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14. ABSTRACT As a consequence of advances in military medical care there are greatly increased numbers of survivors of blast-induced traumatic brain injury (bTBI) sustaining persistent neurosensory dysfunction including hearing loss and balance disorder. The study is to utilize our well-defined shock tube simulation of mild blast-induced traumatic brain injury (bTBI) in rodents to characterize interrelated biomechanical and pathophysiological mechanisms of blast-induced central auditory processing disorders (CAPDs) and central vestibular injuries (CVIs) and to develop an early therapeutic intervention for hearing loss and balance disorder mitigation. The major objectives of the proposed studies and relevant research sub-gaps are: 1) Verify the time course of hearing loss and balance disorders induced by blast exposure and define plasma and CSF TDP-43 as a biomarker related to blast-induced central auditory/vestibular deficits; 2) Characterize blast induced biochemical, functional and morphological alterations in central auditory/vestibular systems and establish that blast-induced altered expression of TDP-43 and its BDPs in these structures play a key pathophysiological mechanism leading to secondary injuries.					
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1. Introduction

With widespread use of improvised explosive devices in recent military conflicts, blast-induced traumatic brain injury (bTBI) and neurosensory dysfunction have emerged as key military medical issues. Auditory and vestibular disorders are particularly prevalent, and the debilitating consequences of these injuries likely progress with age. A comprehensive understanding of the structural and molecular components of the injury is essential for the development of the most appropriate therapies for auditory and vestibular deficits resulting from blast exposure. Existing data indicate that both the inner ear and the structures in the brain responsible for auditory and vestibular function are at high risk of injury following blast exposure. The proposed study will utilize an Advanced Blast Simulator (ABS) to recreate these injuries in rodents in the laboratory. Through comprehensive assessments of the resultant auditory and vestibular deficits using a battery of functional tests in conjunction with characterizations of the underlying biochemical and anatomical changes in these structures, the interrelated biomechanical and pathophysiological mechanisms responsible for blast-induced central auditory processing disorders (CAPDs) and central vestibular injuries (CVIs) will be elucidated and will provide therapeutic targets for hearing loss and balance disorder mitigation.

2. Keywords

TDP43, blast overpressure, mild traumatic brain injury, auditory brainstem response, balance disorder, mouse, pathology, auditory cortex (AU), medial geniculate nucleus (MGN)

3. Accomplishments

○ What were the major goals for the project?

The major objectives for the project were: 1) to verify the time course of central auditory processing disorders (CAPDs) and vestibular injuries (CVIs) induced by blast exposure and to define time-dependent changes in TDP-43 in plasma and CSF as a biomarker related to blast-induced central auditory/vestibular deficits; 2) to characterize blast injury to primary auditory cortex and brainstem/cerebellum associated with CAPDs and CVIs and to define blast-induced altered expression of TDP-43 as a key pathophysiological mediator leading to the secondary central auditory and vestibular processing injuries.

Milestones:

- Year 1: Obtain IACUC and ACURO approval of animal use protocol, define time-course of blast-induced auditory function deficits, and define the role of TDP-43 in neuronal development.
- Year 2: Assess time-course of vestibular functional disruptions, determine TDP-43 levels in serum and CSF, examine morphological alterations in specific neurons in AU, identify blast impaired functional connection between MGN and AU, and examine the regulation of TDP-43 target genes.
- Year 3: Examine morphological alterations of Purkinje neurons in the cerebellum and demonstrate blast impairments of functional connections between FL and Lat.

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

Bulleted list of key research accomplishments emanating from this research

- The effects of blast exposure on TDP43 levels have been investigated successfully in mouse plasma and brain tissue.
- TDP43 levels were quantified in rat's CSF, plasma, cortex, cerebellum and brainstem at 1 and 28 day post-injury using ELISA kits.
- A blast-induced TDP43 change in brain of rat was verified by immunoblotting anti-TDP43 antibodies to the C-terminal.
- Immunoprecipitation and Mass Spectrometry analysis were performed to verify correct identification of TDP43 peptides in rat brain tissue.
- A stereotaxic brain injection employing a digital manipulator arm and an auto syringe pump for microinjections has been successfully used in a pilot study. A standard operating procedure (SOP) has been established for this procedure.
- We have verified expression of green fluorescent protein (GFP) in the medial geniculate nuclei (MGN) following injection of AAV-CAG-ChR2-GFP or Lentiv-Cag-GFP, as well as expression of GFP in the cerebellum after injection of Letv-L7-GFP.
- The functional neural connection between the MGN and AU was successfully demonstrated using combined optogenetics assay with whole-cell patch-clamp electrophysiological recording in a pilot study.
- Injury-induced morphological alterations were revealed through analysis of dendritic spines in the auditory cortex (AU) using transgenic Thy1-YFP mice.
- Time- and severity-dependent blast-induced auditory functional deficits were quantified in rats.
- Blast-induced pathological and biochemical changes in brain were investigated and revealed a consistent, significant increase in expression of synaptophysin (SYP) in multiple brain regions after blast TBI in mice and rats.
- The platform presentation "Characterization of Blast Shockwave on Auditory Deficits in Rodent" was delivered at the Japan-US Technical Information Exchange forum on Blast Injury (JUFBI) in Tokyo, Japan, April 2017.
- The poster "Effects of blast shockwave exposure on ears and auditory signal processing centers in rodents" was presented at Neuroscience 2017 – The Society for Neuroscience (SfN) meeting in Washington DC in November 2017
- The abstract "Structural plasticity in brain auditory signal processing centers following blast wave exposure in mice" was submitted to the National Capital Area TBI symposium, December 2017.

Detailed experimental methods and results

➤ **Methods**

- **Blast-induced neurotrauma in rodents:** 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 experimental groups included single blast exposure (BOP), double blast exposures (BOP*2), triple blast exposures (BOP*3) and sham control (Sham). The blast overpressure (peak static pressure of 17 - 19 psi and 4 msec positive phase duration) was generated by Valmex membrane rupture in the 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. Animals were secured in the ABS in a prone position face or right face toward the oncoming shockwave immediately after administration of 4% isoflurane gas anesthesia in an induction chamber for 8 min (O₂ flow rate 1.5L/min). BOP*2 and BOP*3 experimental subjects received exposures to 1 or 2 additional blast shockwaves, respectively, with a 2 min interval separating blast exposures during which time additional isoflurane anesthesia was delivered. Sham control animals were included in all individual experiments and were handled in the same fashion without exposure to blast shockwaves.

- Mouse bTBI model: CBA male mice, 23 – 26 g (which represents an age of 9 – 10 weeks), were separated randomly into the experimental groups that were described above.
- Rat bTBI model: SD male rats, 320 – 350 g (which represents an age of 8 – 10 weeks), were separated randomly into groups of BOP*2 or sham that were described above.
- **Auditory function assessment:** Auditory Brainstem Response (ABR) and Distortion Product Otoacoustic Emissions (DPOAE) testing were used to evaluate auditory function. Each rat was tested under Ketamine/Dexdomitor anesthesia. 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 after blast exposure.
- **Tissue protein extraction:** Tissues were lysed in lysis buffer (20 mM Tris-HCl, pH7.5, 150 mM NaCl, and 2% SDS) followed by sonication. The lysate was clarified by centrifugation at 15000 g for 10 min. The protein concentration of the supernatant was determined by a BCA reducing reagent compatible assay kit (Thermo Scientific, Rockford, IL).
- **Enzyme-Linked Immunosorbent Assay:** Plasma, CSF and brain tissue levels of TDP-43 were determined using a commercially obtained ELISA kit (Mybiosource, San diego, CA 92195) in accordance with the manufacturer's instructions. All samples were assayed in duplicate. Standards and control samples were run simultaneously for validation.
- **Western blotting:** Twenty micrograms of the protein extracts were fractionated by an SDS-PAGE Electrophoresis System and were electrophoretic transferred to polyvinylidene difluoride membrane using an iBlot apparatus according to the manufacturer's protocol (Thermo Fisher Scientific). The membranes were blocked for 1 hour at room temperature with 5% nonfat milk in TBST (TBS containing 0.05% Tween 20), and then probed with the primary antibodies identified in the proposal overnight at 4°C. The appropriate HRP-conjugated secondary antibody was then added for 1 hour at room temperature. The protein

bands were detected by the Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific).

