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PRINCIPAL INVESTIGATOR: Dr. Joseph B. Long

CONTRACTING ORGANIZATION: The Geneva Foundation
Tacoma, WA 98402

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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 : 1) 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, 2) 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) determine the cell-type-specific changes in gene expression that occur within auditory supporting cells and hair cells after repeated blast exposure, and 4) 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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Table of Contents

	<u>Page</u>
1. Introduction.....	4
2. Keywords.....	4
3. Accomplishments.....	4
4. Impact.....	11
5. Changes/Problems.....	11
6. Products.....	11
7. Participants & Other Collaborating Organizations.....	13
8. Special Reporting Requirements.....	13
9. Appendices.....	13

1. Introduction

With the widespread use of improvised explosive devices in recent military conflicts, blast-induced neurosensory dysfunctions have emerged as a key military medical issue. The debilitating 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 the transcription factor *Atoh1* and inhibition of Notch signaling may convert supporting cells into hair cells in adult organs. We utilize an air-driven shock tube simulation of blast to: 1) preclinically evaluate blast-induced auditory and vestibular injuries in mice and characterize structural, physiological and molecular changes in the inner ear and brain, 2) determine whether gene therapy using a transcription factor can be used to induce functional recovery in blast-damaged inner ears.

2. Keywords

Injury, blast overpressure (BOP), auditory brainstem response (ABR), distortion product otoacoustic emission (DPOAE), vestibular evoked potentials (VsEP), inner ear, cochlea, utricle,

3. Accomplishments

○ What were the major goals for the project?

The major objectives for the project were: (a) 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; (b) 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; (c) to determine the cell-type-specific changes in gene expression that occur within auditory supporting cells and hair cells after repeated blast exposure; (d) 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.

Milestones:

Year 1: Obtain IACUC and ACURO approved protocol, establish blast-induced auditory injury model; start auditory function assessment.

Year 2: Examine cochlear and vestibular tissue at 1d, 7d, 1 m after blast exposure.

Year 3: Analyze RNA expression in blast-injured mice; evaluate the efficacy of hair cell regeneration.

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

Bulleted list of key research accomplishments emanating from this research

- In this reporting period, we continue to increase the sample size for the different blast conditions at multiple injury intervals.
- We have longitudinally investigated the impact of single blast exposure (BOP), 3-repeated blast exposures (BOP*3) and the combined blast and weight drop injuries (BW), respectively on auditory functional deficits defined by DPOAE and ABR evaluations.
- For auditory organ evaluation, we have applied the methods of immunohistochemistry on whole-mount cochlear, cryostat and plastic sections. Antibodies distinguishing phalloidin (f-actin), Pou4f3 (nucleus), Myo7a (hair cell) were used and were visualized by confocal microscopy. The hair cells were counted.
- In this period, we received an approval letter from NIH ACUC for the animal protocol 'Effects of exposure to blast on inner ear function'.
- A collaboration to characterize vestibular evoked potential (VsEP) changes in mice after blast was established and a protocol amendment requesting delivery of mice to Stanford University for this purpose was approved by the WRAIR and Stanford IACUCs. Required documents were reviewed and accepted by the animal facility of Stanford University.
- A total of 48 mice were successfully shipped from WRAIR to Stanford University on the day of and 21 days after blast exposure to allow VsEP testing 3 days and 28 days post-injury.
- Mice were euthanized after VsEP testing at Dr. Alan Cheng's Laboratory at Stanford University and the ear specimens were delivered to NIDCD for pathological evaluation.
- One platform and 4 poster presentations were delivered during this reporting period based upon the findings of this project. These include:
 - a. Poster "Characterization of Auditory Injury in Mice Exposed to Blast Overpressure in an Advanced Blast Simulator," presented at MHSRS in August 2016 in Kissimmee, FL.
 - b. Poster "Auditory functional deficits and structures changes following blast shockwave exposure in mice," presented at the Society for Neuroscience meeting in November 2016 in San Diego, CA.
 - c. Poster "Characterization of Auditory Injury in Mice Exposed to Blast Overpressure in an Advanced Blast Simulator," presented at the Association for Research in Otolaryngology in February 2017 in Baltimore, MD.

- d. Poster “Blast shockwave induced auditory functional and structural changes in mice,” presented at the National Capital Area TBI symposium in March 2017 in Bethesda, MD.
- e. Platform presentation “Characterization of Blast Shockwave on Auditory Deficits in Rodents” delivered at the second Japan and US technical information exchange forum on blast injury in April 2017 in Tokyo, Japan.

Detailed experimental methods and results

➤ **Methods**

Animal models: All animal experiments were conducted in accordance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhered to principles stated in the Guide for the Care and Use of Laboratory Animals with an Institutional Animal Care and Use Committee approved protocol. The blast overpressure (peak static pressure of 19 psi and 4 msec positive phase duration) was generated by Valmex membrane rupture in an advanced blast simulator (ABS) which consists of a 0.5 ft long compression chamber that is separated from a 21 ft long transition/expansion test section. For a single blast treatment (1BOP), mice (male, 23 - 28 g) were secured in the ABS in a prone position facing the oncoming shockwave immediately after administration of 4% isoflurane gas anesthesia in an induction chamber for 8 min (O₂ flow rate 1.5L/min). For three blast exposures (3BOP), mice immediately received additional isoflurane anesthesia for 2 min separating the second and third blast exposures. Sham control animals were included in all individual experiments and were treated in the same fashion without exposure to blast shockwaves. For the combined blast and weight-drop injury (BW), the blast exposed mice (described above) was secured to the foam bed treated immediately afterwards by the 80 g cylindrical Plexiglas dropped from a 1 m height to the head

Auditory functional assessment: Auditory Brainstem Response (ABR) and Distortion Product Otoacoustic Emissions (DPOAE) testing were used to assess auditory function. Each mouse was tested under Ketamine/Dexdomitor anesthesia (60 mg/kg and x 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 and 14 days, then each month up to 6 months after blast exposure.

Vestibular functional assessment: Mice were anesthetized by intraperitoneal injection of a mixture of Ketamine/Dexdomitor (60 mg/kg and x 0.4 mg/kg, respectively). Vestibular evoked potentials 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.

Pathology: 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. With whole-mount sections, cochleae and utricles were dissected from decalcified inner ear for immunostaining. For cryosectioning, the decalcified inner ear was embedded in OCT after cryoprotection in a sucrose gradient (10%–30%). Serial sections were cut and labeled using antibodies for immunohistochemistry. For plastic sections, the labyrinth and cochlea were isolated and post-fixed in 1% osmium-tetroxide in cacodylate buffer for 2 hrs at room temperature. Tissue was sectioned serially after dehydration in a graded ethanol series and embedding in araldite. Specimens were then stained with Epoxy Stain.

For immunostaining, specimens were washed in PBS and then permeabilized and blocked for 1 hr at RT in PBS with 0.2% Triton X-100 (PBS-T) and 10% normal goat serum. Samples were then incubated with the appropriate primary antibodies (described below) in PBS-T with 2% NGS overnight, followed by 3 rinses in PBS-T and labeling with AlexaFluor-conjugated secondary antibodies in PBS-T for 3 hrs at RT. Where indicated, AlexaFluor-conjugated phalloidin (5 U/mL) and/or DAPI (1:1000) and anti-myosin 7a were included with the secondary antibodies to detect F-actin and nuclei, respectively. Specimens were then rinsed in PBS 3 times, mounted in SlowFade, and imaged on a Zeiss LSM 710 microscope.

➤ **Results**

1. Time-course changes in ABR testing after blast exposure:

In this reporting period, we continued investigation on blast-induced auditory deficits

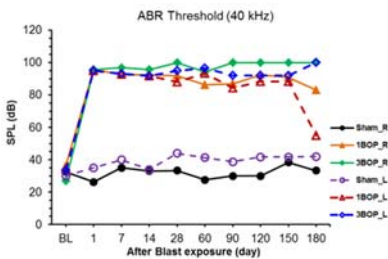


Fig. 1a. Left (L) or Right (R) ear of mouse ABR to 40 kHz stimulus

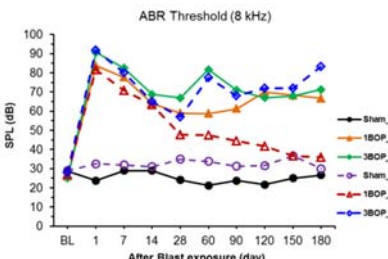


Fig. 1b. Left (L) or Right (R) ear of mouse ABR to 8 kHz stimulus

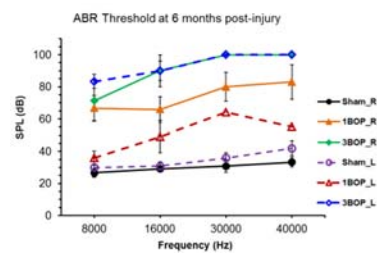


Fig. 1c. Left (L) or Right (R) ear of mouse ABR at 6 months after injury

by assessing ABR and DPOAE up to 6 month post-injury. 24 mice were separated into three experiment groups of Sham, BOP and 3BOP. As showed in the Fig .1, the timing of auditory functional testing ranging 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 17 psi impacts 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 exposed to blast overpressure which persisted over several months (Fig. 1). Compared to sham controls at 28 days post exposure, significantly elevated ABR thresholds and decreased wave amplitudes were evident

in mice with BOP ($p < 0.05$) and 3BOP ($p < 0.0005$) (Fig.2). Mice receiving 3-repeated blast exposures lost hearing to high frequency sound (32k and 40 kHz) for over six months, while 50% of them recovered partially to low frequency sound (8 kHz) during this timeframe.

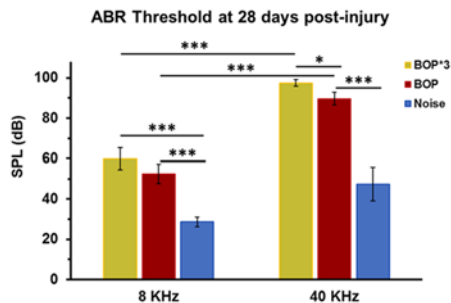


Fig. 2. ABR threshold changes at 28 days after blast injury

We also investigated the effects of various intensities of blast overpressure on ABR threshold (Fig. 3). The functional responses have been recorded under anesthesia at 1, 7, 14, 28 and 60 days after a single blast at 4, 8, 12 and 17 psi, respectively. ABR threshold increased significantly with a high frequency (40 KHz) stimulus which was observed at 1 day post-injury and persisted over 28 days in all mice with blast exposure (Fig. 3a). ABR threshold to a low frequency (8 KHz) stimulus increased significantly at 1, 7 and 14 days after blast exposure (Fig. 3b).

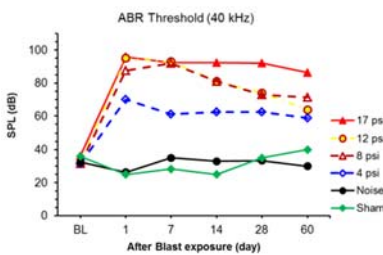


Fig. 3a. ABR threshold to 40 kHz stimulus in mice that received a BOP at 4, 8, 12 and 17 psi.

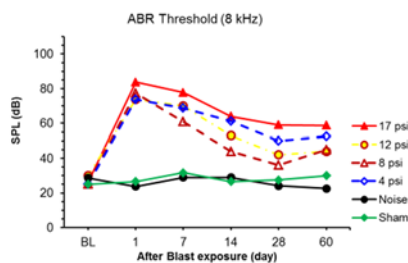


Fig. 3b. ABR threshold to 8 kHz stimulus in mice that received a BOP at 4, 8, 12 and 17 psi.

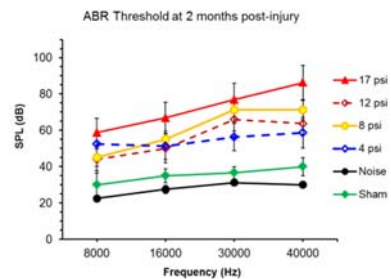


Fig. 3c. ABR threshold at 2 months after exposed to a single BOP at 4, 8, 12 and 17 psi.

2. Effects of blast exposure on DPOAE testing: Adult male CBA/J mice showed a significant loss in distortion-product otoacoustic emission (DPOAE) response following blast overpressure exposure. Data (Fig. 4) showed a persistent complete loss of DPOAE in 3BOP mice at 3 month, whereas 53% and 60% of mice recovered in BOP and BW treatment groups, respectively. At 6 months post exposure, 65% (11) mice had a DPOAE when tested. Out of the 11 mice who showed some level of recovery, 7 of them regained some signal only in one ear and 4 of them regained DPOAE signal in both ears. DPOAE signal recovered usually around low-mid frequencies (9 – 16 kHz). Following blast exposure, damage to the inner ear is evident through the 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 DPOAE signal was not seen following exposure to a 17 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 17 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 than were recorded in sham animals. Overall, these results reveal intensity-dependent blast damage to the inner ear.

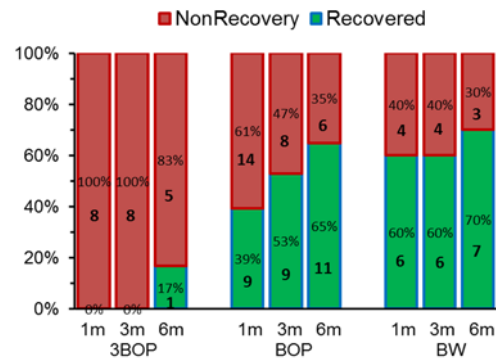


Fig. 4. DPOAE testing at 1, 3 and 6 months after blast exposure.

- Blast-damaged middle ear structures:** To observe the blast injuries to tympanic membrane and middle ear structures, we utilized the digital macroview otoscope to photograph anesthetized mice. Compared to BOP, 3BOP increased bilateral ear damage. Tympanic membranes were disrupted immediately

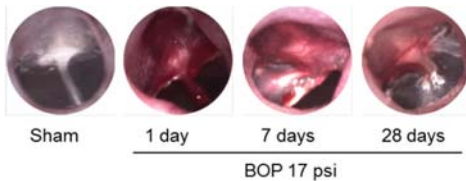


Fig. 5. Blast-induced tympanic membrane and middle ear damage.

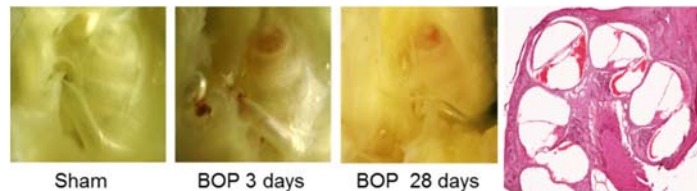


Fig. 6. Blast-induced inner ear hemorrhage.

following the blast exposure. Labyrinthine hemorrhage was prominent from 1 day up to 14 days post-injury. Intensity-dependent blast-induced damage to middle and inner ears was evident with no significant differences between left and right ears. However, the quality of images acquired from mice ears were generally not very good (data were not shown). Therefore, a group of 18 SD rats (350g) were used and handled in a manner identical to the mice. As displayed in the Fig. 5, tympanic membranes and middle ear structures were damaged at 1 and 7 days after blast exposure (17 psi). At 28 days post injury, the tympanic membrane recovered, while middle ear showed a reduction in edema. The images were acquired under a dissecting microscope which displayed clearly the inner ear hemorrhage which was confirmed in cross sections with H&E staining (Fig. 6).

- Blast damage to cochleae:** Experimental mice were euthanized at 1, 7, 14 and 28 days after blast injury, respectively. The whole-mount cochlea was dissected and underwent immunostaining with anti-Myo7a (red) and phalloidin (green), and was then visualized by confocal microscope. The hair cells were counted using

FIJI software. Data (sham n=10, and n=7 for each blast group) showed that blast impacted the cochlear structure (Fig. 7). Hair cell counting revealed that the blast-induced outer hair cell loss occurred at base, mid and apex regions of cochleae. Significant decreases in hair cell number were observed in the basal region of cochleae. Blast damage to cochlear structures and the loss of hair cells were observed at 1, 7, 14 and 28 days post-injury.

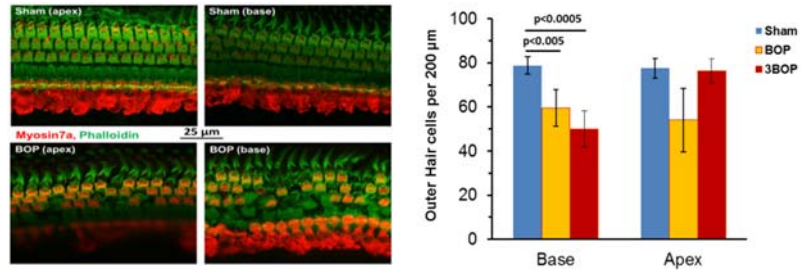


Fig. 7. Blast-induced cochlear damage and outer hair cells loss.

5. **Effects of blast exposure to spiral ganglia:**

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 using a confocal microscope. Velocity software was used for quantification of spiral ganglion neurons. Initial data reveal a decrease in the density of neurons in the spiral ganglia at 28 days after blast exposure in comparison to that seen in the sham controls (Fig. 8).

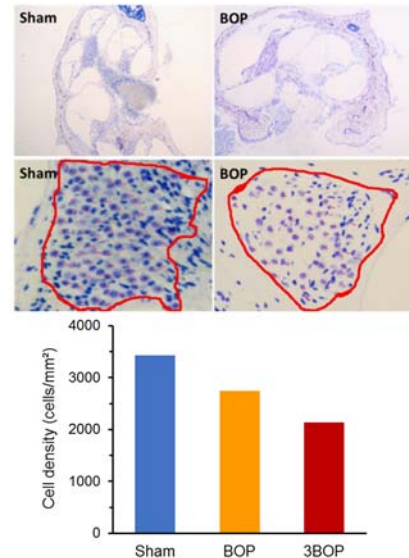


Fig. 8. Effect of blast exposure on spiral ganglion neurons

6. **Effects of blast exposure on vestibular evoked potentials (VsEP):**

During this period, Dr. Alan Cheng, an Associate Professor in Otolaryngology at Stanford University School of Medicine assisted us with VsEP testing in mice. At 3 days after blast exposure, 50% of BOP mice and 66% of 3BOP mice

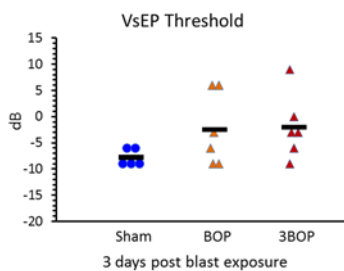


Fig. 9a. VsEP threshold changes at 3 days after Blast exposure

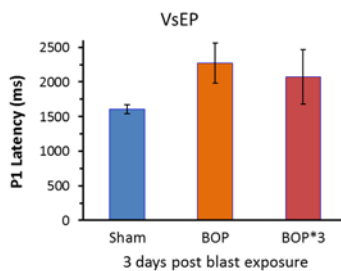


Fig. 9b. P1 latency changes at 3 days after Blast exposure

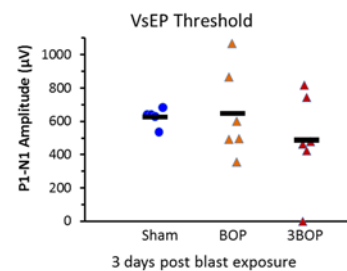


Fig. 9c. Effect of blast exposure to P1-N1 amplitude

showed significant increases in VsEP threshold (Fig. 9a). P1 latency increased in blast exposed mice (Fig. 9b). There was no difference between BOP and 3BOP. For the P1-N1 amplitude, 50% of BOP mice and 66% of 3BOP mice showed reduction, while 4 out of 12 mice receiving blast exposure presented with an increase (Fig. 9c).

