AD _____

Award Number: W81XWH-15-2-0024

TITLE: Assessment and Treatment of blast-Induced Auditory and Vestibular Injuries

PRINCIPAL INVESTIGATOR: Dr. Joseph B. Long

CONTRACTING ORGANIZATION: The Geneva Foundation
Tacoma, WA 98402

REPORT DATE: June 2016

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution is unlimited.

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
June 2016	Annual	30 May 2015 - 29 May 2016

4. TITLE AND SUBTITLE

Assessment and Treatment of blast-Induced Auditory and Vestibular Injuries”

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-15-2-0024

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Dr. Joseph B. Long

E-Mail: Joseph.b.long.civ@mail.mil

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) AND ADDRESS(ES)

The Geneva Foundation
917 Pacific Ave., Suite 600
Tacoma, WA 98402

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR’S ACRONYM(S)

11. SPONSOR/MONITOR’S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited.

13. SUPPLEMENTARY NOTES

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.

15. SUBJECT TERMS
traumatic brain injury

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU		19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

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

Blast overpressure (BOP), auditory brainstem response (ABR), distortion product otoacoustic emission (DPOAE), inner ear, vestibular, injury

3. Accomplishments

Specific Aims:

1. To determine whether exposure to a single blast, repeated blasts, or blast in combination with blunt head trauma causes deficits in vestibular function that are matched by damage to the vestibular organs within the mouse inner ear.
2. To determine whether functional and morphological changes within the auditory organs of the mouse inner ear differ after exposure to single blast, repeated blasts, blunt head trauma or blast in combination with blunt head trauma.
3. To determine the cell-type-specific changes in gene expression that occur within auditory supporting cells and hair cells after repeated blast exposure.
4. To determine whether overexpression of 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: Q 1 – 2. Obtain IACUC and ACURO approved protocol
Q 3. Establish brain injury model; start auditory function assessment

Q 4. Vestibular function assessment

Year 2: Q 1 – 2. Examine cochlear and vestibular tissue at 1d and 7d after bTBI

Q 3 – 4. Examine cochlear and vestibular tissue at 30d and 60d after bTBI

Year 3: Q 1 – 2. Establish transgenic mouse; analyze RNA on BOP-injured mice

Q 3 – 4. Effect of hair cell regeneration

Methods:

Currently, two types of devices [conventional shock tube (Fig. 1) and an Advanced Blast Simulator (ABS, Fig. 2)] are used to simulate blast in the laboratory. To calibrate injury conditions and to determine a proper TBI treatment and appropriate system settings for auditory functional tests, we included 24 mice in a pilot study after obtaining the IACUC and ACURO approved animal protocol and meeting with veterinarians and animal facility members at WRAIR. A conventional shock tube is limited in its ability to simulate blast waveforms. The ABS is designed to intrinsically replicate all the key features of blast wave flow conditions, including the negative phase and secondary shock. However the ABS has not been previously utilized to expose mice to blast. Therefore a pilot study to validate the damage to auditory system is essential. Utilizing this device, we investigated the damage to brains of CBA mice and associated hearing loss by placing mice side-on and front head-on toward the blast shockwave, as well as single and repeated exposures, respectively.

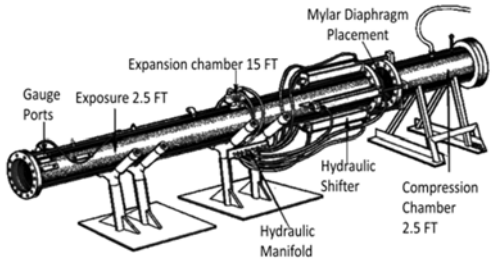


Fig. 1. The conventional shock tube

Fig. 2. The Advanced Blast Simulator (ABS)

For assessments of auditory function, anesthesia is required during Auditory Brainstem Response (ABR) and Distortion Product Otoacoustic Emissions (DPOAE) recording. Inhalation of isoflurane was used in our previous research in rat's ABR testing. The dose of isoflurane can be easily adjusted by the flow control. However, due to movement artefact and nosecone constraints, it is a sub-optimal anesthetic for recordings. A mixture of Ketamine and Dexdometer has been found to be a more effective anesthetic combination in mice. The procedure protocol for ABR and DPOAE testing has been defined and two research assistants have been trained to conduct this task.