- **Immunoprecipitation:** Immunoprecipitation kit Dynabeads / Protein G (Thermo Fisher Scientific) were used to isolate targeted proteins. Following manufacturer's instructions, antibody was incubated with prepared Dynabeads for 10 min at room temperature and the supernatant was then removed after exposure to the magnet. Selective protein extraction was achieved through incubation with the Dynabeads-Ab complex for 10 min. The Dynabeads-Ab-antigen complex was washed with the buffer and eluted by adding 20 ul of NuPAGE LDS sample buffer and reducing agent, followed by heating at 70°C for 10 min and protein analysis by SDS-PAGE.
- **Mass Spectrometry:** The bands excised from the silver stained gel were de-stained with ammonium bicarbonate buffer with acetonitrile. The bands were digested using an In-Gel Tryptic Digestion Kit (Thermo Fisher Scientific) following manufacturer's instructions. The trypsin digestion was conducted at 30 °C and was carried out overnight. The following morning, the digest was spike with 0.1% formic acid to halt the digestion and analyzed on a Xevo G2-XS LC/MS system (WATERS). The samples were separated on a C18 column with a 3%-40% acetonitrile gradient over 40 min. The resulting mass spectrum was imported into UNIFI (WATERS) for analysis and compared to a custom protein library.
- **Brain virus injections:** Mice were anesthetized with isoflurane (4% induction, 1.5–2.0% maintenance) and heads were carefully secured in the stereotaxic apparatus (David Kopf instruments, Inc). After establishing a sterile operation field, craniotomy holes of 1 – 2 mm diameter was made in the skull by a dental drill over the region of interest. The AAV-CAG-ChR2 -YFP (University of North Carolina Vector Core) was administered into the medial geniculate nuclei (MGN) at –3.28 mm from Bregma, 1.9 – 2.1 mm lateral from the midline and 2.9 – 3.1 mm deep from the cortical surface bilaterally, using a NANOFIL syringe with a 33 G needle (World Precision Instruments, Inc). An injection rate of 1 microliter over 10 minutes was controlled by the Model UMP3-1syringe pump (World Precision Instruments, Inc). The scalp was sutured and sealed with tissue glue. Mice recovered for 3 - 5 weeks to allow for expression before further experimentation.
- **Electrophysiology with optogenetics assay:** The brain of mouse was quickly removed after being administered with isoflurane and slice (400 µm) was cut in coronal section using a vibrating blade microtome (Leica VT1000S, Leica Systems), then transferred to a submersion recording chamber perfused with 32°C oxygenated artificial cerebrospinal fluid (ACSF). Whole-cell patch-clamp recordings from pyramidal neurons in AU with a holding potential at -70 mV was obtained with Axoclamp 200B amplifier (Molecular Devices). Recording was under visual guidance using an Olympus BX51 microscope equipped with both transmitted light illumination and epifluorescence illumination which connected a single-wavelength ($\lambda = 470$ nm) LED system (CoolLED Ltd). Light pulses of 2 ms, triggered by a TTL (transistor-transistor logic) signal from the Clampex software (Molecular Devices) was used to evoke synaptic transmission.
- **Pathology:** Brains of mice was dissected after euthanasia and fixed in 4%PFA solution at the designed days post-injury. Coronal brain sections (40 - 100 µm)

were prepared using a vibrating microtome (Leica VT-1000S). Brain sections were processed for silver staining and immunohistochemistry.

➤ Results

- Effects of blast overpressure on TDP43 levels in mouse plasma and brain**
 We have investigated TDP43 protein expression levels in brain tissue and plasma following blast exposures of mice. ELISA data showed that compared to the sham group, TDP43 increased in mouse cortex at 28 days after blast exposure ($p < 0.05$, $n = 5$). TDP43 levels in the brainstem and cerebellum didn't show significant differences. There was no significant change in TDP43 expression in the plasma between BOP*3 group and Sham controls at 28 days post blast exposure in mice (Fig. 1). In the acute phase (1 day) after blast exposure, TDP43 was not found to be significantly changed, which was determined by Western blotting (results not shown).

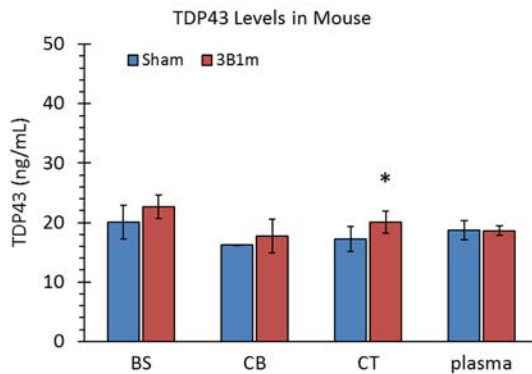


Fig. 1. Changes in TDP43 expression following blast exposures in mice

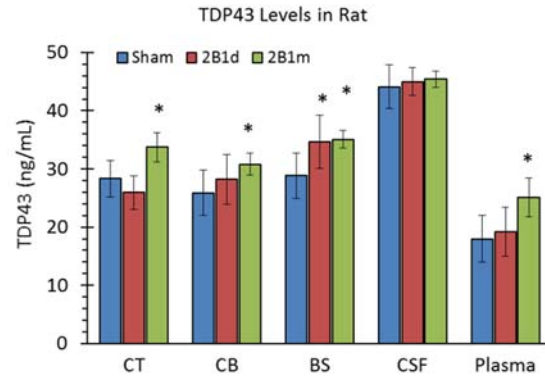


Fig. 2. Changes in TDP43 expression following blast exposures in rats

- Effects of blast exposure on TDP43 levels in rat plasma, CSF and brain**
 Current tools for detecting TDP43 protein level are ELISA and Western blotting. Based on our previous pilot study, 50 ul of CSF and/or plasma was required for the assay. Consequently to determine the effect of blast TBI on TDP43 level in CSF, we utilized the rat model of bTBI. In this report period, 16 rats were randomly separated in groups of BOP and Sham, $n = 4$ for each group at 1 day and 28 days, respectively. As shown in Fig. 2, TDP43 levels in CSF were detectable, but no significant changes were apparent among experimental groups. Compared to Sham ($n = 8$), BOP group ($n = 4$) TDP43 levels were increased in the plasma ($p < 0.05$) and multiple brain regions (cortex and cerebellum $p < 0.02$, brainstem $p < 0.01$) at 28 days after blast exposures. For verification of the measured blast-induced TDP43 changes in the cortex tissues, the Western blotting technique was applied. Protein extractions were handled and processed at the same time with the same procedure. No significant change was found at molecular weight of 45 kda that immunoblotted with anti-

TDP43 specifically on the N terminal antibody. However, the image displayed (Fig.3) a significant increase of TDP43 in the BOP group that was immunoblotted with anti-TDP43 antibody (PA520408, Thermo Fisher Scientific) which binds to the carboxy-terminal of TDP43. Those changes were apparent at 60 kDa or 25 kDa, prompting questions whether blots might represent cleaved and/or dimeric forms of TDP43.

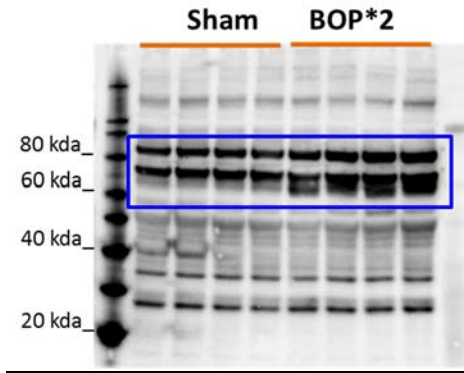


Fig. 3. TDP43 increased in cortex of rat at 28 days after double blast exposures

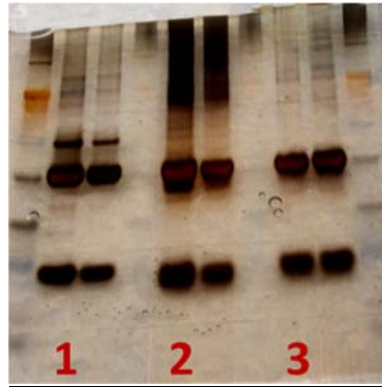


Fig. 4. Gel with silver staining which contains the eluate from protein immunoprecipitation with anti-TDP43

TDP43 isoforms X1 [Rattus norvegicus]

*25kda, 45kda, 60kda

1 – 80 MSEYIRVTED ENDEPIEIPS EDDGTVLLST VTAQFFGACG LRYRNPVSQC MRGVR**LV**EGI **LH**APDAGWGN **LV**YVVNYPKD
 81 – 160 **NKR**KMDEADA **SS**AVKVKRAV **QK**TSDLIVLG **LP**WKTEQDL **KD**YFSTFGEV **LM**VQVKKDLK **TG**HSK**G**FGFV **RF**TEYETQVI
 161 – 240 **VMS**QRHMIDG **RW**CDCKLPNS **KQ**SPDEPLRS **RK**VFVGRCTE **DM**TAEELQQF **FC**QYGEVVDV **FIP**KPFRAFA **FV**TFADDKV
 241 – 320 **QSL**CGEDLII **KG**ISVHISNA **EP**KHNSNRQL **ER**SGRFGGNP **GG**FGNQGGFG **NS**RGGGAGLG **NN**QGGNMGGG **MN**FGAFSINI
 321 – 400 **AM**MAAAQAAL **QSS**WGMMGML **AS**QQNQSGPS **GNN**QSQSGMQ **REP**NQAFGSG **NNS**YSGSNSG **AP**LGWGSASN **AG**SGSGFI
 401 – 414 **FG**SSMDSK**SS** **GW**GM

Fig. 5 Mass spectrometry for cleaved TDP43 identification

During the past two decades, mass spectrometry has become established as the primary method for protein identification from complex mixtures of biological origin, so we applied these analytical techniques. The protein extractions underwent immunoprecipitation with anti-TDP43 antibodies (1) ab190963 (abcam), (2) PA520408 and (3) P21958 (ThermoFisher scientific). The eluate was run to a SDS-PAGE gel and was silver stained using a complete kit (24600, ThermoFisher Scientific). The bands (Fig.4) at 25, 45 and 60 kda were cut and digested with trypsin and analyzed on Xevo G2-XS (WATERS) LC/MS system by Dr. Anthony Vortherms from the Bacterial Diseases Branch/WRAIR. The peptides were mapped to the expected amino acid sequence in the UNIFI (WATERS) software. The results from mass spectrometry analysis (Fig. 5) exhibited the presence of TDP43 peptides, with different coverages for each band. Further experiments are needed to conclusively identify the bands as possible fragments of TDP43. Our hypothesis is that blast TBI-induced increase

in cleaved forms of TDP43 might contribute to neurodegeneration, while the full length of TDP43 expression may not be altered significantly.