7. **Effects of blast exposure on vestibular organs in the inner ear:** In this reporting period, we evaluated blast damage to the hair cells in utricles using anti-Myo7a and anti-4f3 immunostaining. FIJI software was used for quantification of utricle hair cells. Data showed that there was no significant change in hair cell number in utricles among the experimental groups.

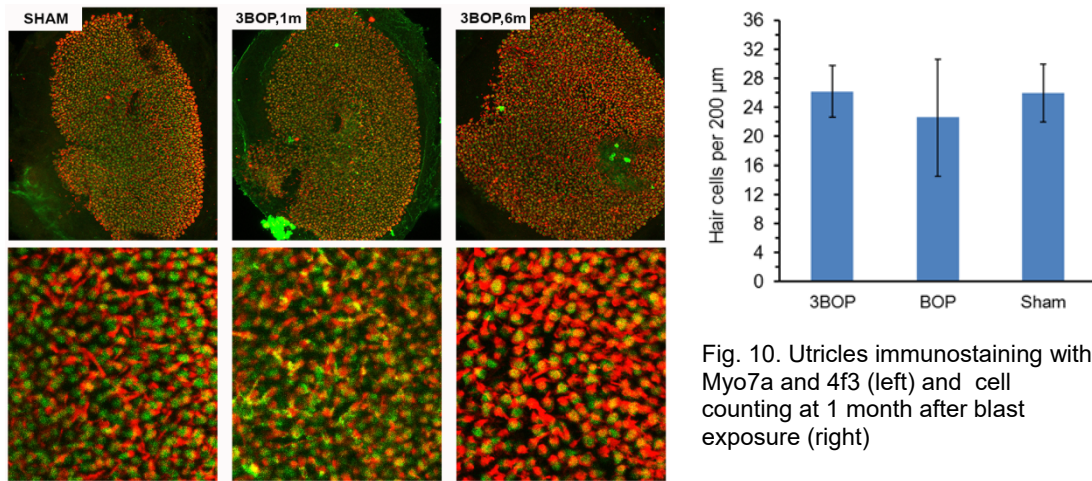


Fig. 10. Utricles immunostaining with Myo7a and 4f3 (left) and cell counting at 1 month after blast exposure (right)

4. Impact

The high energy of explosive devices results in a sharp spike in pressure that emanates as a wave from the source. Those overpressurized air particles can cause significant damage to internal organs. The outcomes from this research will help in identifying the underlying causes of blast overpressure-induced auditory and vestibular deficits. It may provide high-impact diagnostic and medical interventional strategies to benefit the thousands of victims who suffer from hearing loss and balance disorders.

5. Changes/Problems

None

6. Products

Lay Press

None

Peer-Reviewed Scientific Journals

None

Books or other non-periodical, one-time publications

None

Other publications, abstracts, conference papers and presentations

1. Platform presentation, "Characterization of Blast Shockwave on Auditory Deficits in Rodents" at the second Japan and US technical information exchange forum on blast injury in April 2017 at Tokyo, Japan.
2. Ying Wang, MD¹, Yanling Wei, MD¹, Stephen Van Albert, BE¹, Tracy Fitzgerald PhD², Amy Northrop, BS², Rodrigo Urioste, BS¹, Peethambaran Arun, PhD¹, Donna Wilder, BS¹, Sajja Venkatasivasaisujith, PhD¹, Irene D. Gist, BA¹ Stephen McInturff, BS², Weise Chang PhD², Matthew Kelley, PhD² and Joseph Long, PhD¹. Characterization of Auditory Injury in Mice Exposed to Blast Overpressure in an Advanced Blast Simulator, poster presentation at MHSRS in August 2016, at Kissimmee, Florida. (1.BINT/WRAIR, 2.NIDCD/NIH)
3. Ying Wang¹, Yanling Wei¹, Stephen Van Albert¹, Amy Northrop², Rodrigo Urioste¹, Peethambaran Arun¹, Donna Wilder¹, Sajja Venkatasivasaisujith¹, Irene D. Gist¹ Stephen McInturff², Weise Chang², Tracy Fitzgerald², Matthew Kelley² and Joseph Long¹. Auditory functional deficits and structures changes following blast shockwave exposure in mice, Poster presentation at Society for Neuroscience in November 2016, at San Diego, California. (1.BINT/WRAIR, 2.NIDCD/NIH)
4. Kamren Hollingsworth¹, Ying Wang², Stephen McInturff¹, Amy Northrop¹, Yanling Wei², Yan Su², Donna Wilder², Weise Chang¹, Peethambaran Arun², Irene Gist², Rodrigo Urioste², Sajja Venkatasivasaisujith², Stephen Van Albert², Elizabeth C. Driver¹, Tracy Fitzgerald³, Joseph Long², Matthew W. Kelley¹. Characterization of Auditory Injury in Mice Exposed to Blast Overpressure in an Advanced Blast Simulator" poster presentation at Association for Research in Otolaryngology in February 2017 at Baltimore, Maryland. (1.NIDCD/NIH, 2.BINT/WRAIR)
5. Ying Wang¹, Rodrigo Urioste¹, Yanling Wei¹, Yan Su¹, Kamren Hollingsworth², Sajja Venkatasivasaisujith¹, Stephen Van Albert¹, Donna Wilder¹, Driwech Wafae¹, Irene D. Gist¹, Weise Chang², Tracy Fitzgerald², Peethambaran Arun¹, Matthew Kelley² and Joseph Long¹. Blast shockw ave induced auditory functional and structural changes in mice" at National Capital Area TBI symposium in March 2017, at Bethesda, Maryland. (1. BINT/WRAIR, 2.NIDCD/NIH)

Websites or other internet sites

None

Technologies or techniques

None

Inventions, patent applications, and/or licenses

None

7. Participants & Other Collaborating Organizations

Name	Project Role	Percent Effort	Organization
Dr. Joseph Long	PI	10%	WRAIR
Ying Wang	Co-PI	70%	WRAIR
Donna Wilder	Lab Manager	100%	Geneva
Matthew Kelly	Co-Principal Investigator	10%	(NIDCD)
Chang Weise	Co-Investigator	10%	(NIDCD)
Dr. Tessa Sanders	Post-Doctoral Fellow	100%	(NIDCD)
Mr. Scott Haraczy	Post-Bac	100%	(NIDCD)
Ms. Beatrice Mao	Post-Doctoral Fellow	100%	(NIDCD)

8. Special Reporting Requirements

A Quad Chart is attached.

9. APPENDICES

Four posters and one platform presentation are attached.

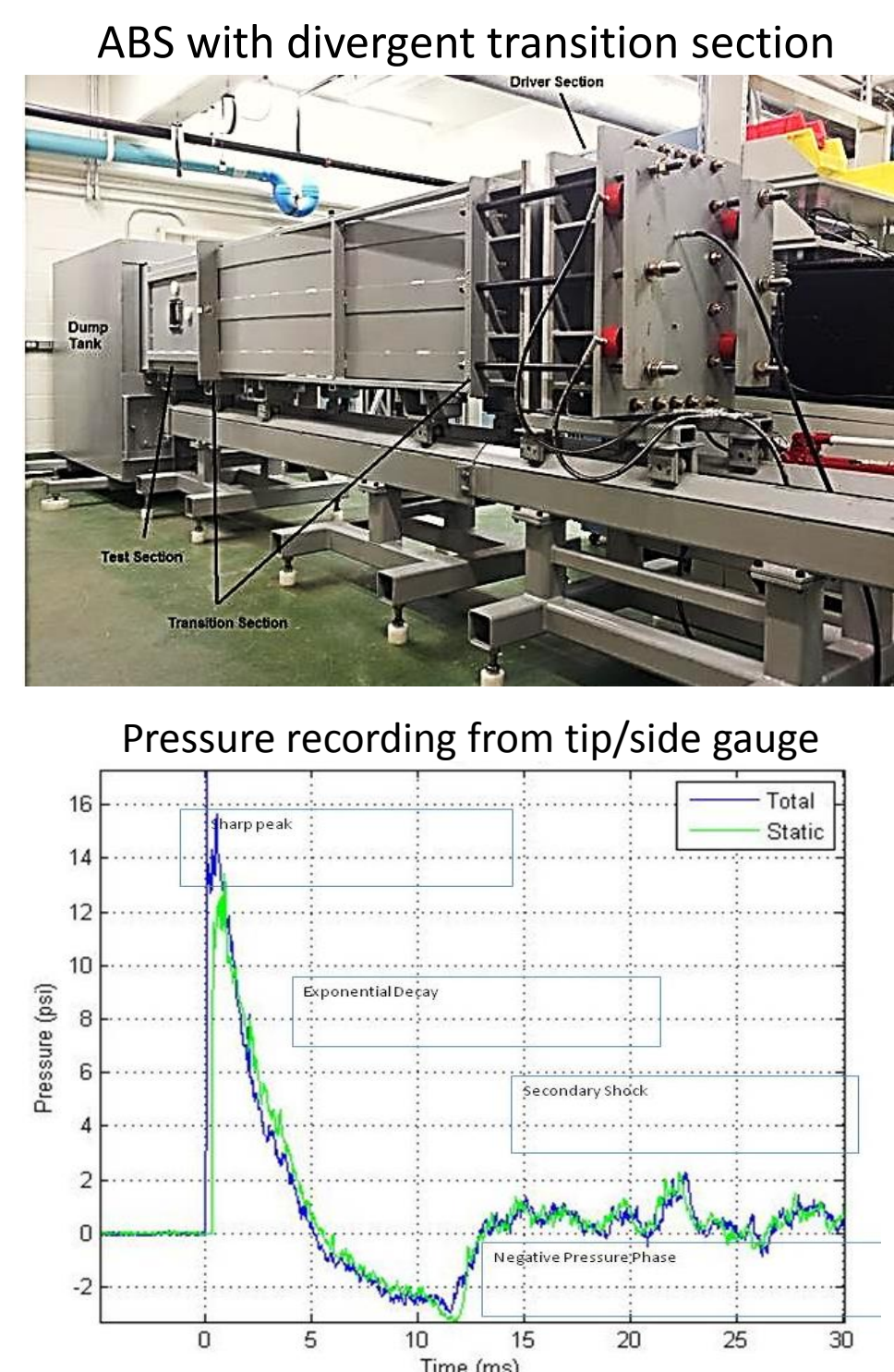


ABSTRACT

A high fidelity animal model is critical to define the mechanism(s) of blast-induced auditory injury and to develop therapeutic strategies. The present research is aimed at producing a comprehensive characterization of auditory functional deficits and associated pathological changes in the peripheral and central auditory signal processing regions disrupted by exposure to blast shockwaves. We have investigated the time-course of blast effects on auditory function and structural changes. Isoflurane anesthetized CBA mice (male, 8 weeks) were exposed to blast overpressure (peak static pressure of 19 psi and 4 msec positive phase duration) generated by the Advanced Blast Simulator (ABS). Auditory function was assessed by analyzing distortion product otoacoustic emission (DPOAE) and auditory brainstem response (ABR) under anesthesia. Data showed that DPOAE signals were undetectable acutely after blast exposure and their disappearance persisted over 14 days that suggests the injuries to the inner ear. Blast exposure caused significant elevations of ABR threshold, increased ABR wave latency, and reductions in ABR wave amplitude immediately following the blast shockwave insult. These changes were observed over the entire acoustic frequency spectrum and persisted over 14 days. Immunostaining of Myo7a and Phalloidin in whole-mount cochlea revealed appreciable damage to hair cells, as well as to other structures in the inner ear. Increases in GFAP, Iba1 and axonal degeneration were detected in the brainstem and cerebellum at 14 days post-injury. The results indicate that both peripheral and central auditory signal processing regions are vulnerable to blast overpressure exposure in the ABS. This mouse model of blast-induced auditory injury should provide a useful experimental tool for studying the mechanisms underlying hearing impairment after blast exposure and for evaluating potential strategies for prevention and cure.

BACKGROUND

Nearly 60% of blast TBI victims exhibit hearing loss, tinnitus, dizziness and balance disorders. Despite the high incidence of auditory dysfunction resulting from blast injuries, the neurobiological mechanisms underlying these blast injuries are largely undefined. A high fidelity animal model is critical to define the mechanism(s) of blast-induced auditory injury and to develop therapeutic strategies. However, conventional shock tubes are limited in their ability to simulate explosive blast waveforms and flow conditions. The Advanced Blast Simulator (ABS) incorporates design features which allow higher fidelity replication of the key features of blast wave flow conditions, including the negative phase and secondary shock. ABS consists of a 0.5 ft long compression chamber that is separated from a 21 ft long transition/expansion test section by rupturable Valmex membranes.



METHODS

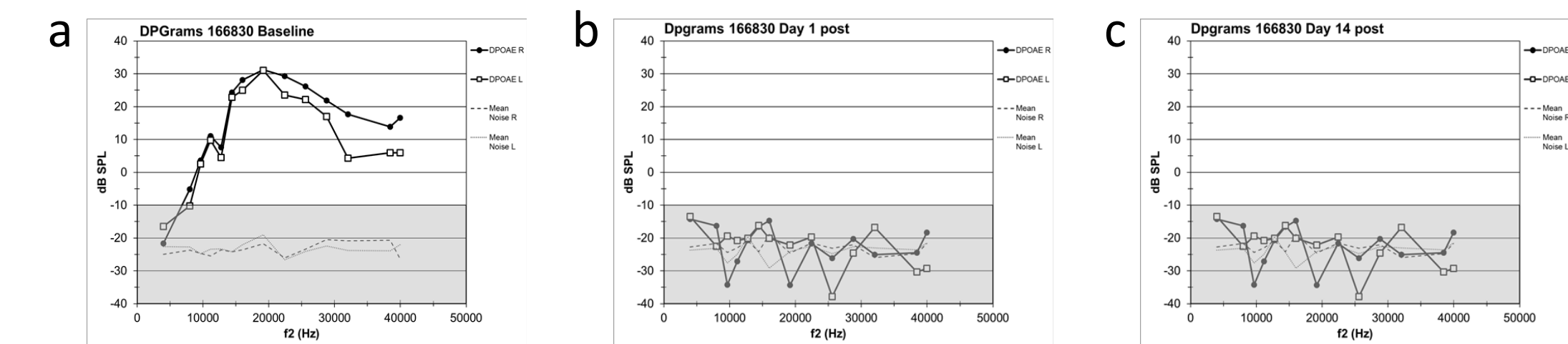
Animals and ABS: After anesthetization with isoflurane for 8 minutes, CBA mice (male, 23 - 28 g) were secured in the ABS in a prone position facing the oncoming shockwave. The blast overpressure (peak static pressure of 19 psi and 4 msec positive phase duration) was generated by Valmex membrane rupture in the ABS. Sham controls were handled similarly but without exposure to the blast.

Auditory functional assessment: A time-course of blast effects on auditory function was assessed by analyzing auditory brainstem response (ABR) and distortion product otoacoustic emission (DPOAE) under Ketamine/Dexdomitor anesthesia.

Pathological investigation: At 14 days post-injury, the whole-mount cochlea were subjected to immunohistochemistry, and brain sections were evaluated after silver staining and immunohistochemistry.

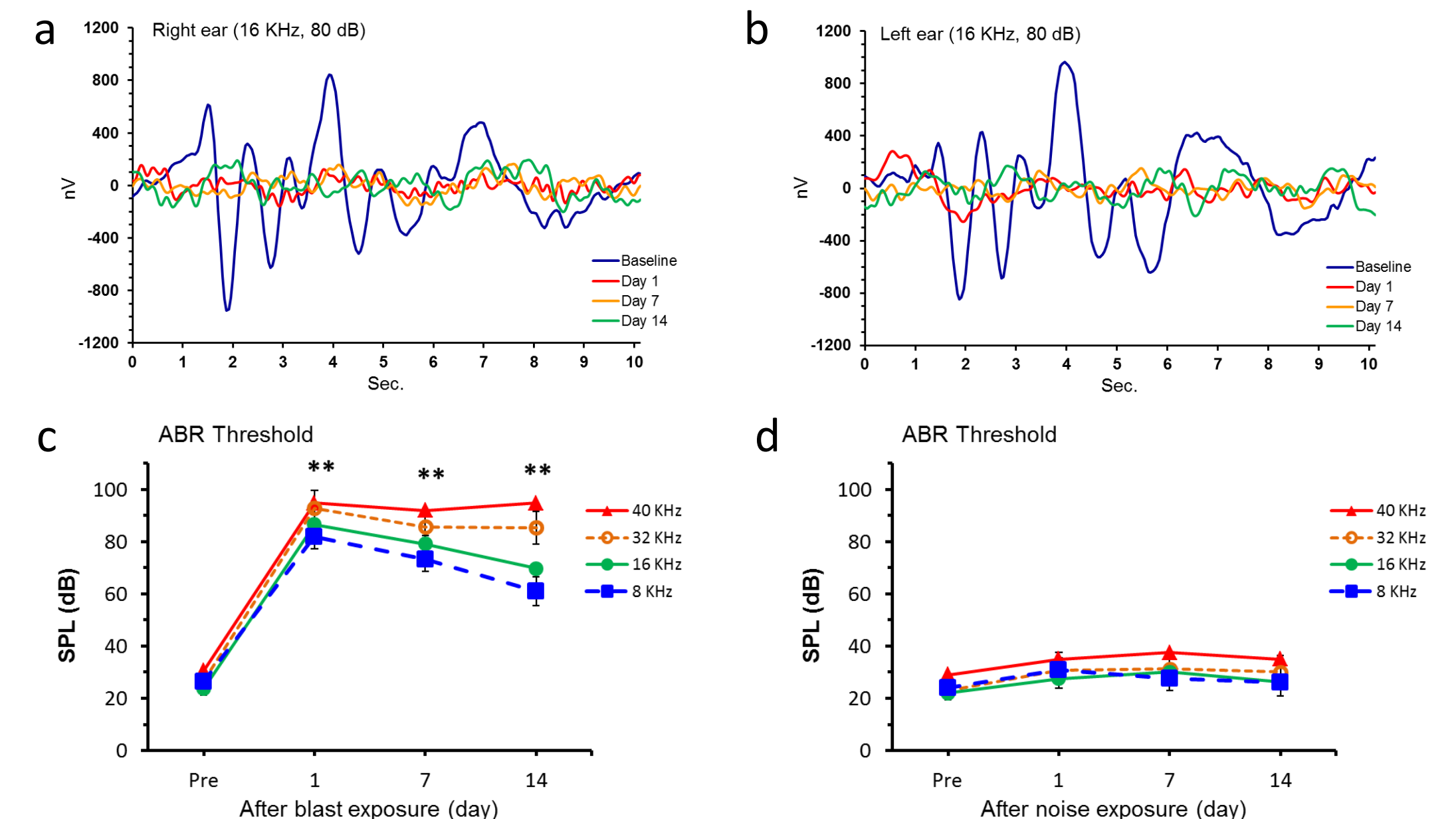
RESULTS

Figure 1. Blast exposure impaired DPOAE



Compared to its baseline (a), DPOAE was undetectable at 1 day (b) and 14 days (c) post-injury.

