate fellows attended scientific meetings and presented our finding at the Society for Neuroscience Symposium, Association for Research in Otolaryngology symposium, Military Health System Research Symposium, National Capital Area TBI symposium as well as the Japan US technical information exchange forum.

4. Impact

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

  The results of Aim 3 may yield insights on what molecular pathways are activated in response to blast damage, or to noise damage generally. They may therefore point to

particular targets for intervention in continuing research, or for development of therapeutic agents and protocols. Results from Aim 4 may allow us to demonstrate robust sensory cell regeneration in response to gene therapy. Additionally, while the cochlea has been the main focus of regeneration science in the inner ear, the mature cochlea cannot be maintained in culture. This makes it impossible to conduct experiments or test therapies using adult tissue, which is problematic because a major goal in our field is to restore hearing in adults. We are using the adult utricle, which can be cultured, for our regeneration experiments. This allows us to evaluate the effectiveness of our therapy in fully mature, post-mitotic tissue, and hopefully will inform future studies on regeneration in adult tissues.

- **What was the impact on technology transfer?**

  N/A

- **What was the impact on society beyond science and technology?**

  This project contributed to our overall understanding of how blast exposure affects the inner ear at the cellular level. They provide further evidence that the development of suitable hearing protection is crucial for individuals, such as service men and women, who risk exposure to blast or other concussive insults in the field. Since sensory cells of the inner ear cannot be replaced in mature tissue, the loss of hair cells that occurs in some areas of the cochlea suggests that prevention is the best, only option for ensuring that servive men and women complete their careers with normal hearing. That being stated, the results of Aim 4 may lead to novel gene therapies that would significantly change treatment options for individuals that have suffered hair cell loss. This would include not only people that have been exposed to concussive blast but also those who have lost hair cells through other processes, including normal aging.

5. Changes/Problems

- **Changes in approach and reasons for change**

  This experiment involved generating a triple transgenic mouse line in the initial design of Aim 4, which would bear the human diphtheria toxin receptor under a sensory cell-specific promoter. This would allow us to selectively kill sensory cells by administering diphtheria toxin, to which the mouse is much less susceptible than humans. Unfortunately, the prior work on this mouse model which showed widespread death of utricular sensory cells in vitro could not be repeated in our hands, and we observed no sensory cell death when administering the toxin, even at very high concentrations. Experiments involving in vivo administration did demonstrate utricular sensory cell death, but concerns about the inconsistency between in vitro and in vivo experiments, combined with uncertainty surrounding whether the diphtheria transgene would work as needed once genetic backgrounds were mixed (as necessary to generate the triple transgenic mouse line) led us to seek a pharmacological alternative. We have since used neomycin, an aminoglycoside antibiotic known to be ototoxic, to selectively kill sensory cells in utricle cultures. Using this method allowed us to generate only double transgenic mice, without the diphtheria toxin transgene, which is much more efficient.

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

  Aim 3: Because differential gene expression identified through RNA sequencing has to be validated using a more quantitative method, we attempted to perform digital-droplet polymerase chain reaction, or ddPCR. Because very little RNA was left after the NIDCD genomics core took samples for sequencing, ddPCR was an attractive alternative to traditional quantitative PCR (qPCR) which requires much more sample. However, given the number of genes we planned to examine, the remaining RNA needed to be amplified before attempting ddPCR. Some samples did not work in any of our assays, which made overall sample sizes too small to draw any conclusions. Instead, we will use smFISH as detailed above.

- **Changes that had a significant impact on expenditures**

  Aim 3: We needed transgenic animals expressing the fluorescent reporter GFP in particular cell types. While these animals could have been bred at NIH and transferred to WRAIR for blast exposure, the latter's animal facility policies would not allow transfer or breeding of those animals. The animals therefore had to be ordered directly from Jackson Laboratories and shipped to WRAIR, which cost more than originally planned.

6. Products:

Conference and presentations

1) Poster presentation entitled "Auditory functional deficits and structures changes following blast shockwave exposure in mice" was given at the Society for Neuroscience Symposium at San Diego, CA, on 15 November 2016.
2) Poster presentation entitled "Characterization of Auditory Injury in Mice Exposed to Blast Overpressure in an Advanced Blast Simulator" was given at the Association for Research in Otolaryngology at Baltimore, MD, on 11 February 2017.
3) Poster presentation entitled "Blast shockwave induced auditory functional and structural changes in mice" was given at the National Capital Area TBI symposium held at the NIH in Bethesda MD, on 9 March 2017.
4) Oral presentation entitled "Characterization of Blast Shockwave on Auditory Deficits in Rodents" was given at Japan US technical information exchange forum on blast injury at Tokyo, Japan, on 15 April 2017.
5) Poster presentation entitled "The Impact of Blast Shockwaves on Ears and Sound Processing Centers in Mice" was given at the Military Health System Research Symposium at Kissimmee, FL, on 18 August 2017.
6) Poster "Effects of blast shockwave exposure on ears and auditory signal processing centers in rodents" was presented at the Society for Neuroscience Meeting in November 2017 at Washington DC.
7) Poster presentation entitled "Transcriptomic and morphological changes after blast exposure reveals a fundamental response to injury in the ear and brain leading to

auditory dysfunction' was given at the Military Health System Research Symposium at Kissimmee, FL, on 21 August 2018.