- **Auditory functional evaluation in rats**

During this reporting period, we have evaluated auditory functions in SD rats since this strain of rat has been used for determining blast-induced TDP43 changes in plasma, CSF and brain tissue. Rats' ABR and DPOAE were assessed at baseline and 1, 7, 14 and 28 days after double blast exposures (Fig. 6). Similar to the CBA mice, rats displayed significant increases in ABR threshold (Fig. 7) and decreases in amplitude (Fig. 8) following blast exposure. Complete hearing loss (threshold > 90 dB) was observed at 1 day after double blast exposures, and these deficits persisted over 28 days. Compared to the sham controls, blast-induced elevation of ABR threshold were evident throughout the whole spectra of sound frequencies. Hearing deficits occurred in both left and right ears. The results indicate that the impact of blast exposure on 32 – 40 KHz (high frequency hearing) was severe and more persistent than on 4 – 8 KHz (lower frequency) stimuli. Significantly increased wave III and V latencies (Fig. 9) revealed blast injuries to brainstem. DPOAE data also indicated that hearing loss occurred throughout 7 days post-injury, and partially recovered at lower frequency stimuli (Fig. 10.) after 14 days blast exposures.

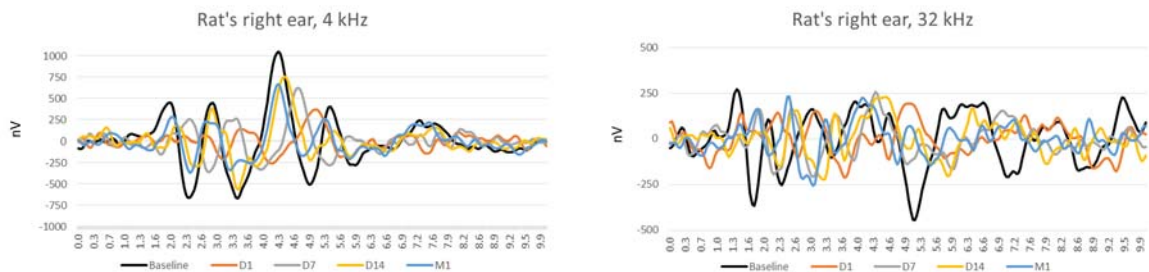


Fig. 6. ABR testing in Rat: brainstem response to an acoustic 4 kHz (left) and 32 KHz (right)

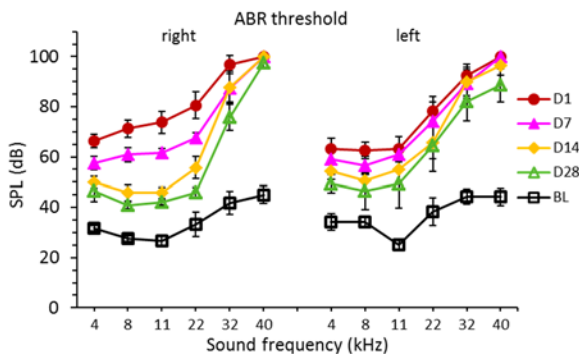


Fig. 7. ABR threshold after blast exposure in rats

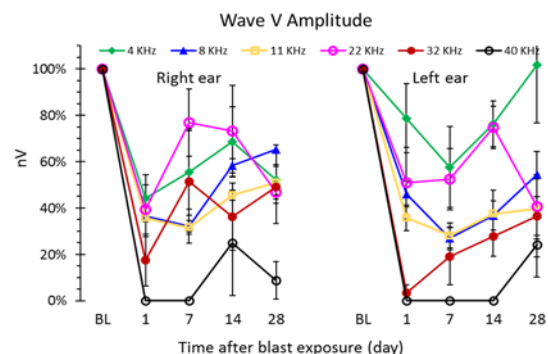


Fig. 8. Reduction in ABR wave V amplitude

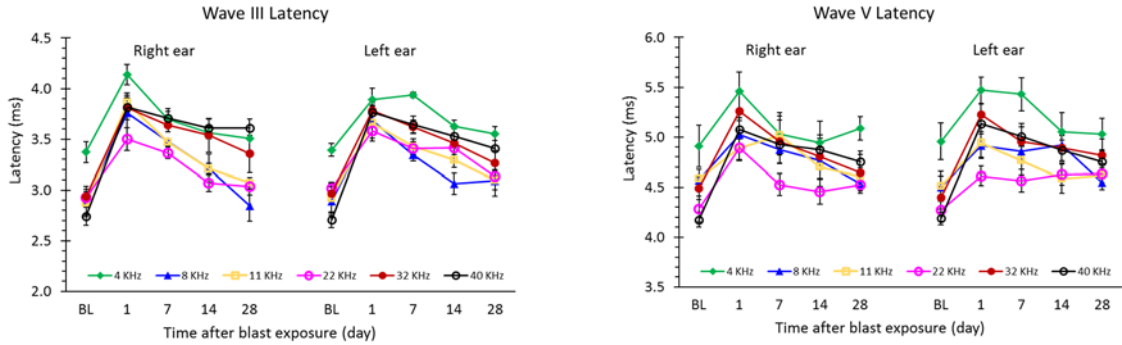


Fig. 9. Blast-induced increase in ABR wave III and wave V latencies

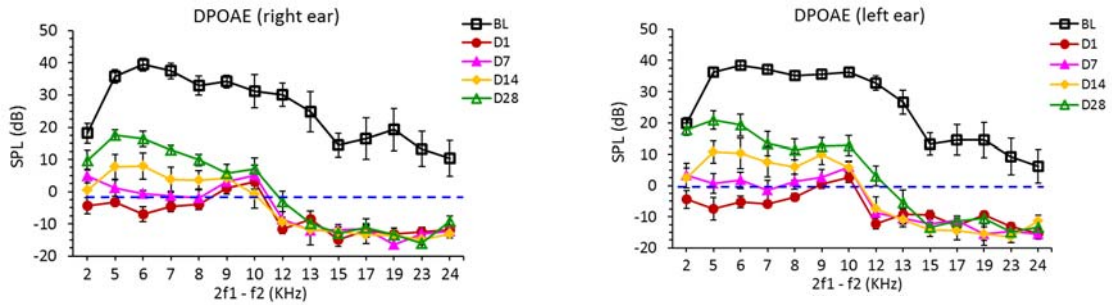


Fig. 10. Blast-induced DPOAE changes

- **Verification of GFP expression in MGN and AU**

In this reporting period, we have established a standard operating procedure (SOP) for stereotaxic brain viral injection.

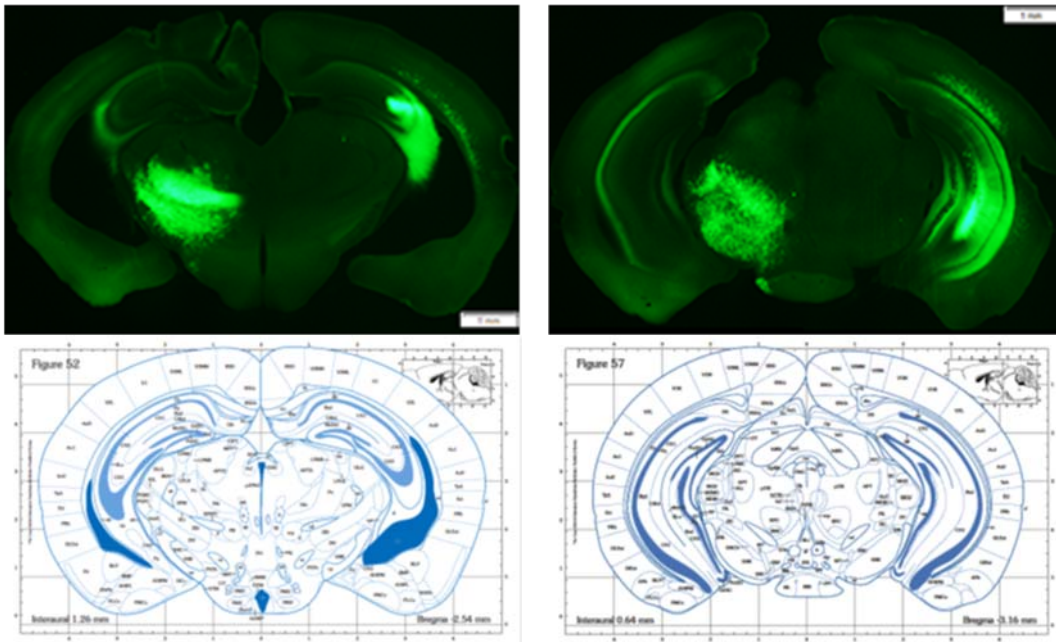


Fig. 11. GFP expression at 6 weeks after brain viral injection

To determine whether alterations in the functional integration and morphology of the medial geniculate nuclei (MGN) and auditory cortex (AU) contribute to blast-induced hearing loss, we injected 1 ul of AAV-CAG-ChR2(H134R)-GFP into the

MGN. Mice were euthanized at 2, 3, 4 and 6 weeks after injection, respectively. Brain serial sections were evaluated under light microscopy for visualized fluorescence. This adeno-associated virus carries channelrhodopsin-2 (ChR2), which can activate neurons in response to 473-nm light. ChR2-GFP was specifically over-expressed in the MGN-AU projections 4 weeks after in vivo injection of this recombinant AAV (Fig. 11). The parameters that include reagent's concentration and amount, location in three dimensions and minimum time for GFP expression have been established.