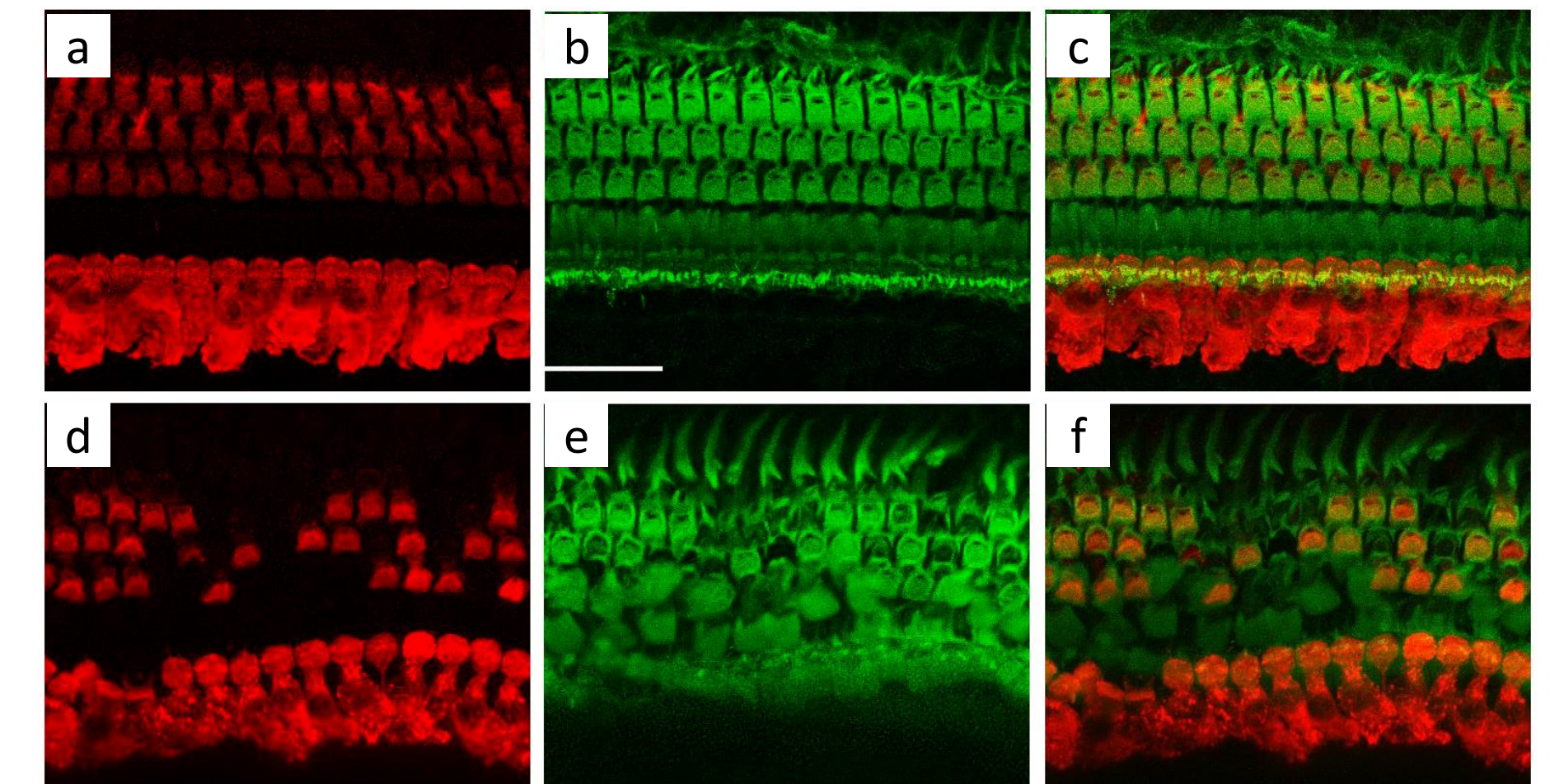
Figure 2. Blast exposure impaired ABR



ABR data showed a significant reduction in wave amplitudes after blast exposure (a and b) along with elevation in thresholds in the frequency range of 8000 to 40000 Hz that was observed at 1 day after blast exposure and persisted over 14 days (c). ABR thresholds did not change in mice placed outside the ABS and exposed to the noise only (d).

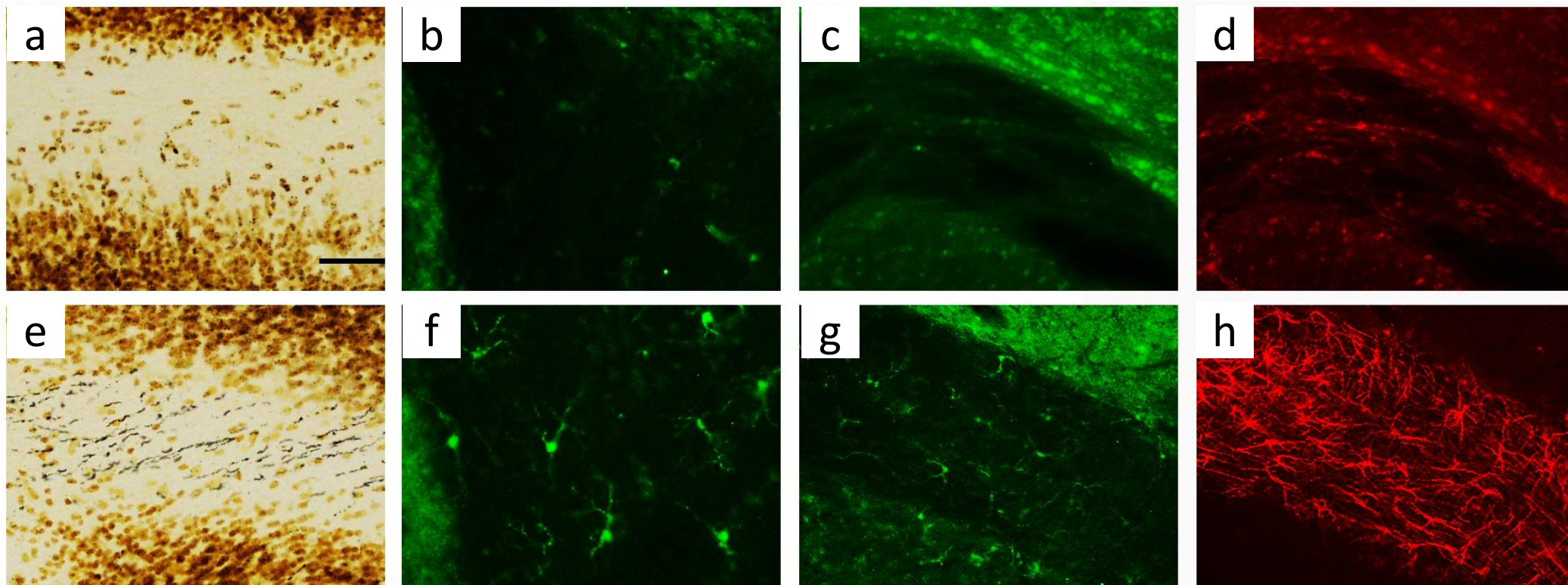
RESULTS

Figure 3. Changes in cochlear hair cells after blast exposure



Blast shockwave damages to outer hair cells was visualized by confocal microscopy; (a - c) sham control, (d - f) blast exposed mouse; (a and d) imaging of Myosin VIIa (red), (b and e) imaging of phalloidin (green), (c and f) merged imaging of myosin VIIa and phalloidin; scale bar 25 μ m.

Figure 4. Changes in CNS after blast exposure



Effect of blast shockwave on axons and glial cells in cerebellum (a, b, e, f) and brainstem (c, d, g, h) at 14 days post-injury. Compared to sham control (a - d), blast exposure induces axonal degeneration (e), increases Iba1 (f, g) and GFAP (h), scale bar 100 μ m.

CONCLUSIONS

- Blast shockwaves (19 psi) produced ABR threshold shifts that persist through 14 days. Compared to high frequency (40 kHz) hearing loss after blast exposure, low frequency (8 kHz) hearing recovered early.
- Appreciable damage to cochlear outer hair cells, inner hair cells, and other structures in the inner ear was observed. Blast overpressure generated by the ABS causes mild axonal degeneration and glial cells proliferation.
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ACKNOWLEDGMENTS

This work was supported by CRM RP award W81XWH-15-2-0024.

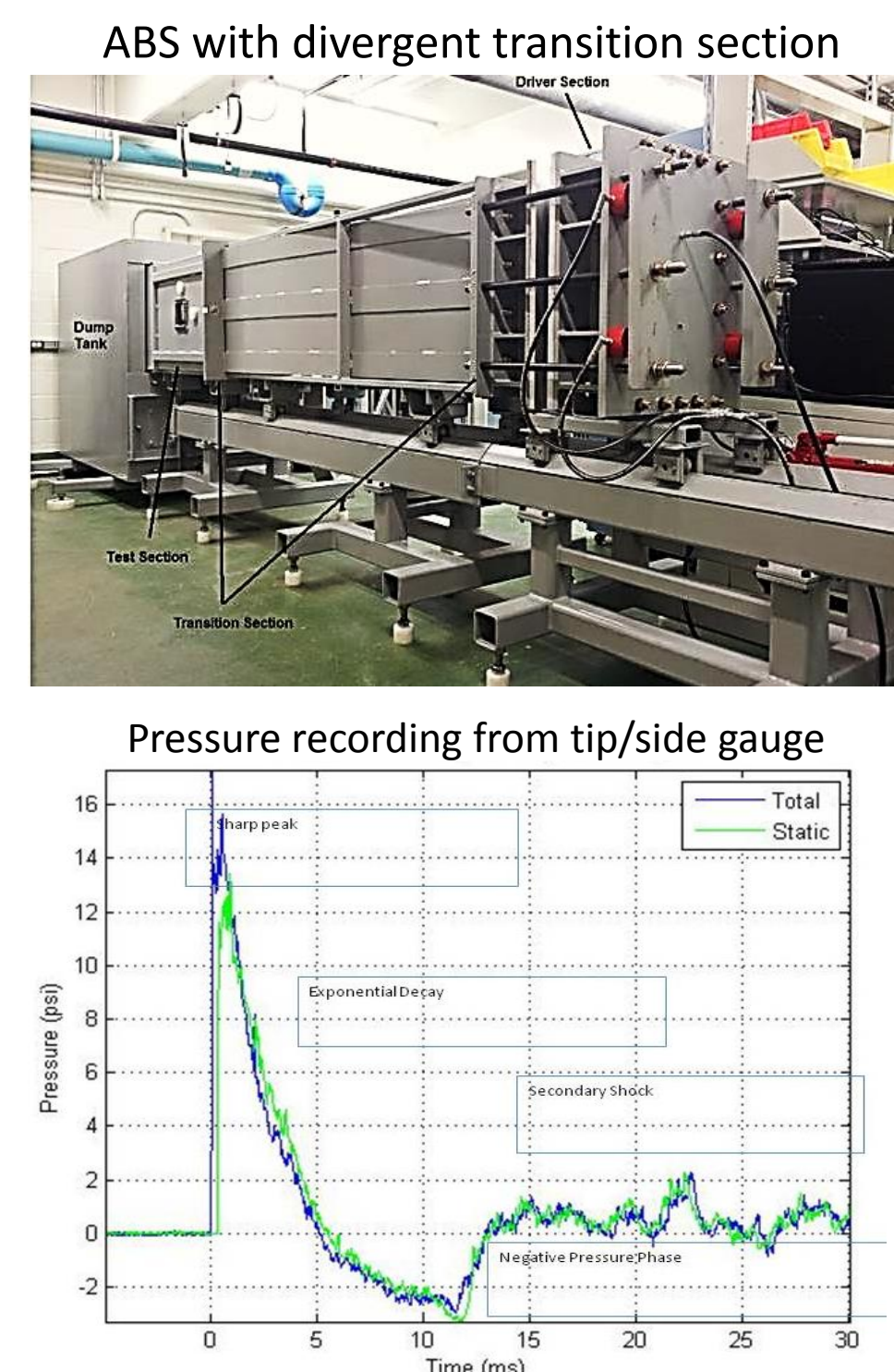


ABSTRACT

A high fidelity animal model is critical to define the mechanism(s) of blast-induced auditory injury and to develop therapeutic strategies. The present research is aimed at producing a comprehensive characterization of auditory functional deficits and associated pathological changes in the peripheral and central auditory signal processing regions disrupted by exposure to blast shockwaves. We have investigated the time-course of blast effects on auditory function and structural changes. Isoflurane anesthetized CBA mice (male, 8 weeks) were exposed to blast overpressure (peak static pressure of 19 psi and 4 msec positive phase duration) generated by the Advanced Blast Simulator (ABS). Auditory function was assessed by analyzing distortion product otoacoustic emission (DPOAE) and auditory brainstem response (ABR) under anesthesia. Data showed that DPOAE signals were undetectable acutely after blast exposure and their disappearance persisted over 14 days that suggests the injuries to the inner ear. Blast exposure caused significant elevations of ABR threshold, increased ABR wave latency, and reductions in ABR wave amplitude immediately following the blast shockwave insult. These changes were observed over the entire acoustic frequency spectrum and persisted over 14 days. Immunostaining of Myo7a and Phalloidin in whole-mount cochlea revealed appreciable damage to hair cells, as well as to other structures in the inner ear. Increases in GFAP, Iba1 and axonal degeneration were detected in the brainstem and cerebellum at 14 days post-injury. The results indicate that both peripheral and central auditory signal processing regions are vulnerable to blast overpressure exposure in the ABS. This mouse model of blast-induced auditory injury should provide a useful experimental tool for studying the mechanisms underlying hearing impairment after blast exposure and for evaluating potential strategies for prevention and cure.

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METHODS

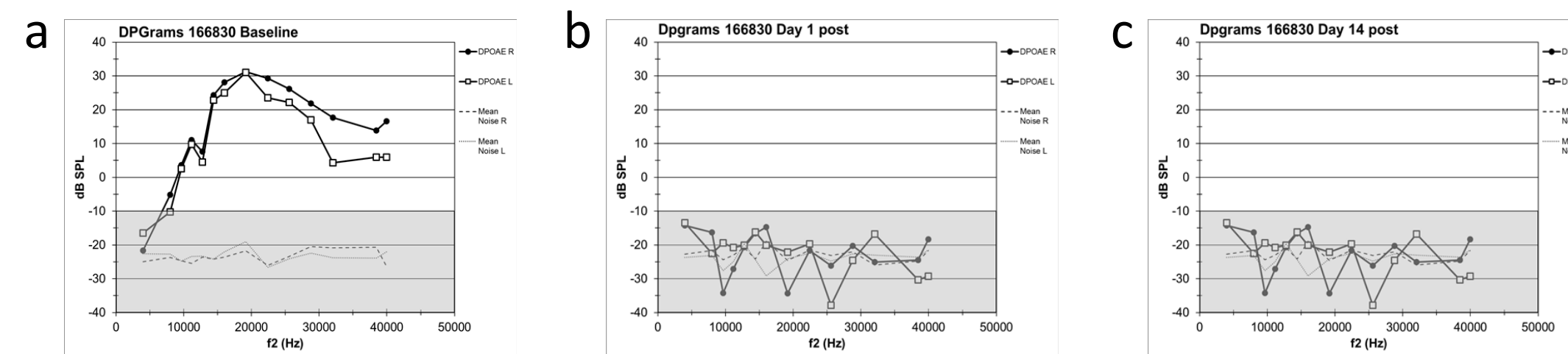
Animals and ABS: After anesthetization with isoflurane for 8 minutes, CBA mice (male, 23 - 28 g) were secured in the ABS in a prone position facing the oncoming shockwave. The blast overpressure (peak static pressure of 19 psi and 4 msec positive phase duration) was generated by Valmex membrane rupture in the ABS. Sham controls were handled similarly but without exposure to the blast.

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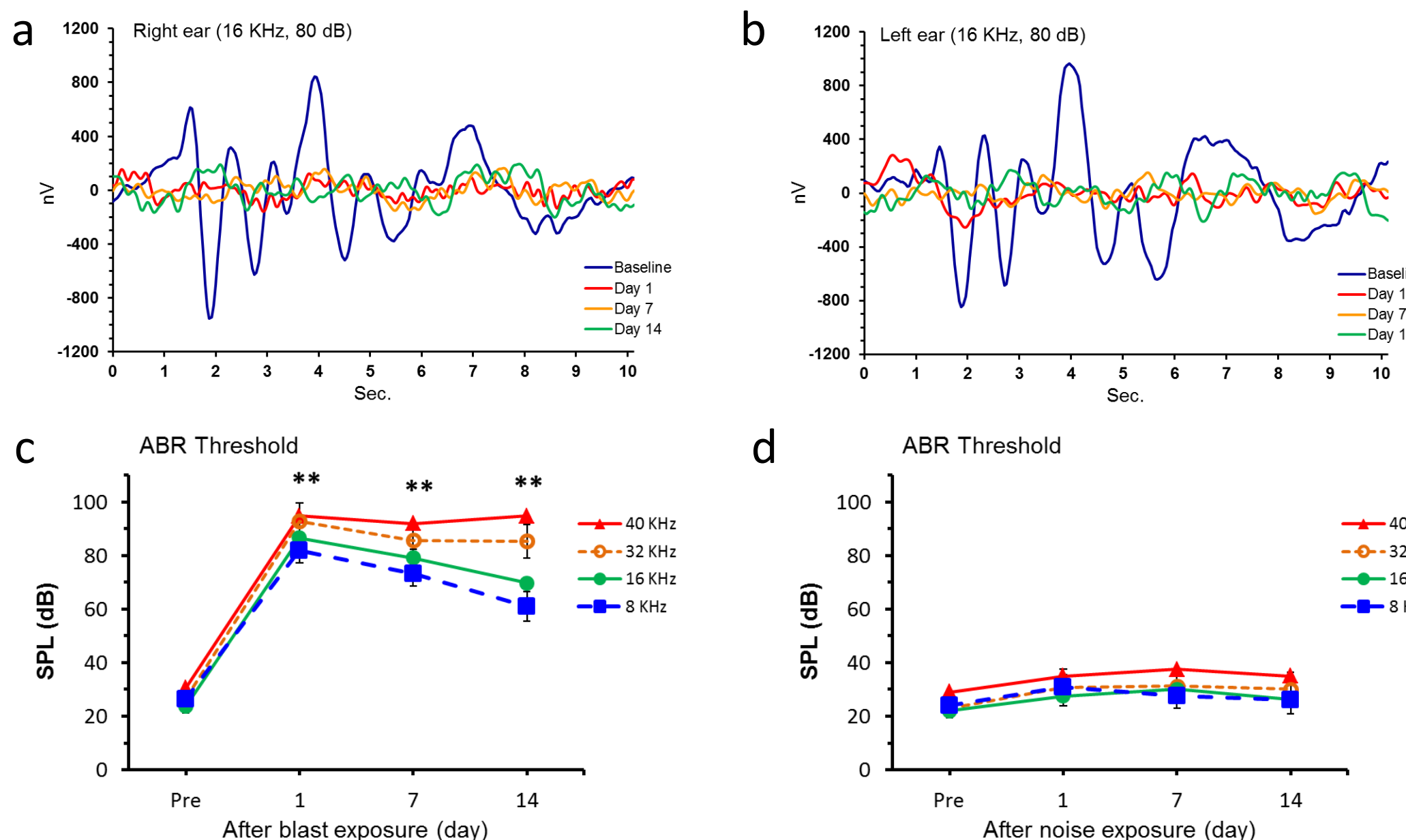
RESULTS

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Compared to its baseline (a), DPOAE was undetectable at 1 day (b) and 14 days (c) post-injury.