8) Poster presentation entitled "Concussive blasts result in loss of outer hair cell and inner hair cell synapses in mice' was given at the Association for Research in Otolaryngology at Baltimore, MD, on 13 February 2019.
9) Poster presentation entitled "Examination of transcriptional changes in cochlear hair cells and supporting cells following blast was given at the Association for Research in Otolaryngology at Baltimore, MD, on 13 February 2019.

7. Participants & Other Collaborating Organizations

- What individuals have worked on the project?

Name	Project Role	Percent Effort	Organization
Dr. Joseph Long	PI	2%	WRAIR
Ying Wang	Co-PI	50%	WRAIR
Donna Wilder	Lab Manager	50%	WRAIR
Rodrigo Urioste	Research Assisstant	50%	WRAIR
Yanling Wei	Lab Tech	50%	WRAIR

- What other organizations were involved as partners?

Organization Name	Location	Contribution
National Institute on Deafness and Other Communication Disorders	Maryland	Collaboration

8. Special Reporting Requirements

Quad Chart is attached.

9. Appendices

Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

Assessment and treatment of blast-induced auditory and vestibular injuries

MR130592

W81XWH-15-02-0024

PI: Joseph B. Long **Org:** WRAIR/The Geneva Foundation **Award Amount:** $1,482,039

Study/Product Aim(s)

The etiology of blast-induced hearing loss and balance disorders is largely undefined and there are no FDA-approved drugs for treatment. Using adult mice exposed to blast overpressure, we propose to describe blast-induced structural and cellular damage, including loss of hair cells, within the auditory and vestibular organs at acute and chronic phases. Our hypothesis is that delivery of Atoh1 will induce the formation of replacement hair cells in the vestibular organs with a resulting recovery of vestibular function.

Approach

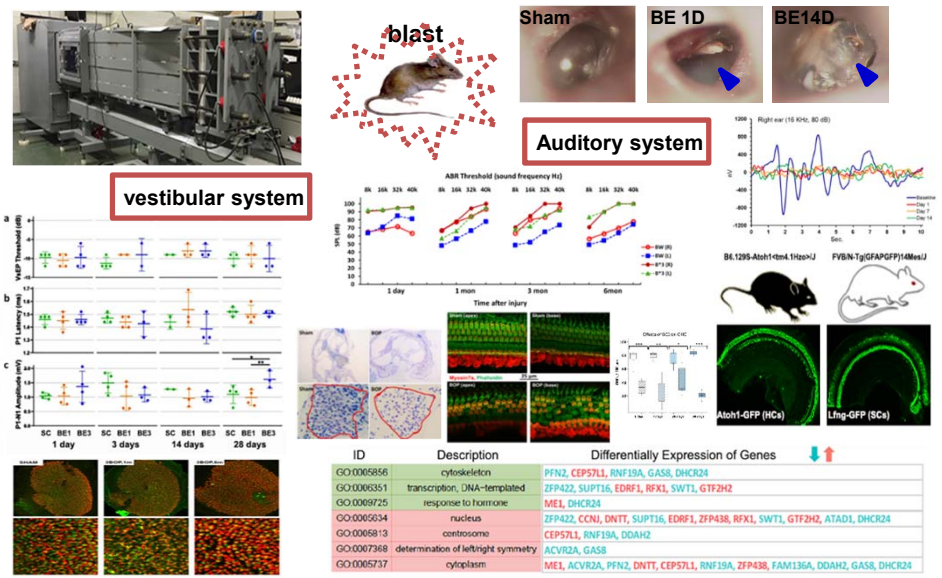
Blast injury model using well-characterized shock tube exposures of mice to repetitive blast overpressure.

Combined functional, morphological, and neurobiological assessments.

Functional testing using ABR, DPOAE, Rotarod test and VEPs.

Morphological changes identified using immunohistochemistry, histological sections, TEM and SEM.

Biochemical alterations evaluated by qPCR, and in situ hybridization analysis.



Timeline and Cost

Activities CY	15	16	17	18	19
Animal protocol writing and approval					
Auditory and vestibular functional assessment					
Auditory and vestibular morphological and biochemical evaluation					
Wrap up data and then write final reports and publications					
Estimated Budget ($K)	**$549**	**$462**	**$470**		

Updated: (09/19/19)

Goals/Milestones

CY15 -16 Goal – Functional assessment

- ☑ Obtain approval of animal use protocol
- ☑ Establish a mouse auditory injury model
- ☑ Assessment of blast exposure injury to auditory function
- ☑ Assessment of blast injuries to vestibular function

CY16 - 17 Goals – Pathological assessment

- ☑ Determine effects of blast damages to auditory structure
- ☑ Determine effects of blast damage to vestibular structure
- ☑ Establish the transgenic mice

CY17 - 18 Goal – Therapeutic Efficacy

- ☑ Efficacy of viral-mediated expression of Atoh1 on hair cell formation
- ☑ Efficacy of induced hair cell regeneration

Budget Expenditure to Date

Actual Expenditure: $1, 482,038.99