- **Blast-induced changes in functional connectivity between MGN and AU**
Auditory functional deficits can result from any disruption of neural connections from brainstem to thalamus and cortex. However, conventional experimental technology cannot readily address abnormalities in long-range connectivity induced by blast exposure. In this reporting period, we conducted a pilot study using optogenetics combined with whole-cell patch-clamp electrophysiological recording (Fig. 12). A group of 6 mice received AAV-CAG-ChR2 (H134R)-GFP injection in MGN, were exposed to blast overpressure at WRAIR, and were subsequently transported to LIBD at JHU. Mice were euthanized to prepare MGN-AU slices at 1, 3 and 7 days after blast exposure. Quantitative aspects of repetitive evoked postsynaptic currents (EPSCs) elicited by 473-nm light pulses were studied in layer IV granular neurons in the MGN slices of bTBI and sham mice. The initial data showed that the input-output relations of optically evoked EPSCs were substantially decreased at day1 and day3 post-injury. The impairment of MGN-AU projection was largely recovered after 7 days post-injury (Fig. 13).

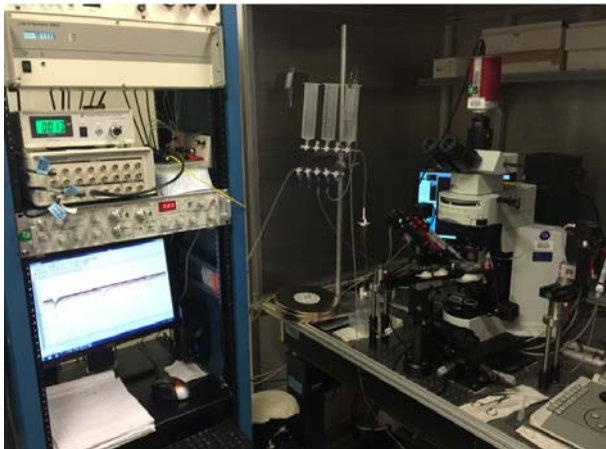


Fig. 12. Electrophysiological recording system

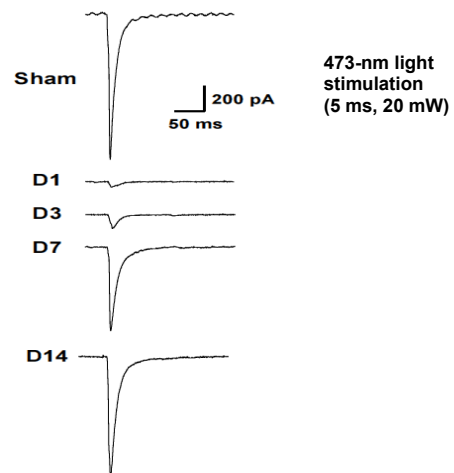


Fig.13. Impaired MGN-AU synaptic transmission

- **Blast-induced dendritic spine alterations in the auditory cortex**

Auditory cortex (Au) is one of the key units of sound processing. Precise sensory information processing requires complex interactions between neurons. In this report period, we continued the evaluation of morphological changes in dendritic spines in the auditory cortex at 4 hrs and 7 days after blast exposure using the Thy1-YFP mouse in which a yellow fluorescent protein is expressed at high levels in the layer V pyramidal neurons. Compared to the sham controls, total number of dendritic spines in auditory cortex increased at 4h and 7d after blast exposure (Fig. 14). Those changes were observed in Stub and Thin phenotypes, but no change was evident for the number of the mushroom type of spines. The increase in the stub type of spine persisted to 7 days post-injury. Dendritic spine numbers and shapes correlate with the strength of synaptic transmissions that are associated with the function of particular neural networks. The results indicated the synaptic transmissions in excitatory neurons were quite sensitive to blast insult.

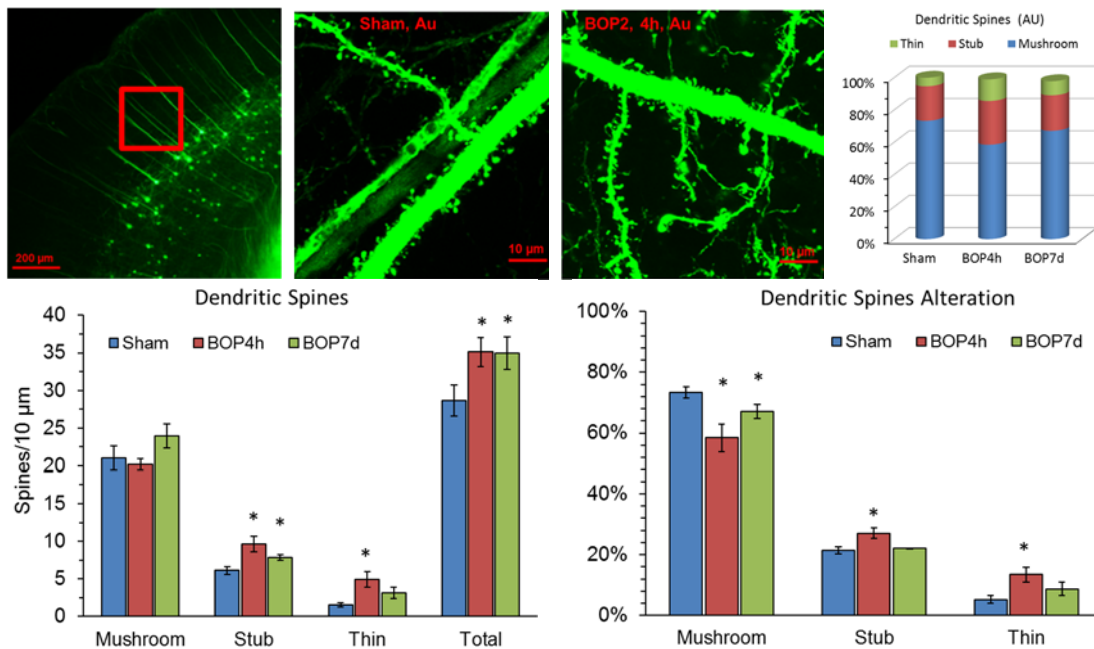


Fig. 14. Effect of blast exposure on dendritic spine in the auditory cortex

- **Increase in synaptophysin (SYP) expression after blast exposures**

In this period, we have investigated effects of blast overpressure on SYP and PSD95 expressions in both animal models. Western blotting data showed significant increases in cortex, cerebellum and brainstem in rats at 1 day after injury (Fig. 15). Figure 16 illustrates increases of SYP in cerebellum and brainstem in mouse at 3 days post-injury. So far, PSD95 was not found to be significantly changed in the brains of rodents after blast TBI.

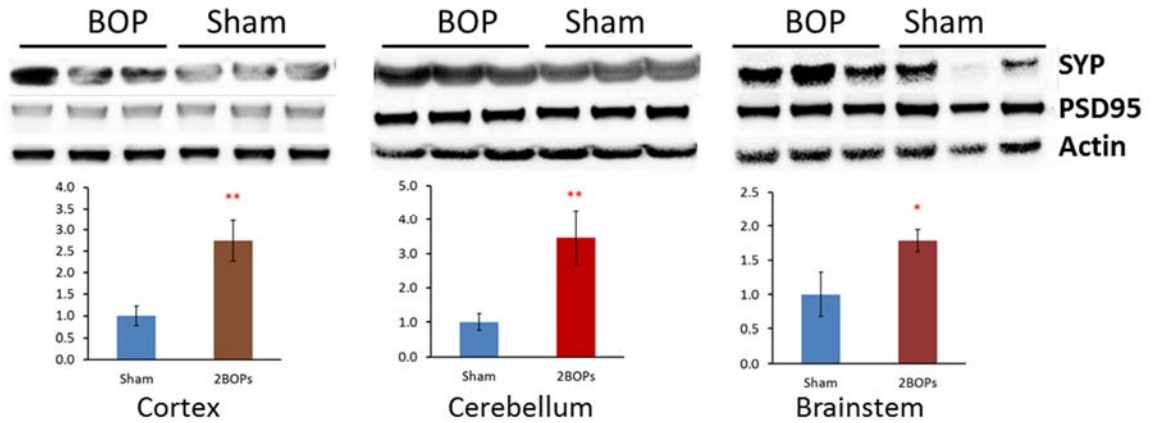


Fig. 15. Blast-induced increase in synaptophysin expression in brain of rat at 1 day post-injury