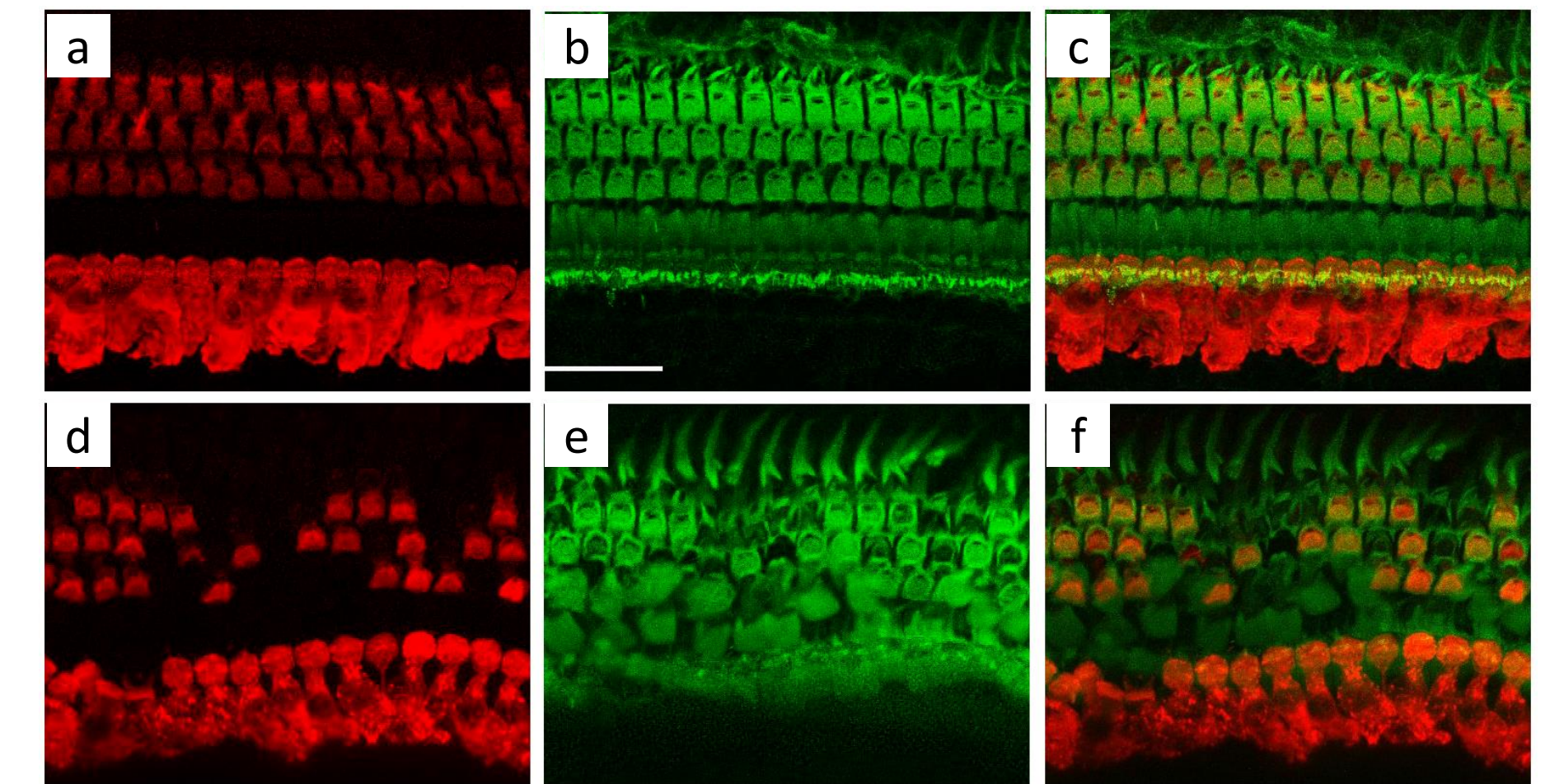
Figure 2. Blast exposure impaired ABR



ABR data showed a significant reduction in wave amplitudes after blast exposure (a and b) along with elevation in thresholds in the frequency range of 8000 to 40000 Hz that was observed at 1 day after blast exposure and persisted over 14 days (c). ABR thresholds did not change in mice placed outside the ABS and exposed to the noise only (d).

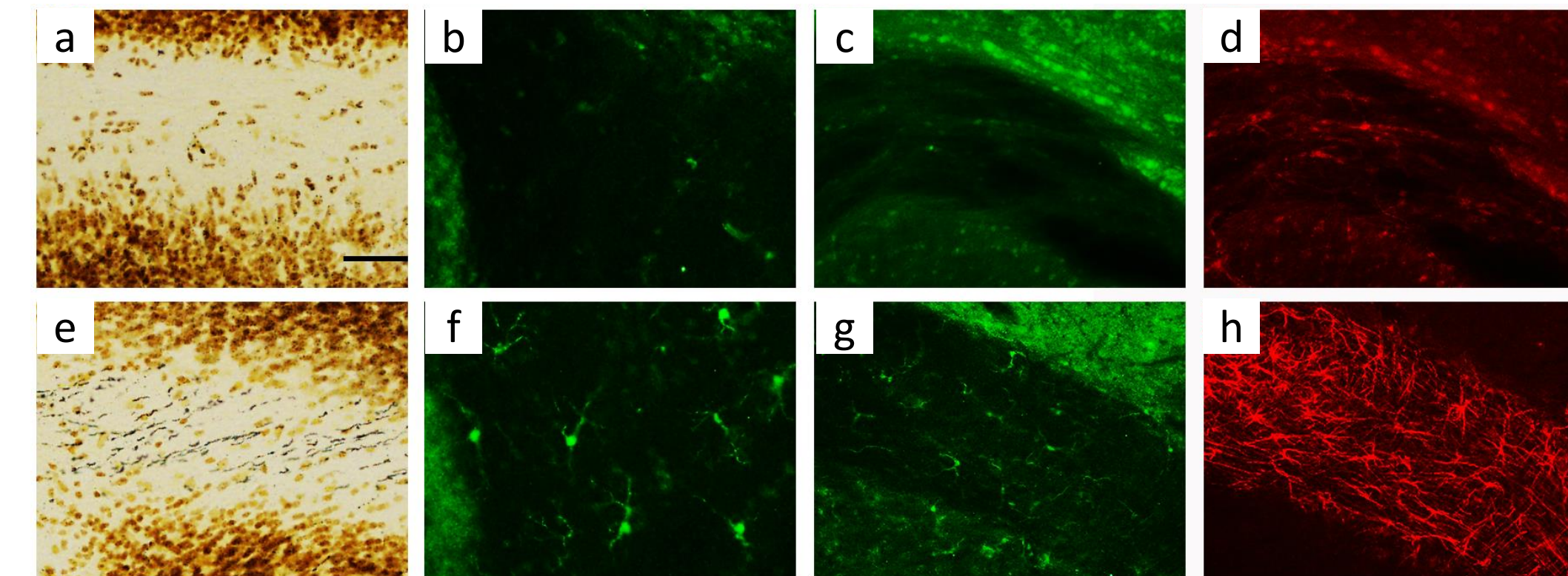
RESULTS

Figure 3. Changes in cochlear hair cells after blast exposure



Blast shockwave damages to outer hair cells was visualized by confocal microscopy; (a - c) sham control, (d - f) blast exposed mouse; (a and d) imaging of Myosin VIIa (red), (b and e) imaging of phalloidin (green), (c and f) merged imaging of myosin VIIa and phalloidin; scale bar 25 μ m.

Figure 4. Changes in CNS after blast exposure



Effect of blast shockwave on axons and glial cells in cerebellum (a, b, e, f) and brainstem (c, d, g, h) at 14 days post-injury. Compared to sham control (a - d), blast exposure induces axonal degeneration (e), increases Iba1 (f, g) and GFAP (h), scale bar 100 μ m.

CONCLUSIONS

- Blast shockwaves (19 psi) produced ABR threshold shifts that persist through 14 days. Compared to high frequency (40 kHz) hearing loss after blast exposure, low frequency (8 kHz) hearing recovered early.
- Appreciable damage to cochlear outer hair cells, inner hair cells, and other structures in the inner ear was observed. Blast overpressure generated by the ABS causes mild axonal degeneration and glial cells proliferation.
- This mouse model of blast-induced auditory injury should provide a useful experimental tool for studying the mechanism of hearing impairment after blast exposure and for evaluating potential strategies for prevention and cure.

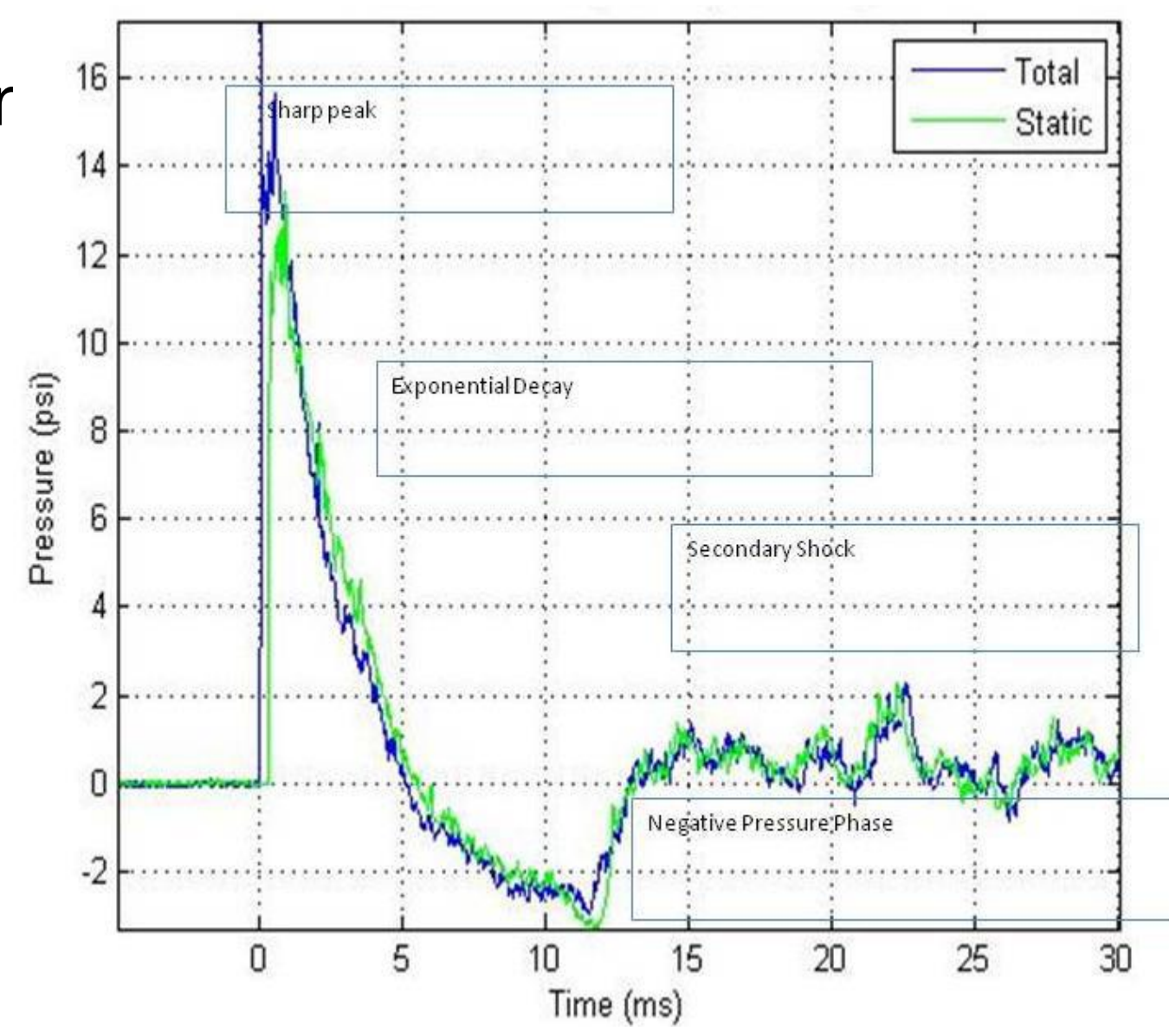
ACKNOWLEDGMENTS

This work was supported by CRM RP award W81XWH-15-2-0024.

of blast exposure in the field. However, small sample sizes in previous studies have limited our understanding of compounding effects to the inner ear from blast exposure. A better understanding of the etiology of vestibular and auditory damage sustained to the inner ear after exposure to blast overpressure could lead to the development of new treatment strategies. Previous studies of the effect of exposure to a concussive blast often used conventional shocktubes to mimic blast wave forms. However, older open ended shock tubes failed to precisely mimic explosive blast waveforms because of exposure of test subjects to additional stimuli arising from reverberation of the blast tube. Therefore, an Advanced Blast Simulator (ABS) with a high fidelity model accurately replicating key aspects of wave forms and flow was developed. Using a mouse model, we have investigated the effects of ABS exposure on the inner ear.



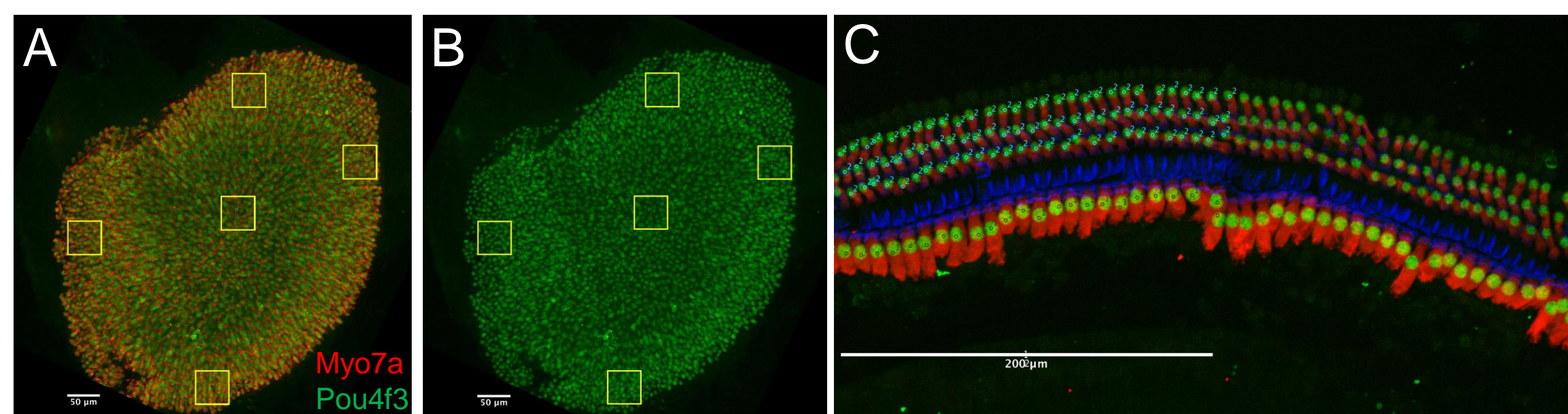
Advanced Blast Simulator (ABS)



Pressure recording measurement from side gauges. Includes total and static pressure, which dynamic pressure can be calculated by the difference of the two.