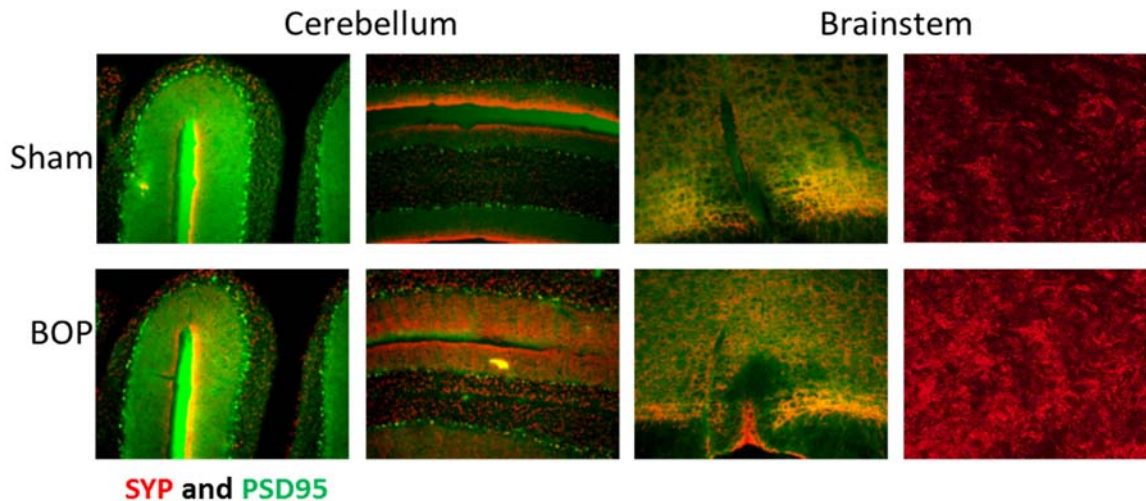


Fig. 16. Blast-induced increase in synaptophysin expression in brain of mice at 3 day post-injury

4. Impact

None to note

5. Changes/Problems

We proposed to determine the impact of repetitive blast exposures to Purkinje cell using Pcp2-EGFP transgenic mouse. The adult Pcp2-EGFP mice have to be bred at WRAIR since The Jackson Laboratory provides only the frozen mouse embryo. However WRAIR has restrictions on animal breeding. We are planning to use alternative approach, injection of AAV-L7-GFP or Lent-L7-GFP into the cerebellum of mouse.

6. Products:

Lay Press- none

None

Peer-Reviewed Scientific Journals -none

None

Books or other non-periodical, one-time publications- none

None

Other publications, abstracts, conference papers and presentations

1. Ying Wang, Rodrigo Urioste, Yanling Wei, Wafae Driwech, Yan Su, Kamren Hollingsworth, Donna Wilder, Sajja Venkatasivasaisujith, Stephen Van Albert, Matthew Wise, Tracy Fitzgerald, Peethambaran Arun, Weise Chang, Matthew Kelley, Irene D. Gist and Joseph Long. Effects of blast shockwave exposure on ears and auditory signal processing centers in rodents. Neuroscience 2017 - Society for Neuroscience (SfN). Washington DC. November 2017

2. Ying Wang, Wafae Driwech, Yanling Wei, Rodrigo Urioste, Sujith Sajja, Donna Wilder, Irene D. Gist, Peethambaran Arun and Joseph B. Long. Structural plasticity in brain auditory signal processing centers following blast wave exposure in mice. Abstract, National Capital Area TBI symposium. December 2017

Websites or other internet sites

Technologies or techniques

Inventions, patent applications, and/or licenses

7. Participants & Other Collaborating Organizations

Name	Project Role	Percent Effort	Organization
Dr. Joseph Long	PI	10%	WRAIR
Ying Wang	Co-PI	30%	WRAIR
Yanling Wei	Research Associate	50%	Geneva
Donna Wilder	Lab Manager	50%	Geneva

Organization Name	Location	Contribution
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Lieber Institute for Brain Development at Johns Hopkins University	Maryland	Collaboration

8. Special Reporting Requirements

A Quad Chart is attached.

9. APPENDICES

A poster and an abstract are attached.

Central mechanisms and treatment of blast-induced auditory and vestibular injuries

MR141274

W81XWH-16-2-0002

PI: Joseph B. Long

Org: WRAIR/The Geneva Foundation

Award Amount: \$1,476,364



Study/Product Aim(s)

The etiology of blast-induced hearing loss and balance disorders is largely undefined. There are no FDA-approved drugs for treatment. This study utilizes a well-characterized, high fidelity rodent blast injury model to evaluate central auditory processing disorders (CAPDs) and central vestibular injuries (CVIs) and target disrupted TDP-43 and PERK-eIF2 α -ATF4 signaling as a likely therapeutic means to mitigate blast-induced auditory and vestibular dysfunction.

Approach

Blast TBI model: repetitive blast overpressure exposures to mice

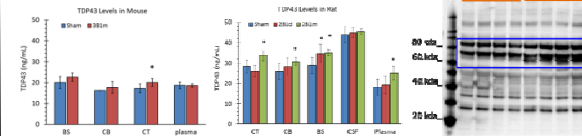
Functional assessment: ABR, DPOAE, VsEP and Rotarod

New technology: optogenetics with whole-cell patch recording to uncover the impaired functional connection between brain regions; CRISPR/Cas9 gene editing and Single cell RNA-seq assay

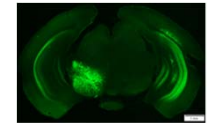
Pathology: silver staining, immunohistochemistry on transgenic mice for specific neuronal plasticity and morphology

Define biomarkers: Western blotting and ELISA

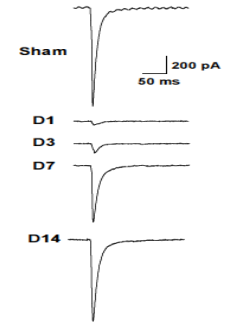
Effects of blast exposure on TDP43 level changes in brain tissues, CSF and plasma



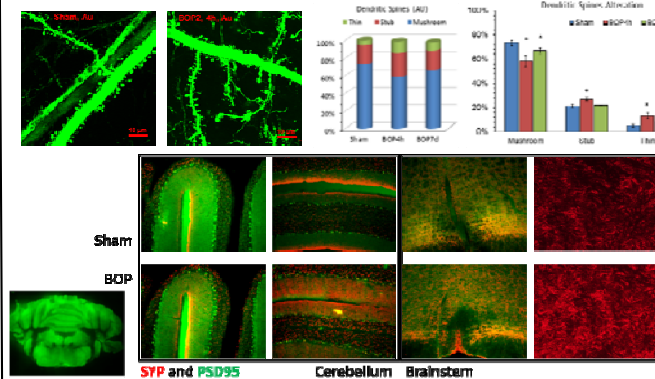
Blast-induced functional connectivity changes between MGN and AU



473nm light st.
(5 ms, 20 mW)



Blast-induced morphological and biochemistry changes



Timeline and Cost

Activities	CY	16	17	18
Verify blast-induced CAPDs and CVIs, define TDP-43 levels in serum and CSF		█	█	
Verify blast-induced morphological alterations in central auditory system and vestibular system			█	█
Estimated Budget (\$K)		\$500	\$500	\$476

Updated: (01/26/2018)

Goals/Milestones

CY16 Goal

- Approval of animal use protocol
- Time-course of blast-induced auditory function deficits
- Define the role of TDP-43 in neuronal development

CY17 Goal

- Time-course of vestibular function assessment
- Determine TDP-43 levels in serum and CSF
- Morphological examination on specific neurons in AU
- Blast impaired functional connection between MGN and AU
- Examine the regulation of TDP-43 target genes

CY18 Goal

- Morphological examination on Purkinje neurons in the cerebellum
- Blast impaired functional connection between FL and Lat

Budget Expenditure to Date

Projected Expenditure: \$ 873,211.66

Actual Expenditure: \$ 750,181.33

Structural plasticity in brain auditory signal processing centers following blast wave exposure in mice

Ying Wang, Wafae Driwech, Yanling Wei, Rodrigo Urioste, Sujith Sajja, Donna Wilder, Irene D. Gist, Peethambaran Arun and Joseph B. Long

Blast-Induced Neurotrauma Branch, WRAIR, Silver Spring, MD 20910

Abstract

Blast exposure-induced auditory deficit is one of the most common disabilities in military personnel and is thought to result at least in part from disrupted connectivity among brain auditory signal processing centers. However, the role of auditory neural signal processing abnormalities in the evolution of auditory impairment and long-term disability has not been extensively characterized. To uncover neurobiological mechanisms underlying these injuries, we have investigated the changes in brain structures involved in auditory signal processing using a mouse model of blast-induced auditory injury. Anesthetized mice were secured in an Advanced Blast Simulator (ABS) and were exposed to blast overpressure (peak static pressure of 16 psi and 4 msec positive phase duration). The effect of shockwaves on hearing was determined by testing auditory brainstem response (ABR). The results revealed significant changes in waveforms and elevations of threshold which suggested that hearing loss occurred immediately after insult and auditory dysfunction typically persisted for several months. It is known that precise sensory information processing requires complex interactions among neurons. In particular, dendritic spine numbers and shape correlate with the strength of synaptic transmissions that are associated with the function of neural networks. Auditory cortex (Au) is one of the key units comprising a sound processing center. To measure the impact of blast injury on dendritic morphogenesis, we employed Thy1-YFP mice that express a yellow fluorescent protein at high levels in the layer V pyramidal neurons of the auditory cortex. The morphological alterations of excitatory neurons were examined at 4hrs, 7 and 28 days post-injury. Data showed that total numbers of dendritic spines in Au region were increased at 4hrs post-injury. Those changes were in the immature types of stub and thin spines, so that the ratio of mushroom type spines decreased. Brainstem/cerebellar and cerebral tissues were also evaluated by immunoblotting or immunohistochemical staining with antibodies against synaptophysin, PSD-95, NKCC1, GFAP and Iba-1. Data collectively indicated that blast shockwaves influence neurotransmission, membrane transport and glial cell proliferation in auditory signal processing centers at acute and/or sub-acute phase after injury. Cortical and brainstem injuries after blast exposure likely play vital roles in auditory dysfunction.

Keywords (< 4 words): blast, auditory dysfunction, neuron

Effects of blast shockwave exposure on ears and auditory signal processing centers in rodents

Ying Wang¹, Rodrigo Urioste¹, Yanling Wei¹, Wafae Driwech¹, Donna Wilder¹, Peethambaran Arun¹, Kamren Hollingsworth², Yan Su¹, Sajja Venkatasivasaisujith¹, Stephen Van Albert¹, Irene Gist¹, Matthew Wise¹, Weise Chang², Matthew Kelley² and Joseph Long¹

1. Blast-Induced Neurotrauma Branch, Center for Military Psychiatry and Neuroscience, WRAIR, Silver Spring, MD
2. Porter Neuroscience Research Center, National Institute on Deafness and Other Communication Disorders, Bethesda, MD



ABSTRACT

Auditory deficits are particularly prevalent in blast victims. To better understand the mechanisms underlying blast related auditory injuries, we longitudinally studied auditory brainstem responses (ABR) to blast overpressure (BOP) exposure and assessed the associated damage to the ear and brain. Isoflurane anesthetized mice (CBA, male, 25 g) and rats (SD, male, 350 g) were secured in an Advanced Blast Simulator (ABS) in a prone position facing the oncoming shockwave. BOP (peak static pressure of 4, 8, 12 and 19 psi and 4 msec positive phase duration) was generated by Valmex membrane ruptures in the ABS. Noise controls were anesthetized and placed immediately outside the ABS and exposed to the noise but not the shockwave associated with membrane rupture. Sham controls were handled similarly without exposure to either the noise or shockwave. DPOAE signals were undetectable immediately after BOP exposure and their disappearance persisted over months, suggesting significant damage to the inner ear. ABR thresholds and latencies increased and amplitudes decreased in BOP-exposed animals relative to preinjury baselines and recordings in the sham and noise controls. These changes were observed over the entire acoustic frequency spectrum and persisted over months. Low frequency (8 kHz) hearing recovered more readily than high frequency (40 kHz) hearing. BOP damage to the tympanic membrane, middle and inner ear structures, as well as brainstem and auditory cortex were observed. Blast intensity-dependent damage to middle and inner ears was evident with no significant differences between left and right ears. Tympanic membranes were ruptured immediately following BOP exposure and resolved within a month. Labyrinthine hemorrhage was prominent from 1 day up to 14 days post-injury. Morphological evaluation of the inner ear showed hair cell loss and decreased neurons in spiral ganglia at 28 days post-injury when compared to the sham control. Brain pathology showed acute changes of dendritic spines in the auditory cortex along with neuronal degeneration and glial cell proliferation in the brainstem. Thus, BOP-induced hearing loss can be associated with disruptions to ear structures and damage to brain regions associated with the sound processing. The model of ABS-induced auditory deficits in rodents can be used for studying the mechanisms of hearing impairment after blast exposure and for evaluating potential strategies for prevention and cure.

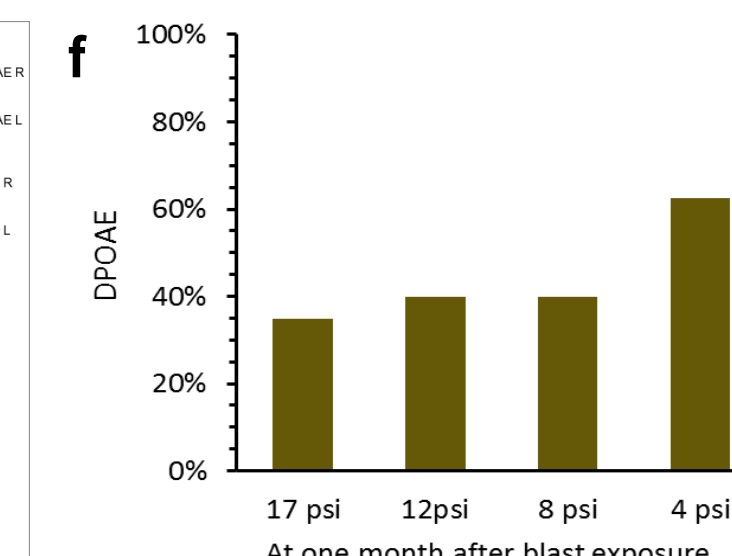
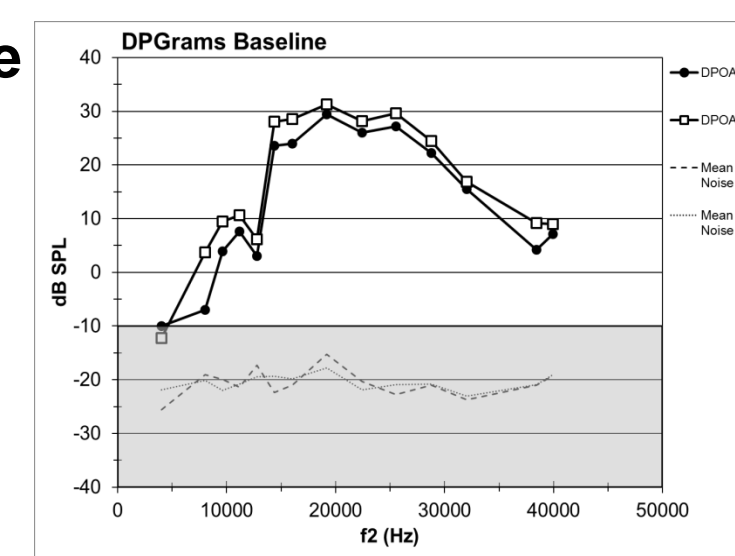
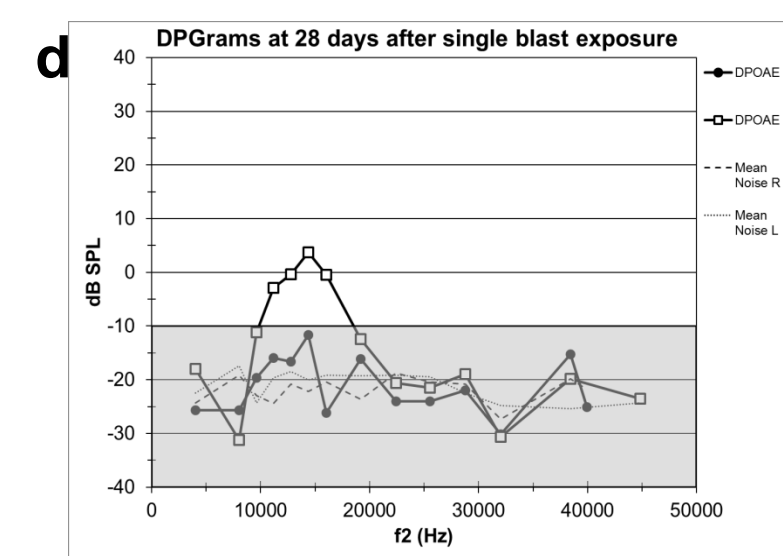
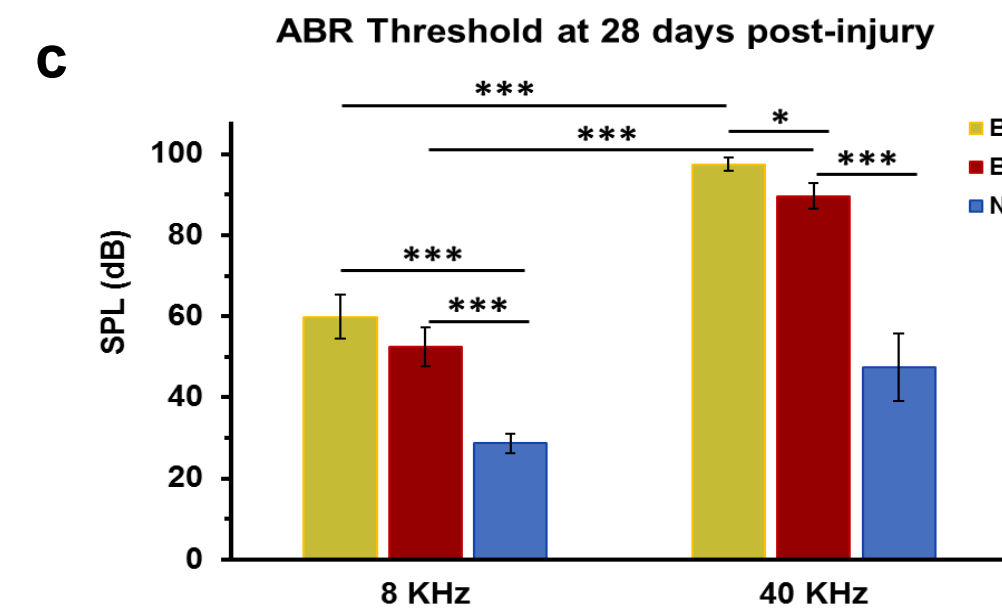
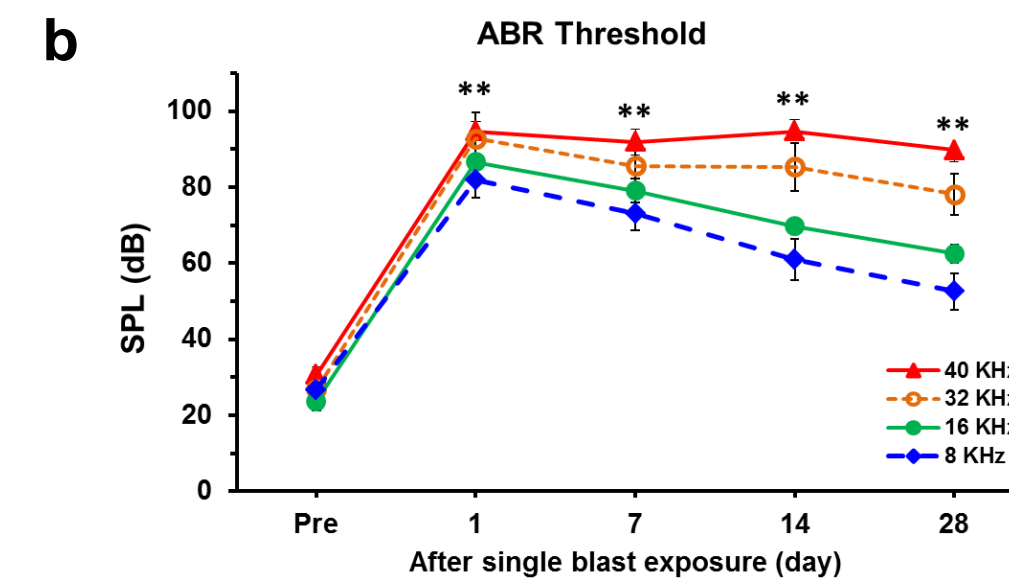
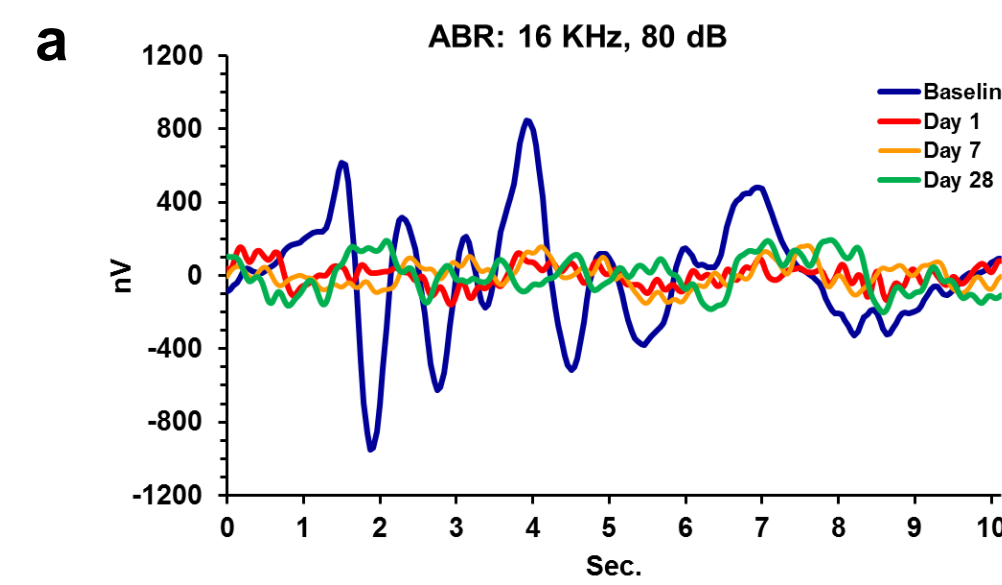
METHODS