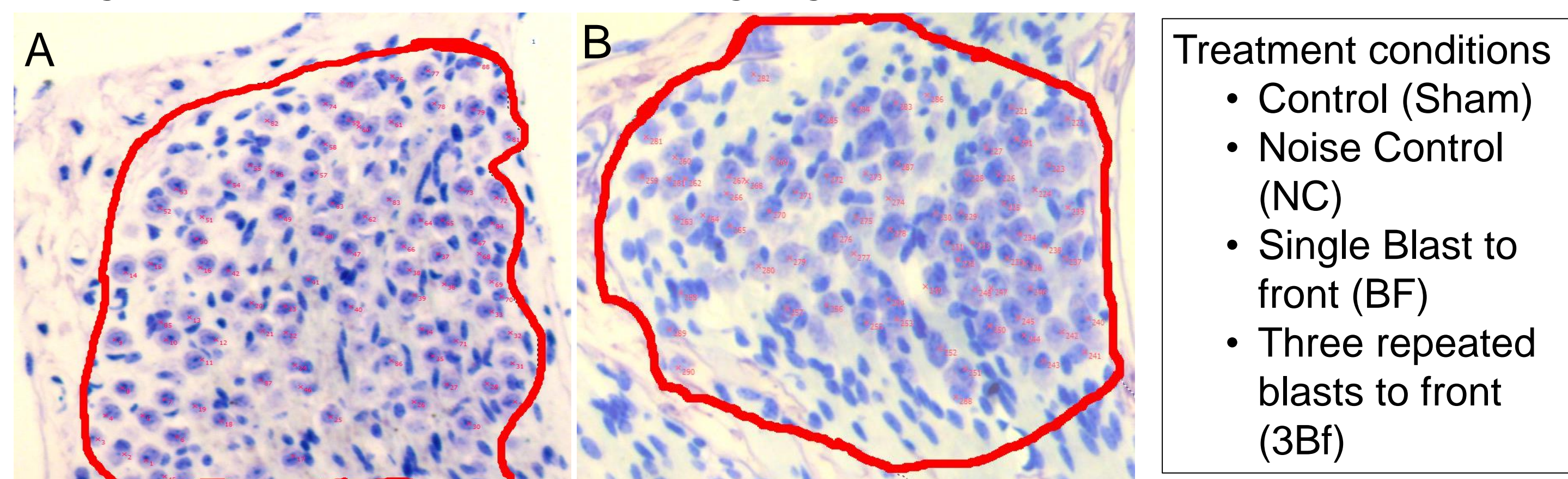
Methods

Figure 1: Quantification of number of hair cells

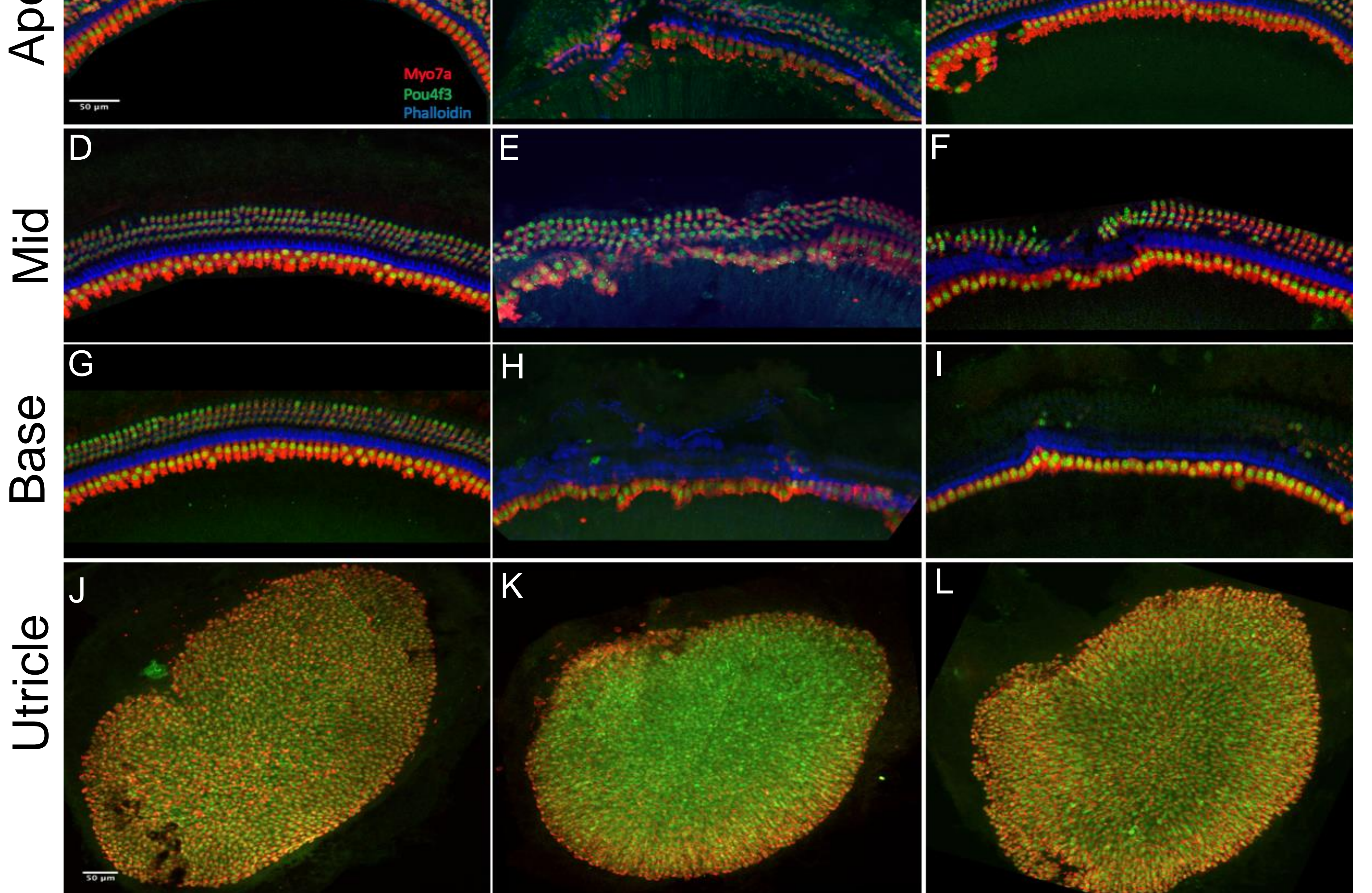


To assess the severity of the physical damage to the hair cells in sensory organs of the inner ear we use FIJI software to quantify number of vestibular and auditory hair cells. Using anti-Myo7a and anti-Pou4f3 staining in (A) & (B) utricular hair cells are counted using 5 boxes of 2500 μm^2 each. Cochlear hair cells (C) are counted per 200 μm , distinguishing inner hair cells (marked in blue) and outer hair cells (cyan).

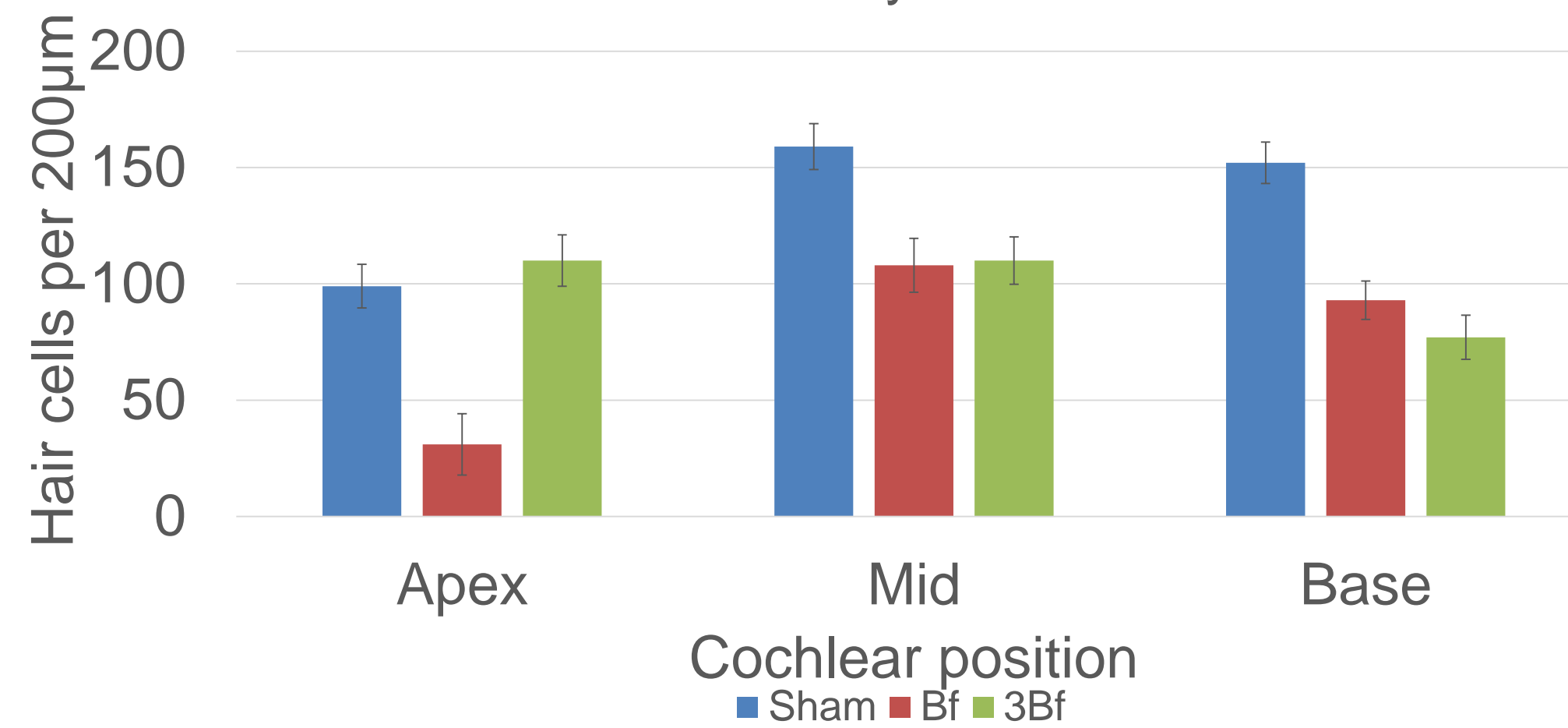
Figure 2: Quantification of spiral ganglion neurons



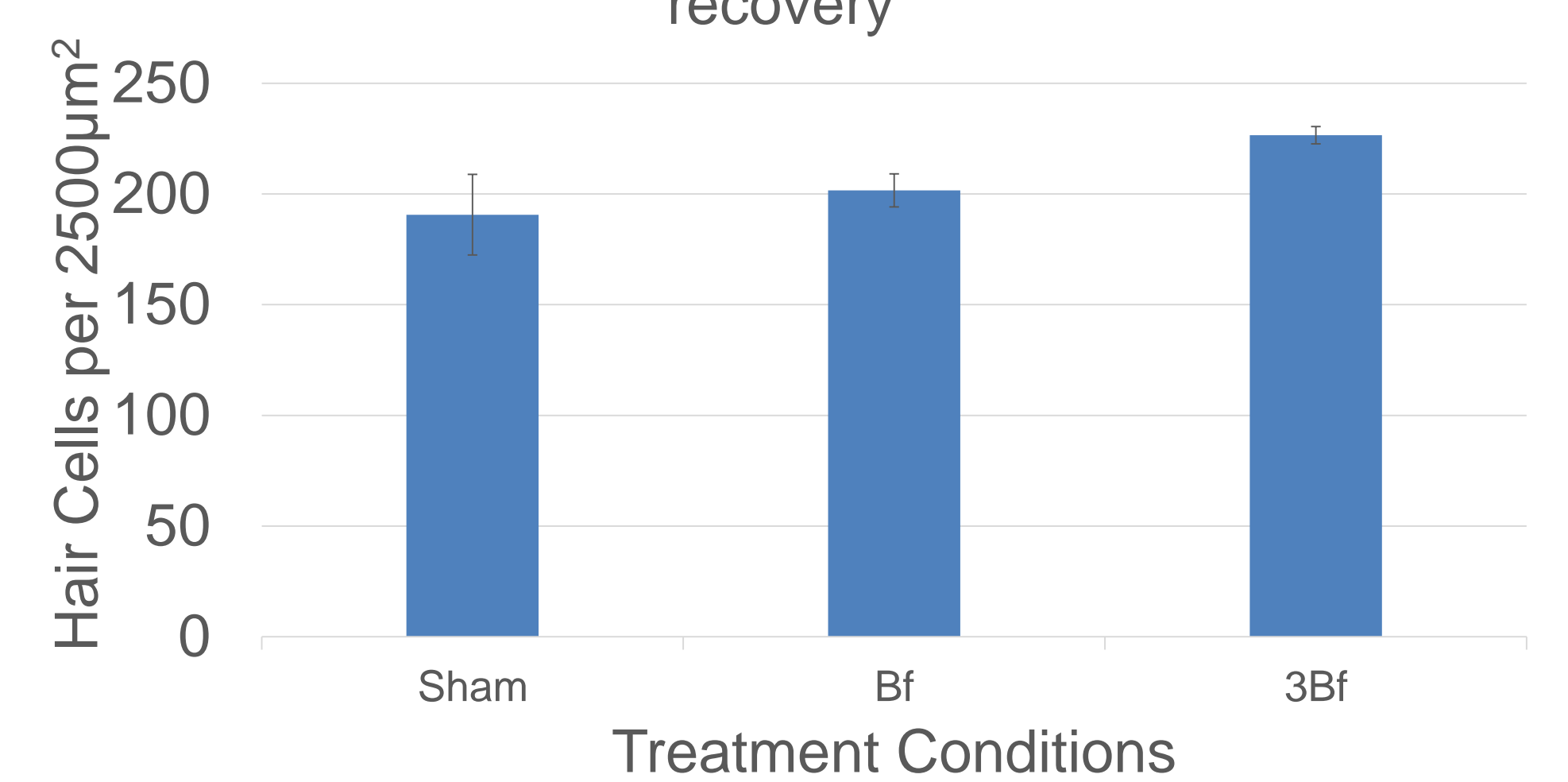
Spiral ganglion neurons, as illustrated in A & B are counted in multiple regions of interest (ROI) to gain an understanding of the density of neurons for each mouse treatment. Using Velocity software for total cell counts within an ROI, we can take the area to find the density of cells/ μm^2 .



M Cochlea hair cell count following 1 month recovery

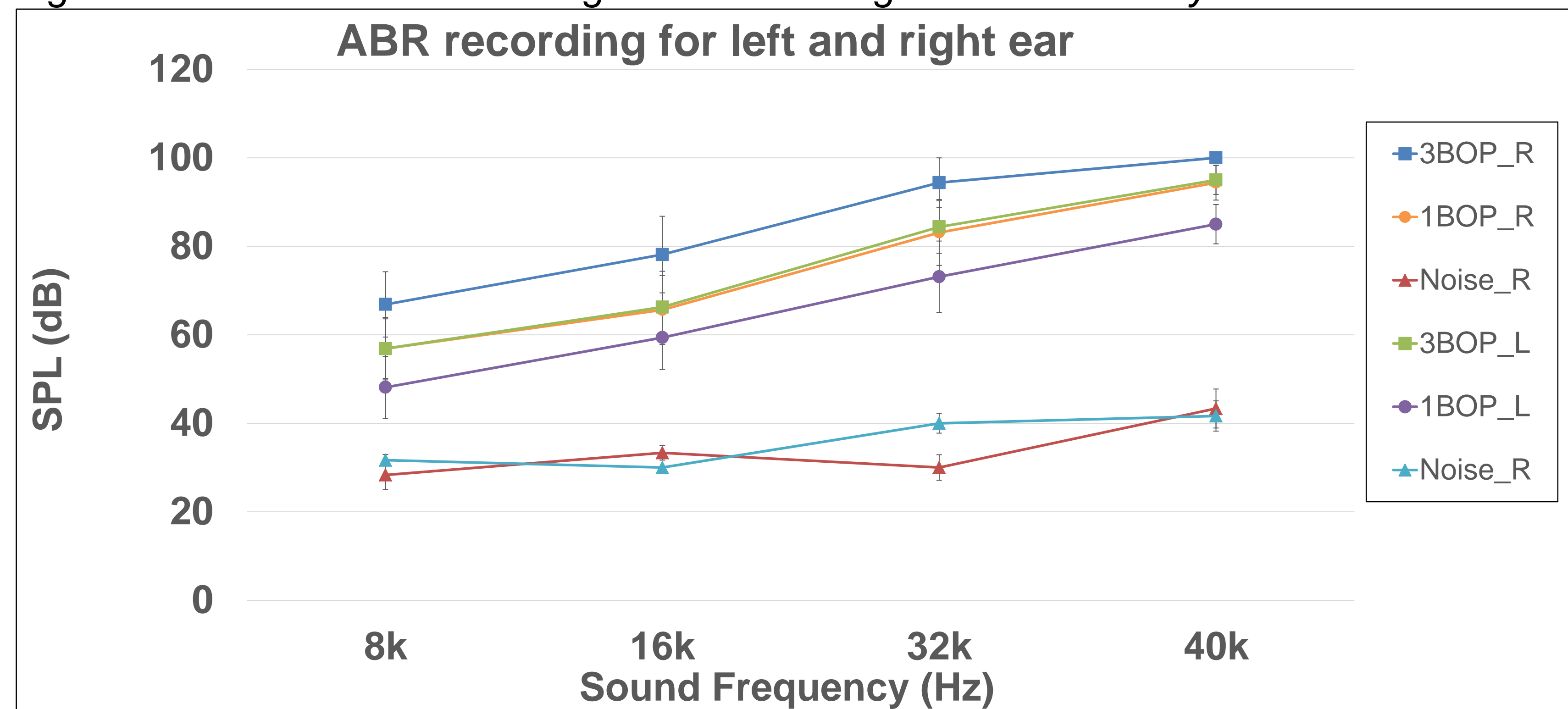


N Utricle hair cell count following 1 month recovery



A-I. Images of the organ of Corti at the indicated positions and for the indicated treatments. All images are following a one month recovery period. Myo7a, Phalloidin, and Pou4f3 are used to visualize any morphological changes to hair cells. J-L. Images of the entire utricle from similarly treated samples. No significant changes in hair cell number are present. M. Summary of changes in hair cell number at the indicated positions and in total for the indicated treatments following a one month recovery. Significant decreases in hair cell number were observed in the basal and middle regions. N. Summary of changes in hair cell number in the utricle, using the sampling paradigm described in Figure 1. No significant changes were observed.

Figure 4: ABR threshold recordings made following 1 month recovery



ABR threshold recordings in mice exposed to a single blast, three repeated blast (3BOP), or no blast control (NC) following 1 month of recovery. Ears exposed to blast showed a significant threshold shift that increased slightly at higher frequencies.



INTRODUCTION

A high fidelity animal model is critical to definition of the mechanism(s) of blast-induced auditory injury and development of therapeutic strategies. The Advanced Blast Simulator (ABS) incorporates design features which allow high fidelity replication of the key features of blast wave flow conditions, including the negative phase and secondary shock. Using this device, the present research was aimed at producing a comprehensive characterization of auditory functional deficits in mice and the associated pathological changes in the inner ear and central sound processing regions disrupted by exposure to blast shockwaves. To determine the effects of sound generated by the ABS on auditory function, a group of anesthetized mice was placed immediately outside the ABS during shock wave generation. Auditory function was assessed 3 days before blast exposure and post-injury at 1, 7, 14 and 28 days by auditory brainstem response (ABR) testing and distortion product otoacoustic emission (DPOAE) testing. Elevations of the ABR threshold and hearing loss were observed at 1 day post-exposure and persisted through 28 days. Pathological outcomes revealed that blast exposure caused appreciable damage to outer hair cells, inner hair cells and other structures in the inner ear, as well as axonal degeneration and glia proliferation in auditory signal processing regions were detected. This mouse model of blast-induced auditory injury should provide a useful experimental tool for studying the mechanisms underlying hearing impairment after blast exposure and for evaluating potential strategies for prevention and cure.

METHODS

Animals and ABS: Isoflurane anesthetized CBA mice (male, 23 - 28 g) were secured in the ABS (Fig. 1) in a prone position facing the oncoming shockwave. The blast overpressure (peak static pressure of 19 psi and 4 msec positive phase duration) was generated by Valmex membrane rupture in the ABS. Noise controls were placed outside of the blast chamber after anesthesia. Sham controls were handled similarly without exposure to the blast.