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 17 psi and 5 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.

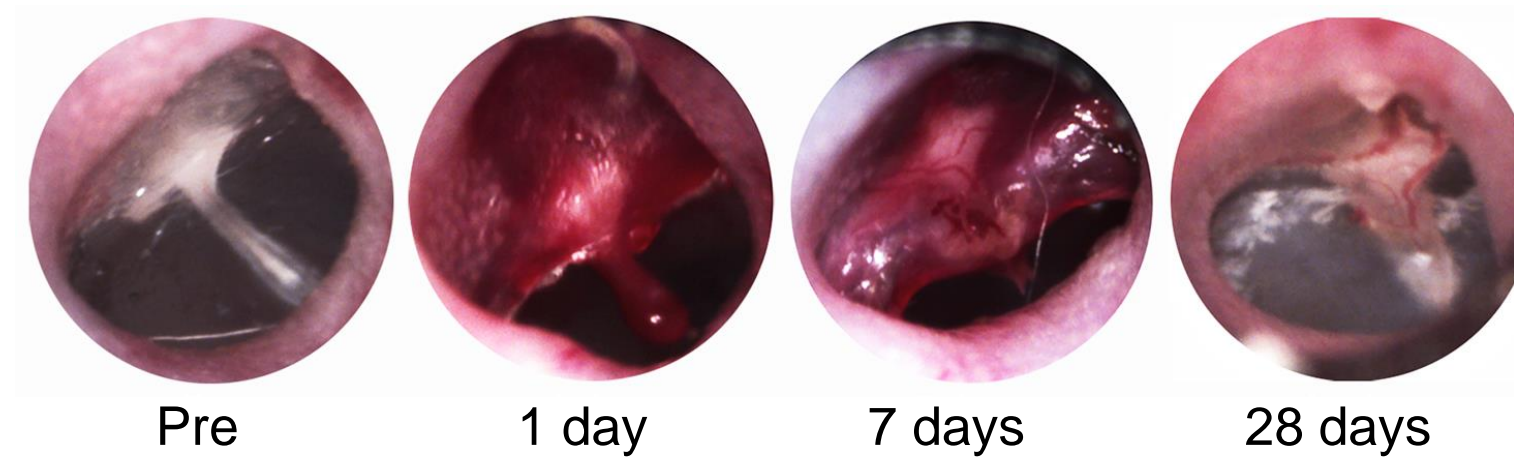
Pathological investigation: The cochleae and brain sections were prepared for light microscopic analysis.

1 Time-course changes in ABR and DPOAE after blast exposure

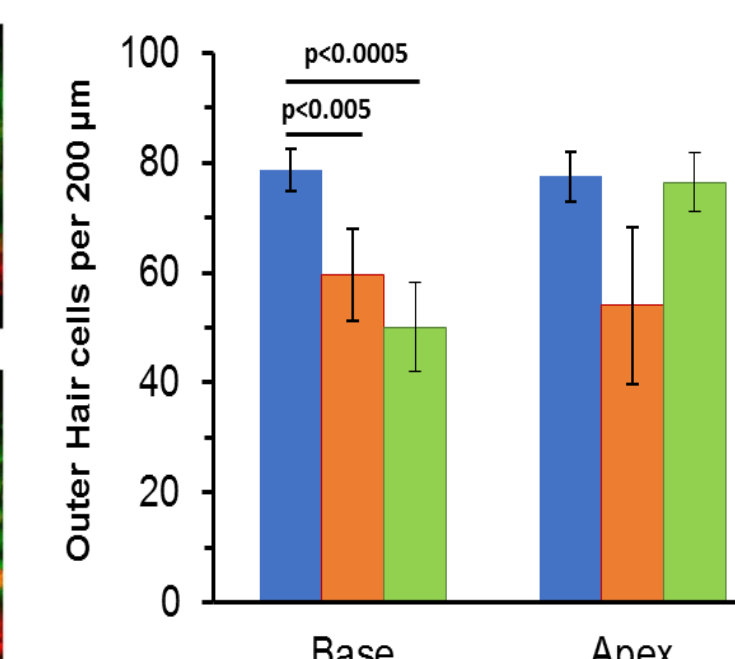
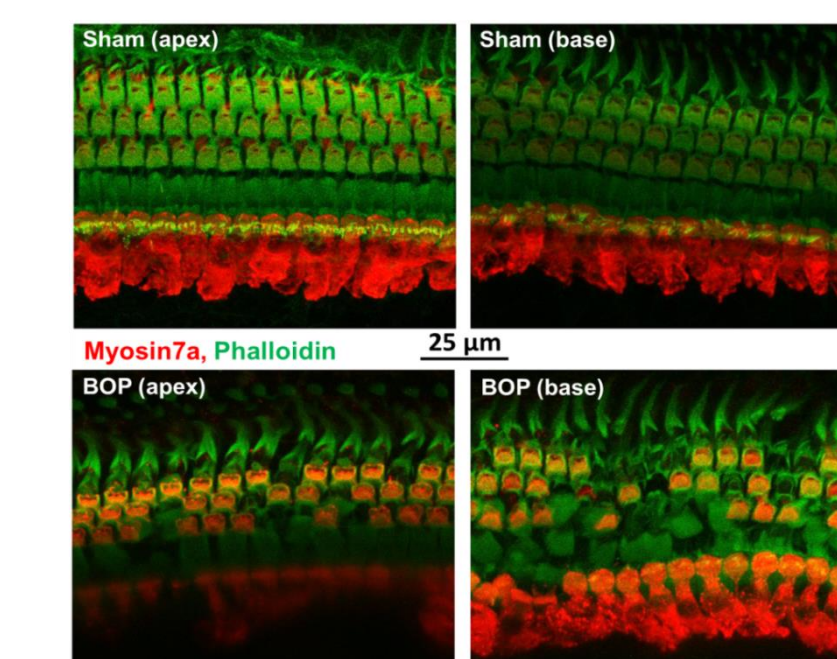