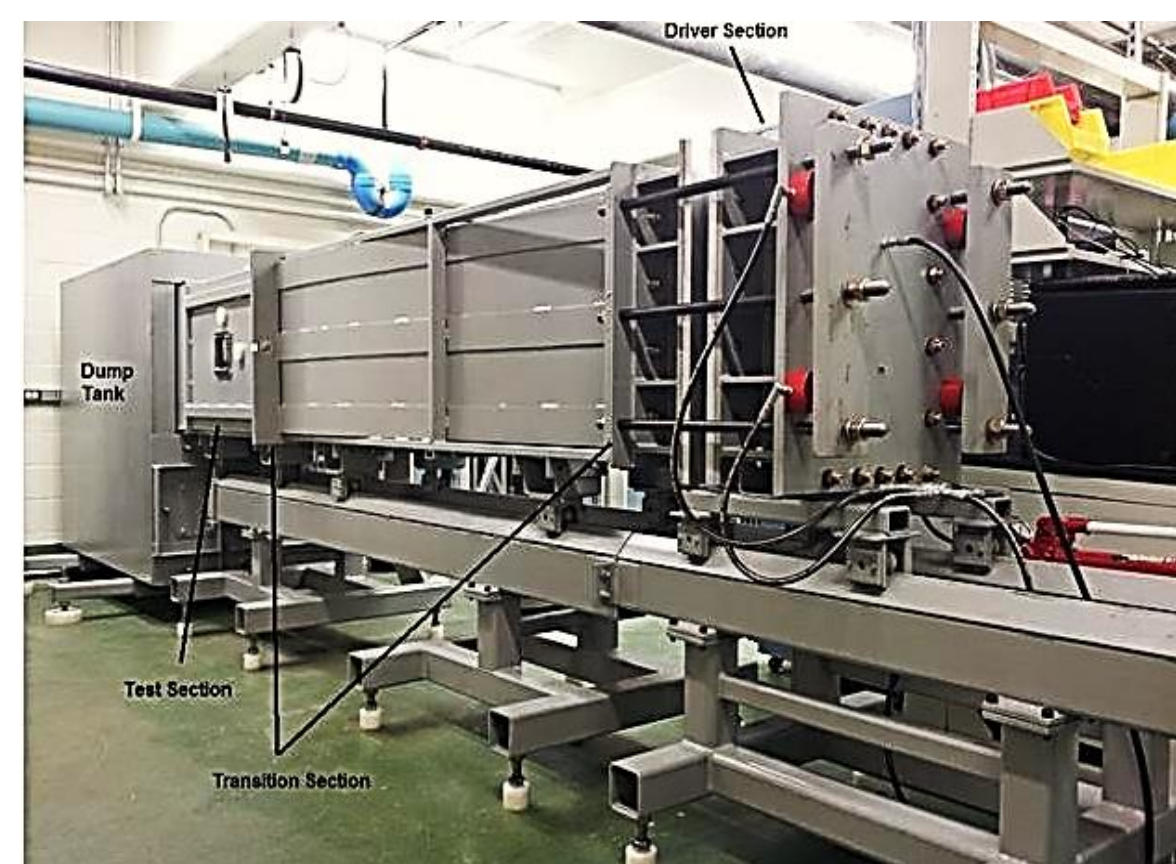


Fig. 1. ABS with divergent transition section

Auditory functional assessment: A time-course of blast effects on auditory function was assessed by analyzing auditory brainstem response (ABR) and distortion product otoacoustic emission (DPOAE) under Ketamine/Dexdomitor anesthesia.

Pathological investigation: At 14 or 28 days post-injury, inner ears were proceeded whole-mount cochlea or cross section for morphological evaluation. Brain sections were evaluated after silver staining and immunohistochemistry.

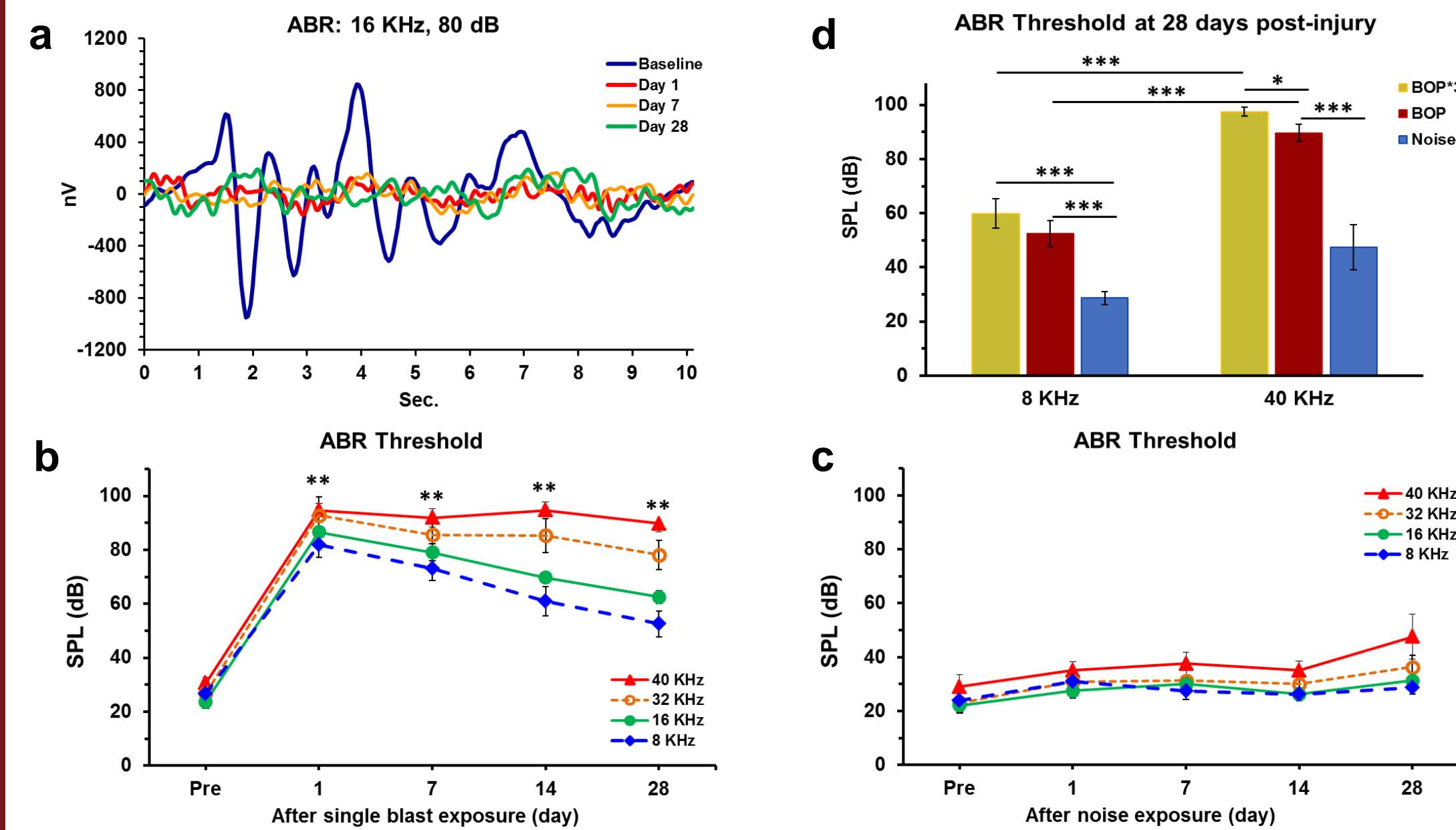


Fig. 2. Blast exposure impaired ABR. ABR data showed a significant reduction in wave amplitudes after a single blast exposure (a) along with elevation in thresholds in the frequency range from 8 to 4 KHz which were observed at 1, 7, 14 and 28 days after blast exposure that were compared with noise controls (c). ABR threshold among the groups of single, 3-blasts and noise exposure at 28 days post exposure. * p<0.05. *** p<0.0005.

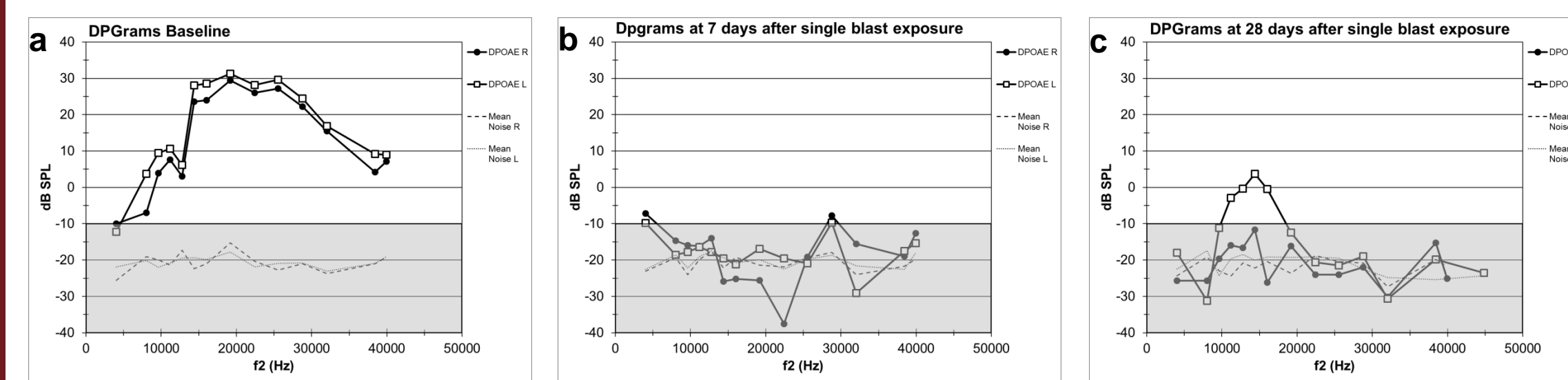


Fig. 3. Blast exposure impaired DPOAE. Compared to its baseline (a), DPOAE was undetectable at 7 days (b) and partially recovered of left ear at 28 days (c) post-injury.

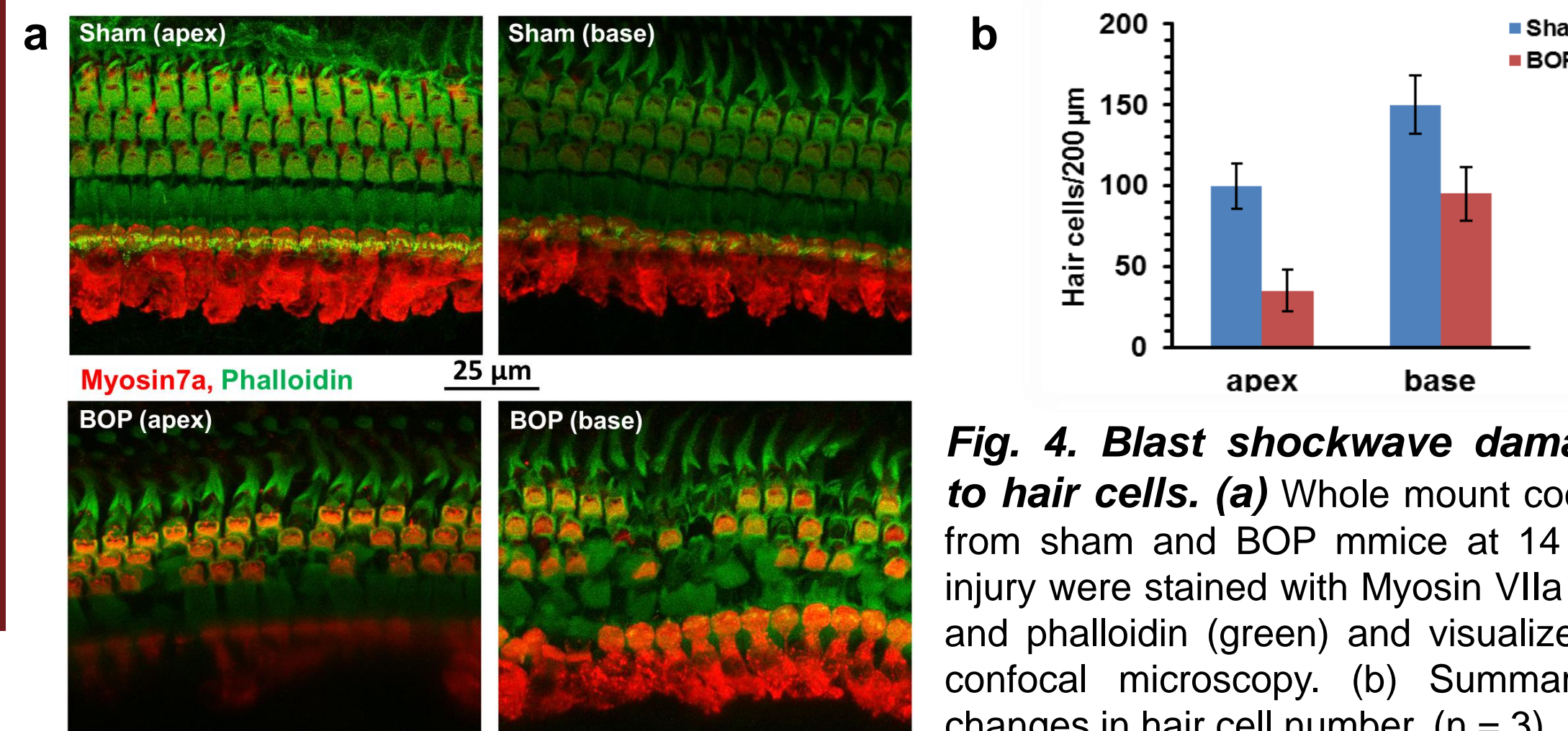


Fig. 4. Blast shockwave damages to hair cells. (a) Whole mount cochlea from sham and BOP mmice at 14 after injury were stained with Myosin VIIa (red) and phalloidin (green) and visualized by confocal microscopy. (b) Summary of changes in hair cell number. (n = 3)

RESULTS

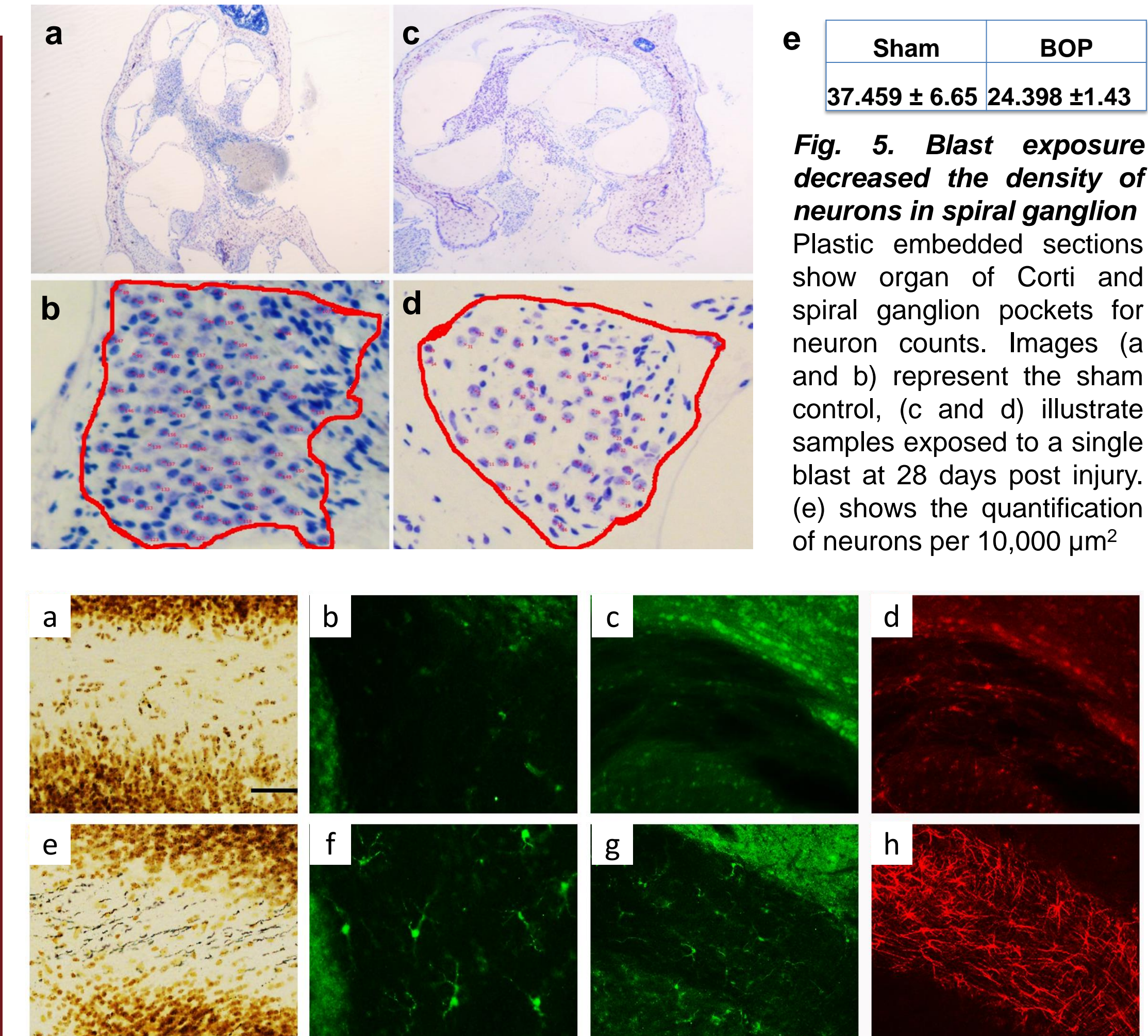


Fig. 5. Blast exposure decreased the density of neurons in spiral ganglion. Plastic embedded sections show organ of Corti and spiral ganglion pockets for neuron counts. Images (a and b) represent the sham control, (c and d) illustrate samples exposed to a single blast at 28 days post injury. (e) shows the quantification of neurons per 10,000 μm²

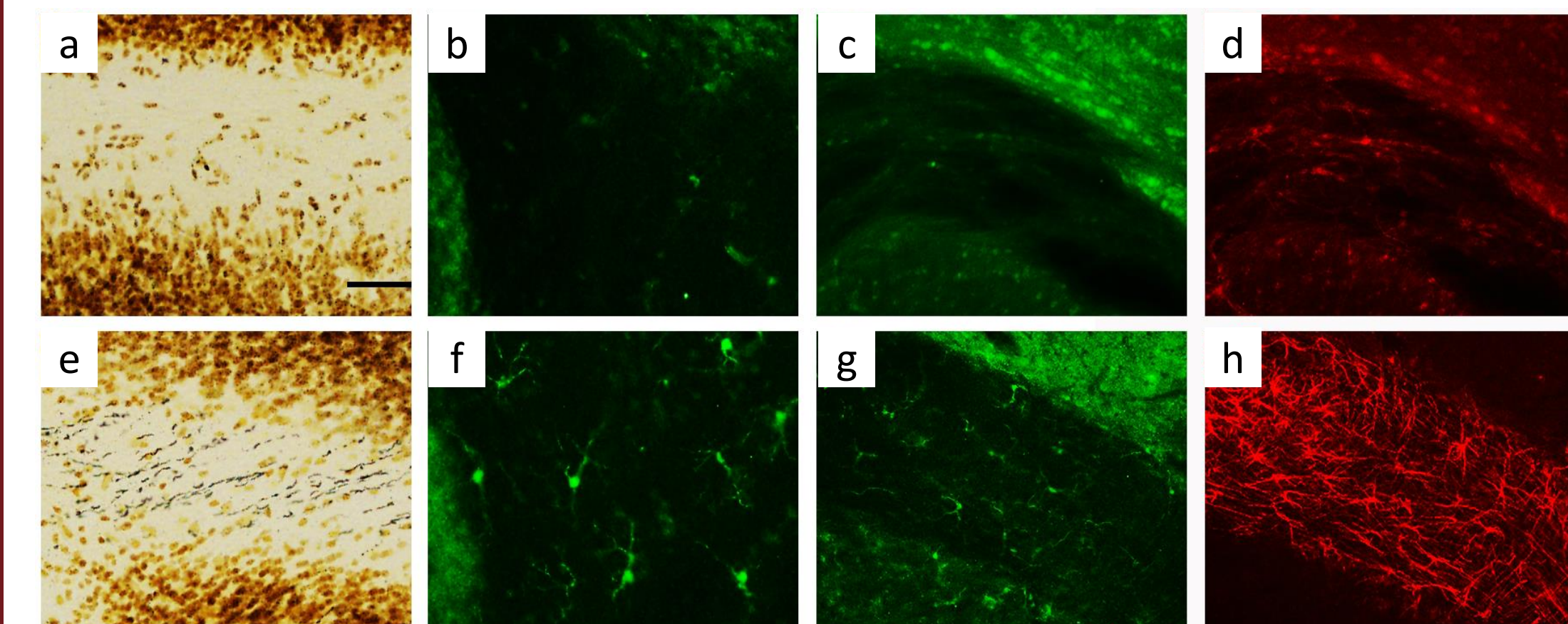


Fig. 6. Changes in CNS after blast exposure. Effect of blast shockwave on axons and glial cells in cerebellum (a, b, e, f) and brainstem (c, d, g, h) at 14 days post-injury. Compared to sham control (a – d), blast exposure induces axonal degeneration (e), increases Iba1 (f, g) and GFAP (h), scale bar 100 μm.

CONCLUSIONS

- Blast shockwaves (19 psi) produced ABR threshold shifts that persist through 28 days. Compared to high frequency (40 kHz) hearing loss after blast exposure, low frequency (8 kHz) hearing recovered early.
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ACKNOWLEDGMENTS

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Characterization of Blast Shockwave on Auditory Deficits in Rodents

Ying Wang, M.D

**Blast-Induced Neurotrauma Branch
Center for Military Psychiatry
and Neuroscience**

WRAIR

Walter Reed Army
Institute of Research

Soldier Health • World Health

April 15, 2017



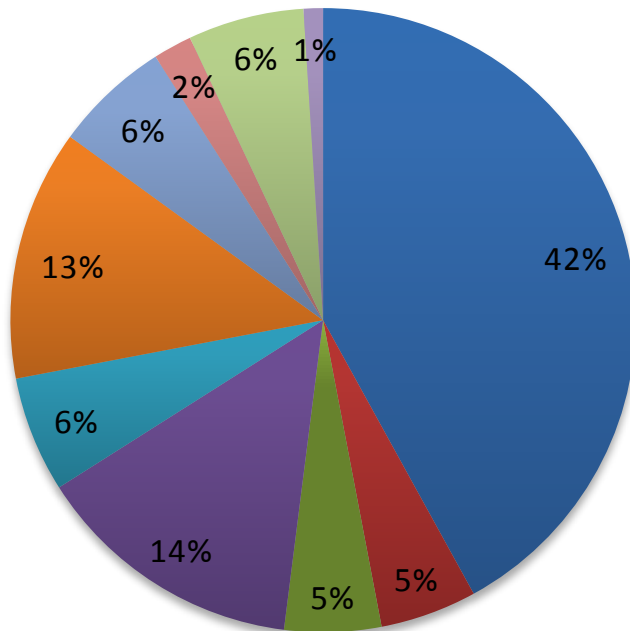
DISCLAIMER

Material has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the author, and are not to be construed as official, or as reflecting true views of the Department of the Army or the Department of Defense. Research was conducted under an IACUC approved protocol in an AAALACi accredited facility in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 2011 edition.

BACKGROUND



Type of hearing loss

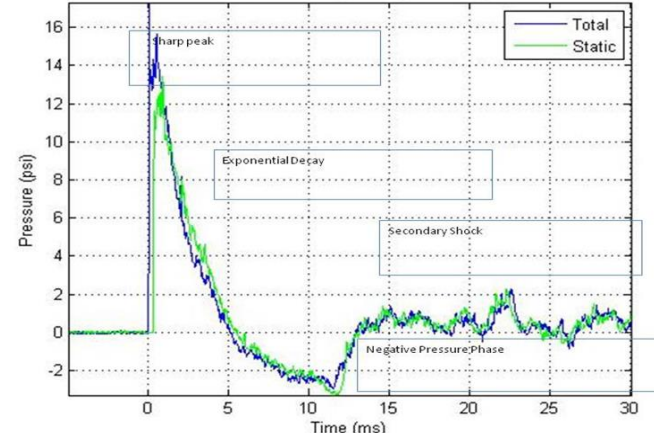
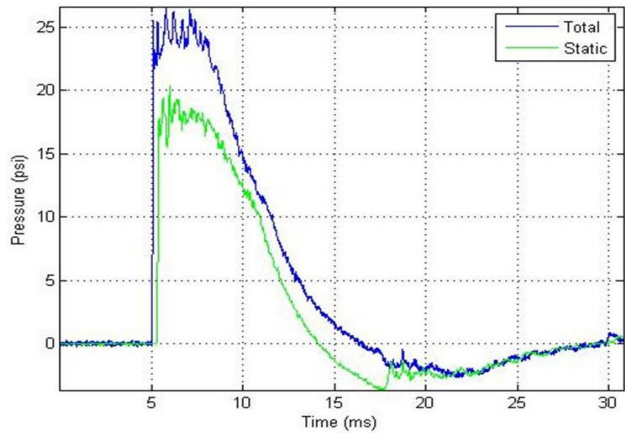


- normal AU
- conductive AU
- mixed AU
- sensorineural AU
- unilateral conductive loss
- unilateral sensorineural loss
- unilateral mixed loss
- sensorineural one ear, conductive one ear
- sensorineural one ear, mixed other ear
- conductive one ear, mixed one ear

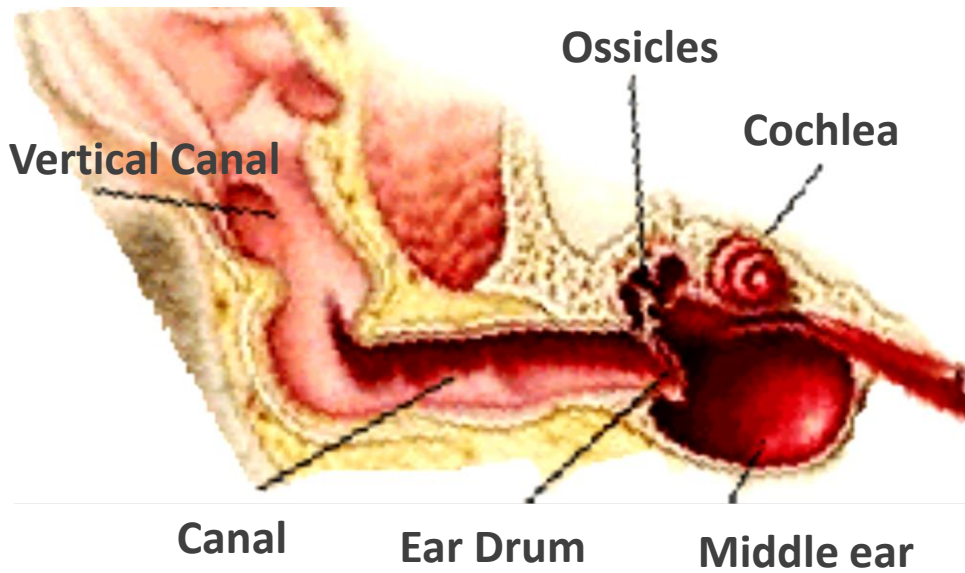
METHODS

- **Animal model: Rat and Mouse exposed to blast overpressure**
- **Auditory function assessment: distortion product otoacoustic emission (DPOAE) and auditory brainstem response (ABR) testing**
- **Microscope and Otoscope: digital macroview otoscope, Olympus SZX16 Stereo microscope, AX-80 and confocal Fv microscope**
- **Brain pathology: immunohistochemistry for brain sections**
- **Inner ear pathology: whole-mount cochlea preparation for viewing the hair cells, H&E staining for the cross-sections**
- **qPCR: RT² Profile PCR array to screen and analyze gene expression**
- **ELISA kits to determine protein levels in cerebrospinal fluid (CSF), plasma and brain tissues**

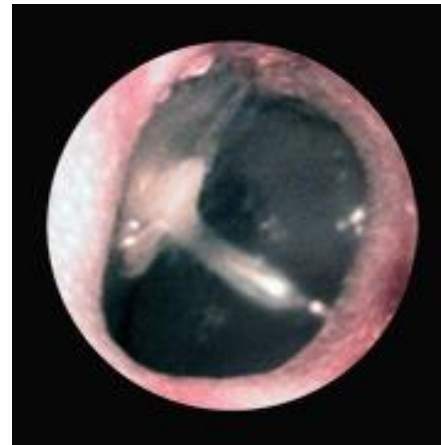
Air-driven shock tube



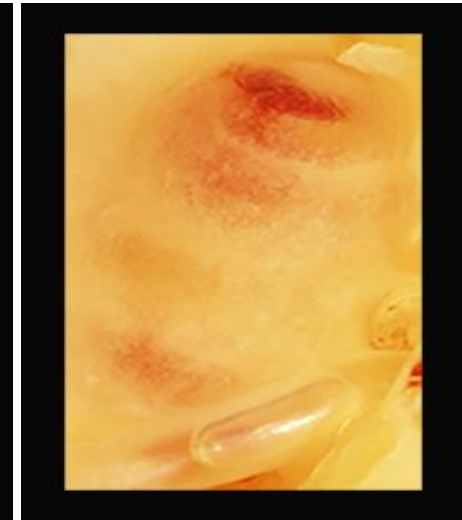
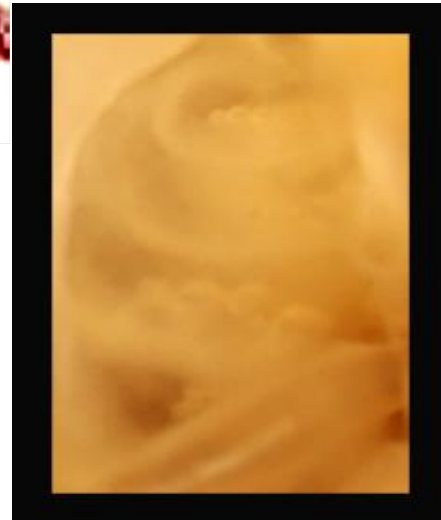
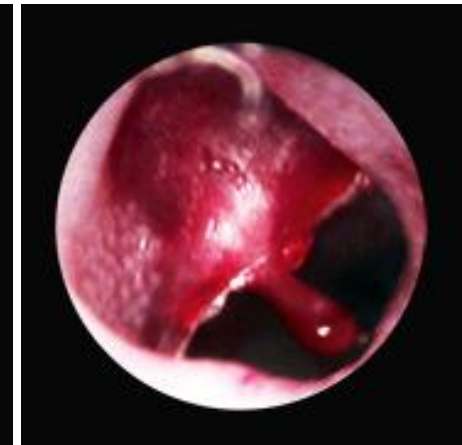
Blast overpressure impaired ear structures



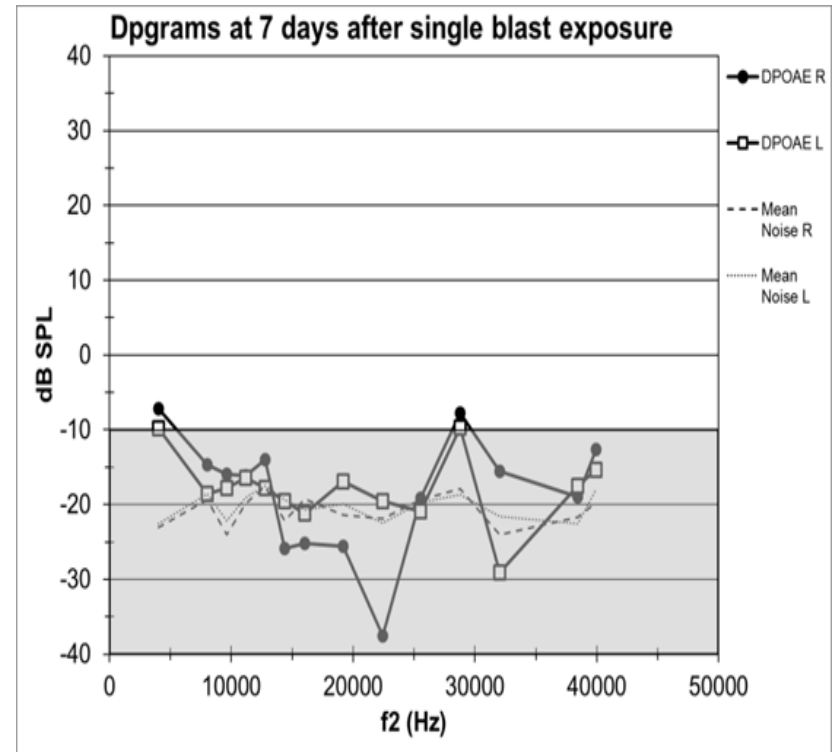
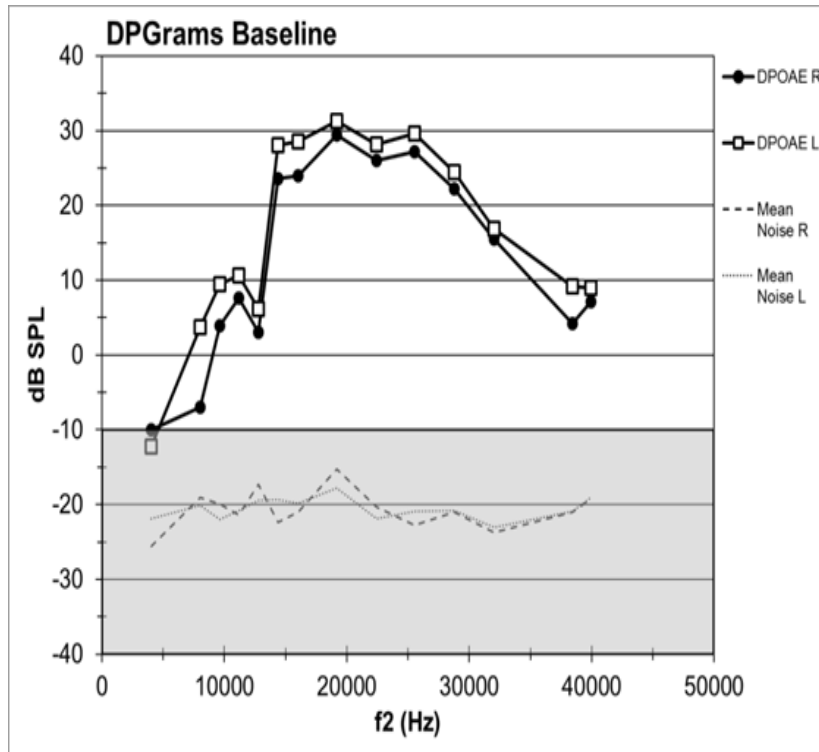
Sham