Mice exposed to BOP (17 psi): (a) a significant reduction in ABR wave amplitudes, (b) elevation in thresholds in the frequency range from 8 to 40 KHz, (c) comparison of ABR threshold among the groups at 28 days post injury, * p<0.05. *** p<0.0005, (d) significant loss in DPOAE response at a month post injury to (e) the baseline, (f) DPOAE at a month after BOPs of 12, 8 or 4 psi.

2 Blast damage to the tympanic membrane and middle ear structures



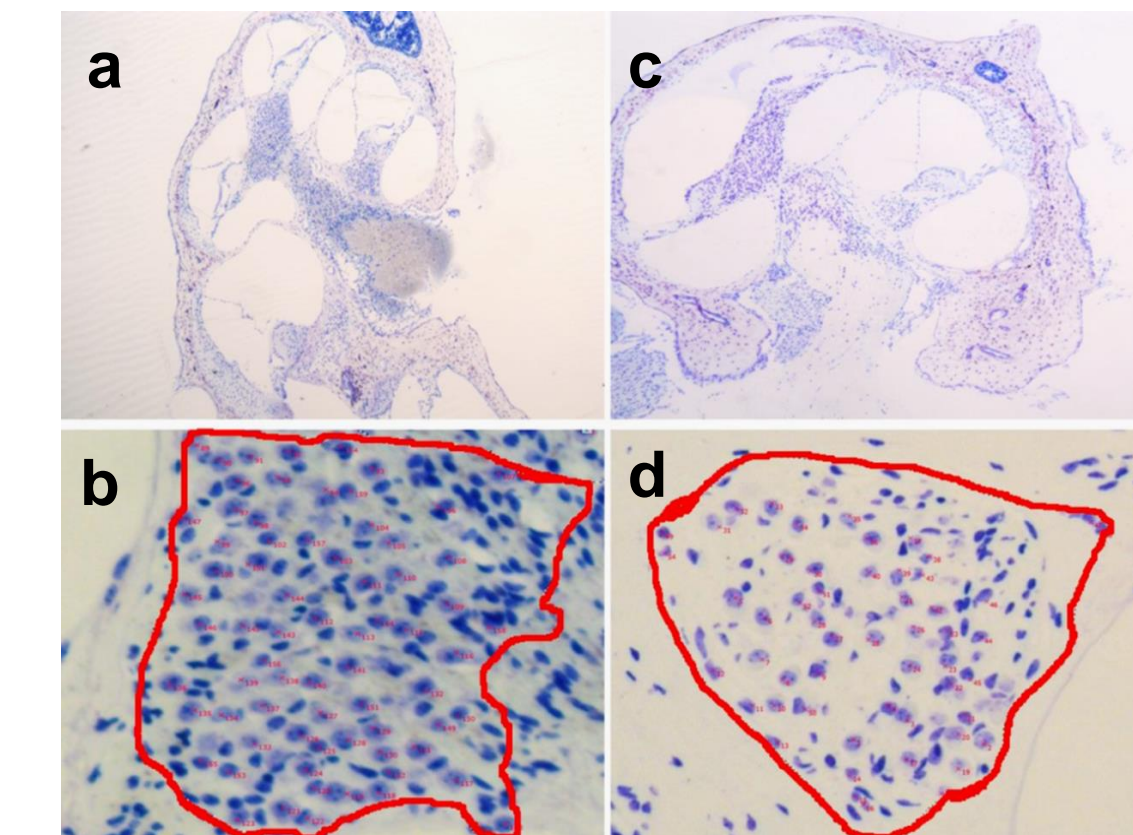
3 Blast shockwave damages to hair cells



Whole mount cochleae from sham (n=10), BOP (n=7) and 3-BOPs (n=7) at 14 days post treatment were prepared with phalloidin (green) and myosin7a (red) antibodies.

RESULTS

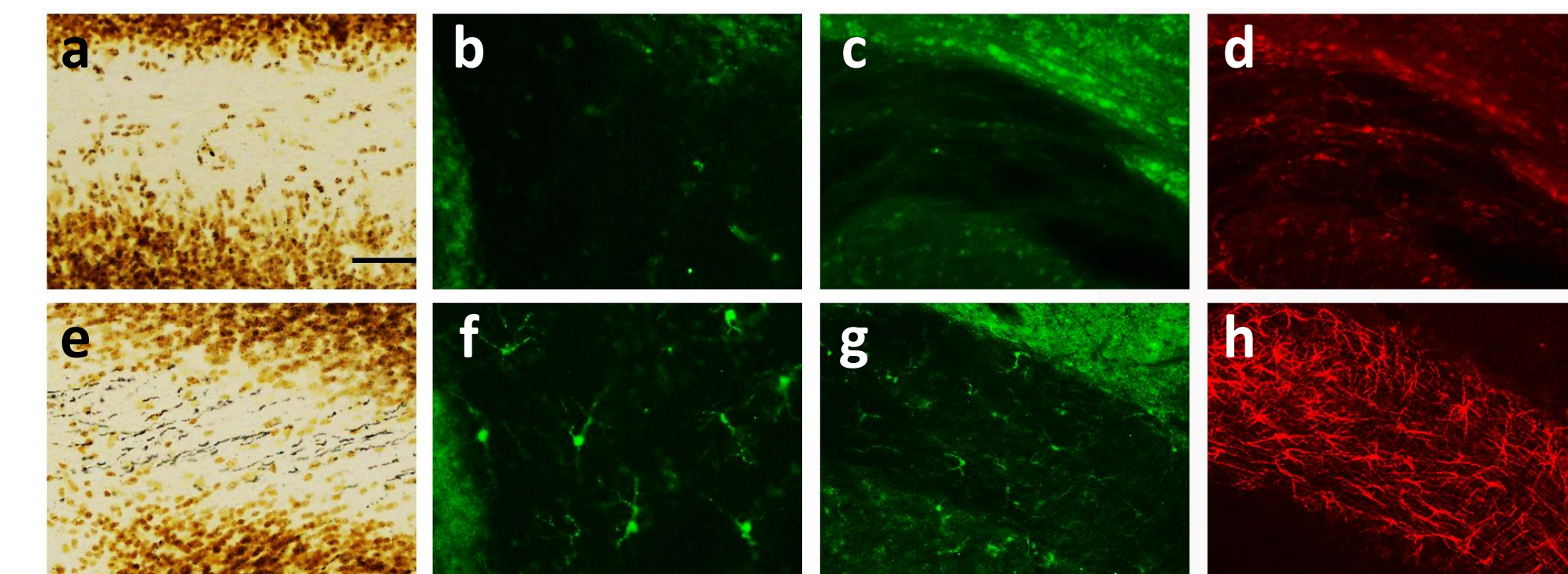
4 Blast exposure decreased the density of neurons in spiral ganglia



Sham	BOP
37.459 ± 6.65	24.398 ± 1.43

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 a mouse exposed to a single blast at 28 days post injury. Table shows the quantification of neurons per 10,000 µm².

5 Blast-induced brain pathological changes



Effect of blast shockwaves 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 triggers axonal degeneration (e), increases Iba1 (f, g) and GFAP (h); scale bar 100 µm.

CONCLUSIONS

- Blast shockwaves (17 psi) produced ABR threshold shifts that persisted through 28 days. Compared to persistent high frequency (40 kHz) hearing loss after blast exposure, low frequency (8 kHz) hearing recovered quickly.
- 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 mitigation.

ACKNOWLEDGMENTS

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MHSRS Poster Format Instructions

Recommended font sizes:

Title: ≥ 50 point (≥ 100 on printed poster)

Authors/Affiliations: 24-40 point (48-80 on printed poster)

Body text: 12-24 point (24-48 on printed poster)

Adjustable text boxes & other elements: These will proportionally enlarge when MAVS enlarges the posters.

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MAVS = save as .pdf, VIOS for printing orders, request prints at 72x48 inches.