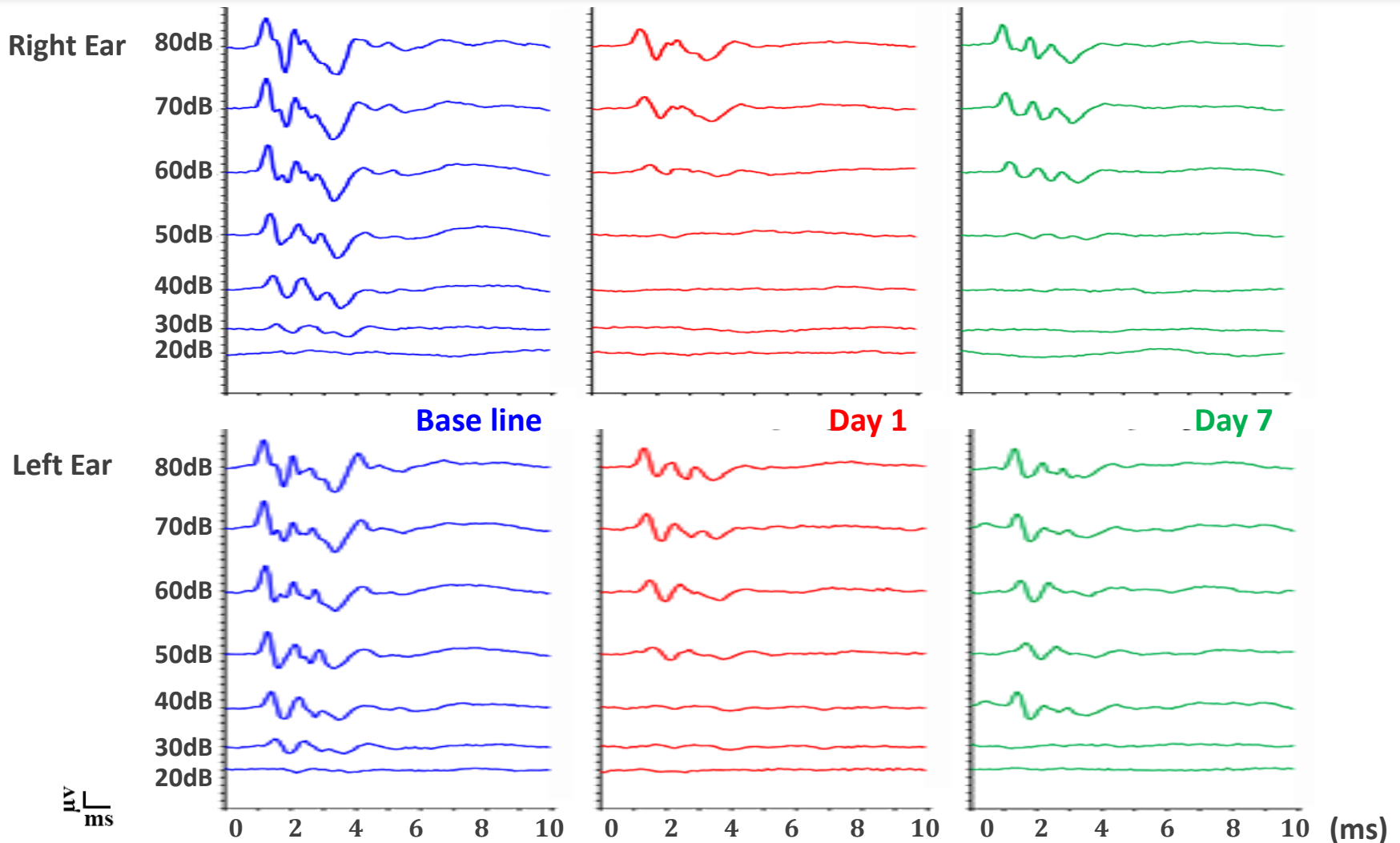
Blast



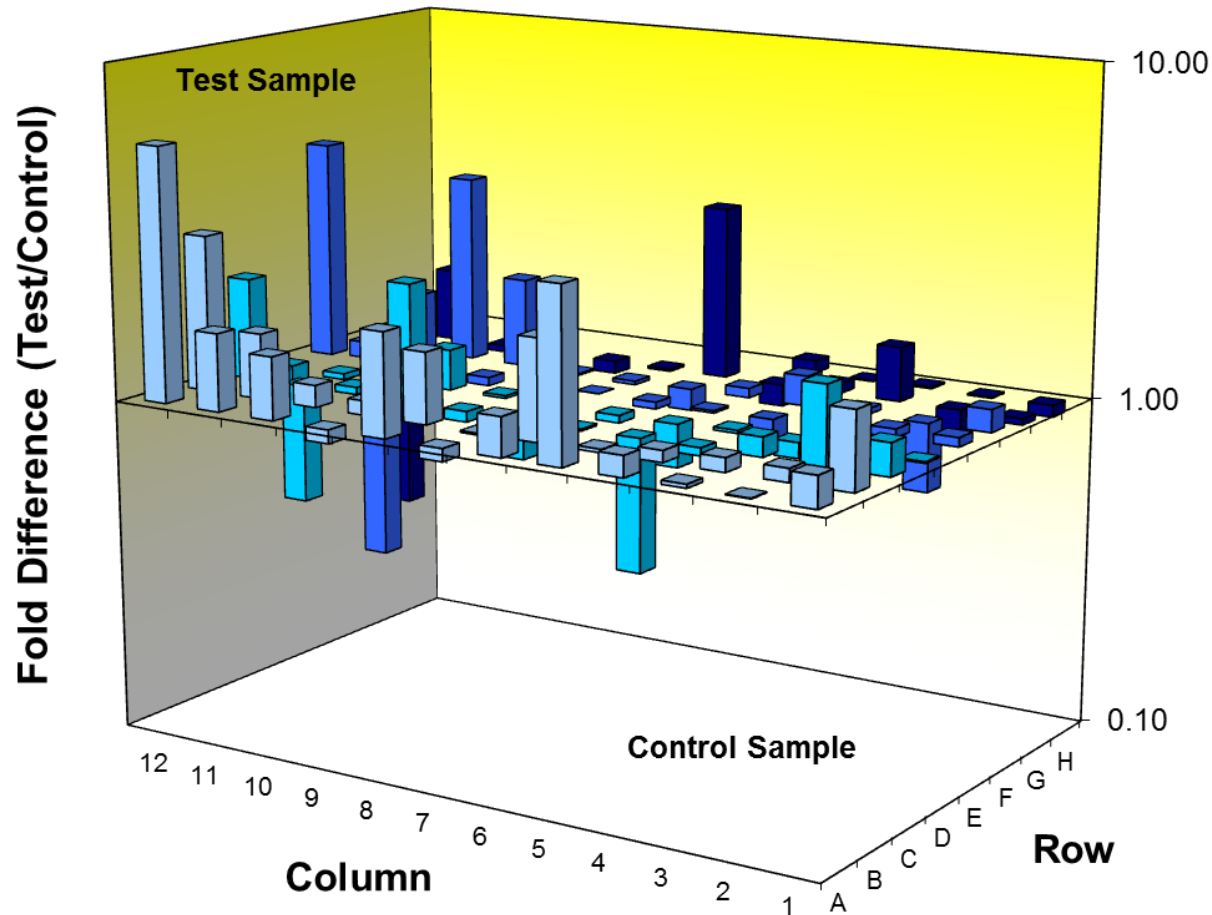
Impact to DPOAE



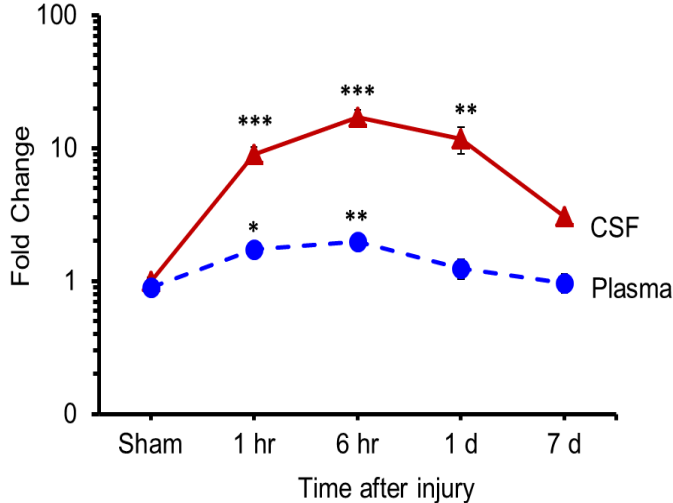
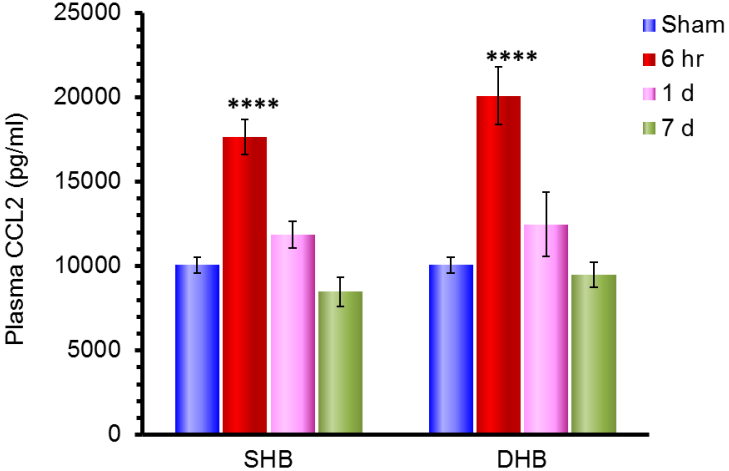
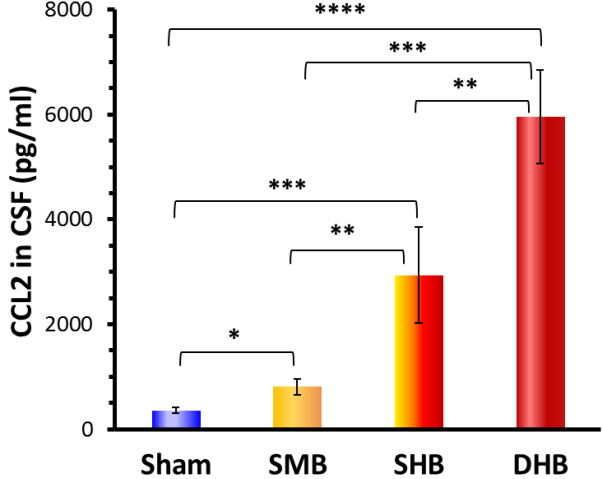
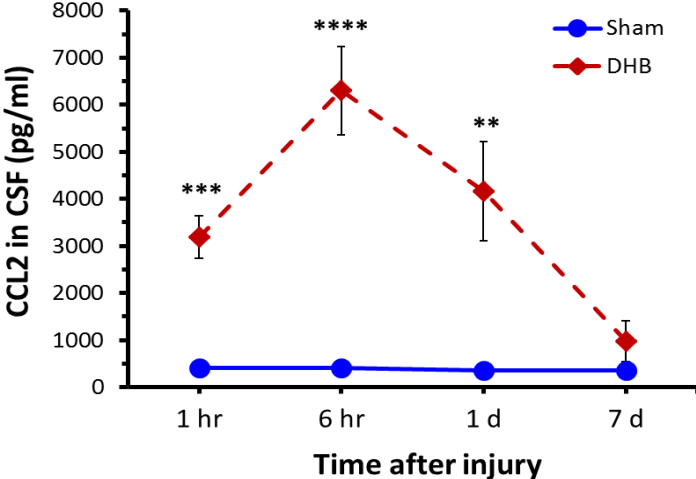
Changes in ABR thresholds



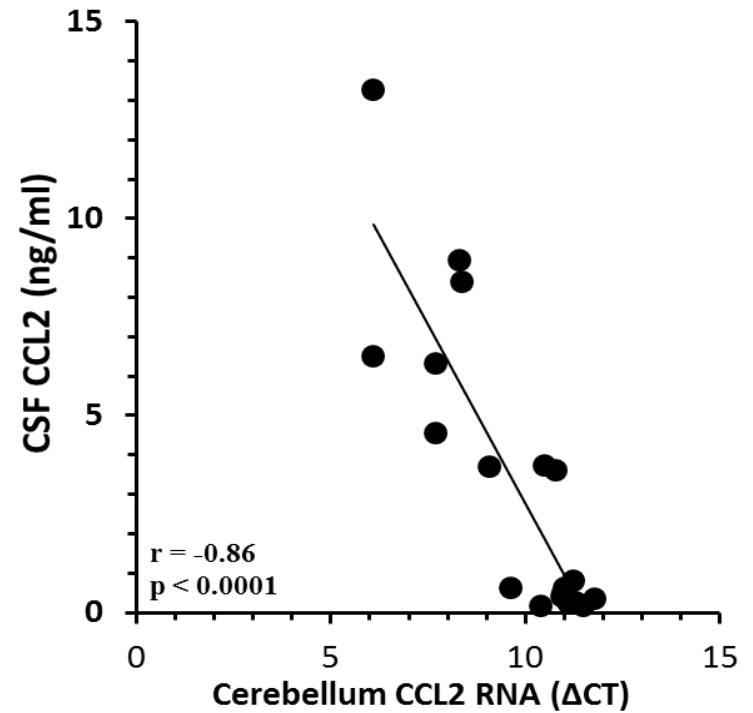
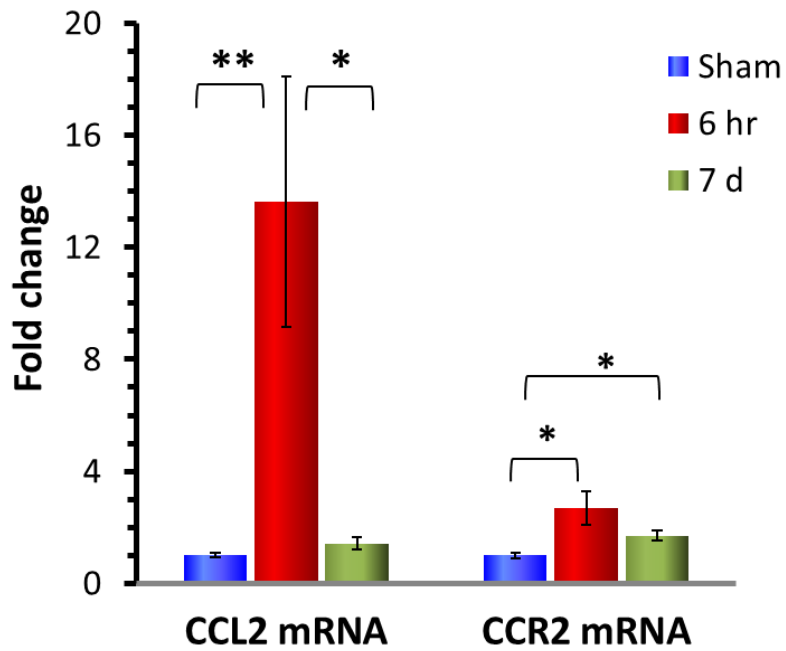
Changes in RNA expression in cochlea and brain



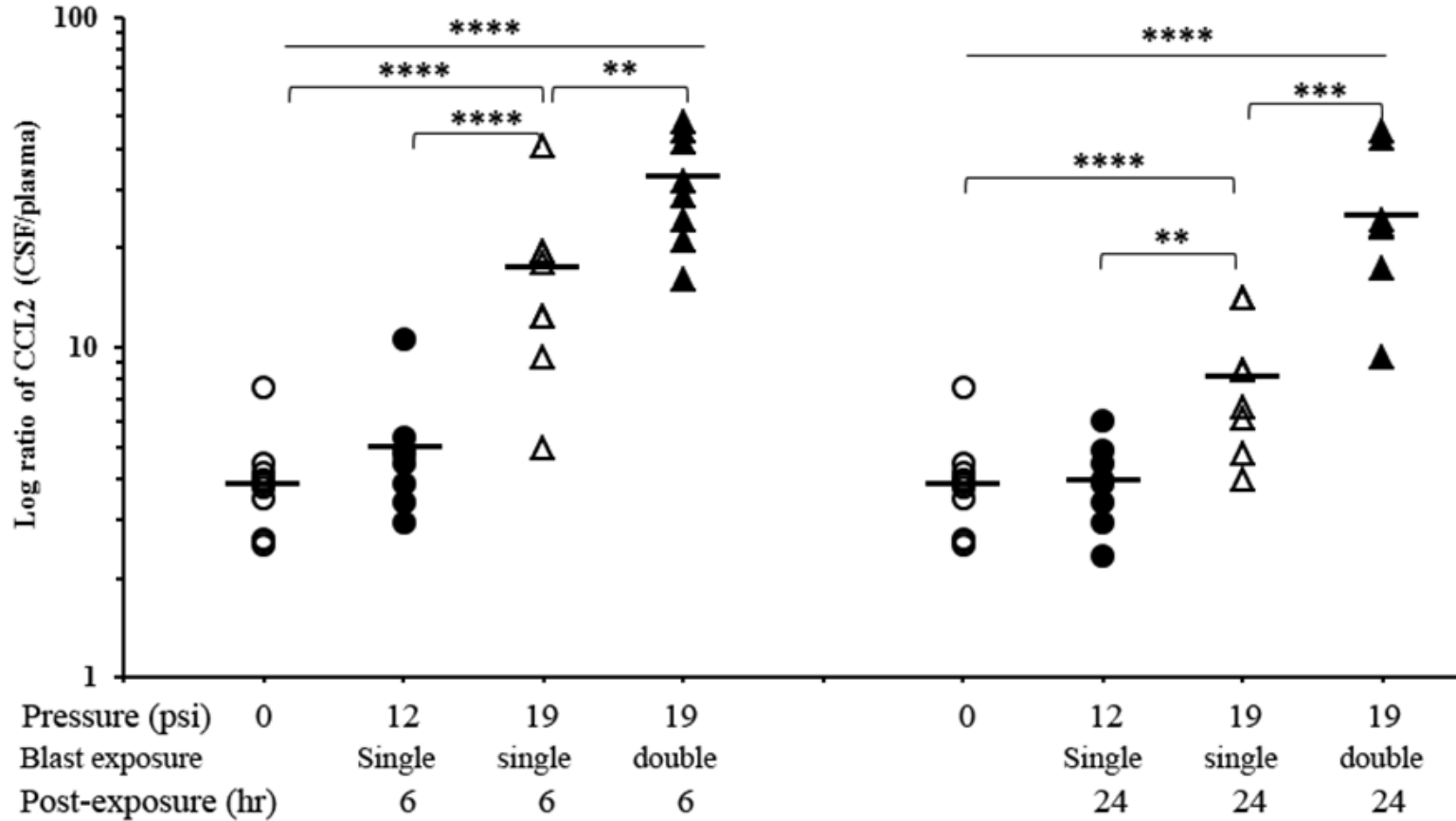
MCP1/CCL2 levels



MCP1/CCL2 levels



Ration of CSF/Plasma



CONCLUSIONS

- Blast exposure induced significant elevations of ABR threshold which were observed over the entire acoustic frequency spectrum and persisted over months. Compared to low frequency (8 kHz) hearing, the impairment on high frequency (40 kHz) hearing was severer.
- Intensity-dependent blast-induced damage to middle and inner ears was evident with no significant differences between left and right ears. Compared to a single blast, double blast exposures increases a damage to ear structures on both sides.
- Tympanic membranes were disrupted immediately following the blast exposure. Labyrinthine hemorrhage was prominent at 1 day up to 14 days post-injury.
- The blast damage to cochlear structure and the loss of hair cells were observed at 1, 7, 14 and 28 days post-injury.
- Inner ear cross sections showed cochlea hemorrhage at 3 days post-injury, and a decrease in density of neurons in spiral ganglia at 28 days after a single blast exposure when compared to the sham control.
- Blast overpressure induced auditory cortex and cochlea inflammation and cell proliferation
- CCL2 levels significate elevated in CSF and plasma with a peak increase around 6 hours post-exposure. Blast-induced the increase in CSF CCL2 was greater than that in plasma. The changes in CCL2 in CSF and in plasma correlated with the levels of CCL2 mRNA in cerebellum, the brain region most consistently neuropathological disrupted by blast insult.



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Thank You!



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

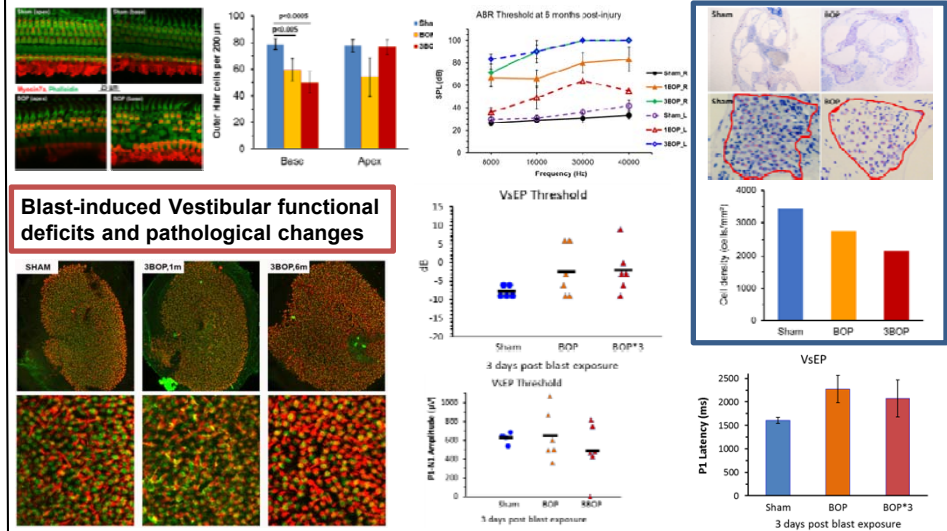
Study/Product Aim(s)

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.

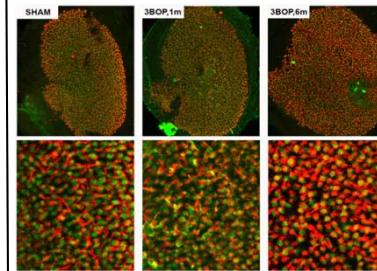
Approach

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

Auditory functional deficits and pathological changes after blast exposure



Blast-induced Vestibular functional deficits and pathological changes



Timeline and Cost

Activities	CY	15	16	17	18
Determine the functional, morphological and biochemical changes in the auditory system in response to blast-related insults.		█			
Determine the functional, morphological and biochemical changes in the vestibular system in response to blast-related insults.			█		█
Determine if specific acute or long-term therapies ameliorate auditory/vestibular deficits resulting from blast insults.				█	█
Estimated Budget (\$K)		\$549	\$462	\$470	

Goals/Milestones

- CY15 Goal – Functional assessment**
 - ✓ Obtain approval of animal use protocol
 - ✓ Auditory/vestibular functional assessment after blast exposure injury
 - ✓ Determine effects of blast damage to auditory structure & morphology
 - ✓ Determine effects of blast damage to vestibular structure & morphology
- CY16 Goals – Pathological assessment**
 - ✓ Alteration of neuronal structure and synapses
 - ✓ Progressive loss of hair cells
 - ✓ Alterations in vibratory membranes within the inner ear
- CY17 Goal – Therapeutic Efficacy**
 - ❑ Efficacy of viral-mediated expression of Atoh1 on hair cell formation
 - ❑ Efficacy of induced hair cell regeneration

Budget Expenditure to Date

Projected Expenditure: \$1,289,741.07

Actual Expenditure: \$795,728.07

Updated: (07/07/17)