After the pilot study, the ABS-induced auditory injuries in mouse models have been refined. Anesthetized mice were placed in a

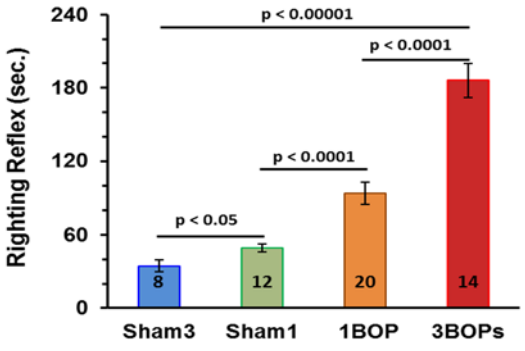


Fig. 3. Righting reflex time after exposure to ABS

headfirst orientation (facing the oncoming shock wave) and received a single blast (BOP, total pressure of 42 psi, static pressure of 18 psi) or three repeated blast exposures (3BOPs) with intervals of 2 minutes. Sham controls were handled in the same manner without exposure to shockwaves. Data showed that righting reflex time (RRT) increased significantly in BOP groups. Compared to a single BOP, 3BOPs caused a significant elevation inRRT (Fig. 3). The mortality was 3% (2 deaths out of 70) after 3BOPs.

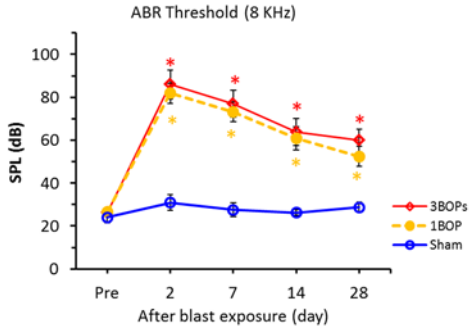


Fig. 4a. ABR to low frequency stimulus

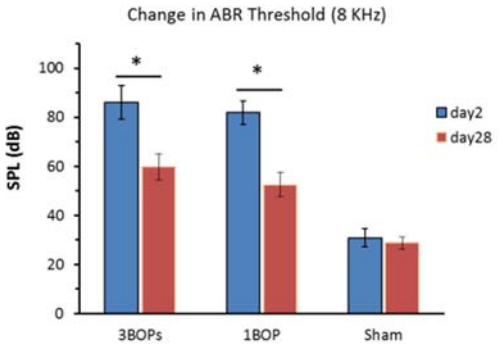


Fig. 4b. ABR to low frequency stimulus after injury

A time-course of auditory functional deficit has been investigated. Each mouse received auditory function tests including Auditory Brainstem Response (ABR) and Distortion Product Otoacoustic Emissions (DPOAE) before injury as its baseline.
Those functional responses have been also recorded under anesthesia at 2, 7, 14 and 28 days after blast exposure. Data showed a complete hearing loss at frequencies of 8, 16, 32 and 40 KHz (threshold > 90 dB)

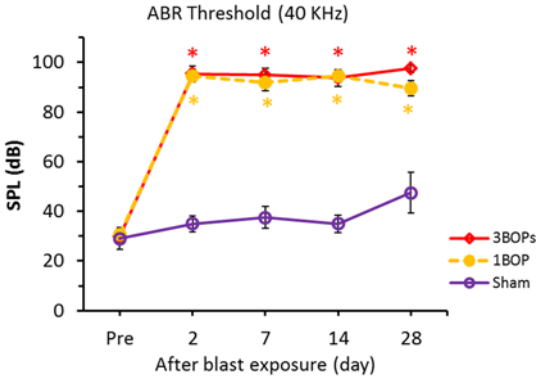


Fig. 5. ABR to high frequency stimulus

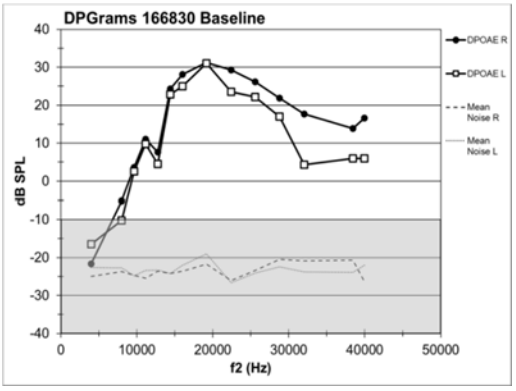


Fig. 6a. DPOAE baseline

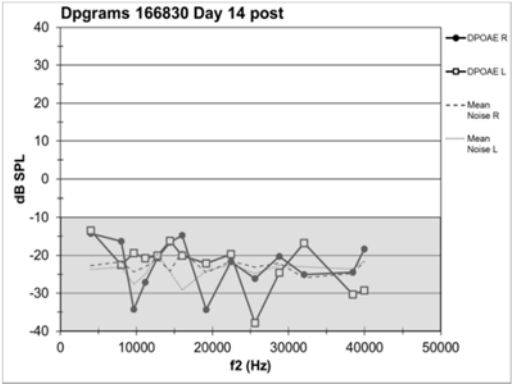


Fig. 6b. DPOAE at 14 days post-injury

immediately after exposure to blast overpressure which persisted over one month (Fig. 4a and Fig. 5). At 28 days after injury, BOP and 3BOPs caused significant decreases in

ABR threshold to lower frequency (8 KHz) stimuli in comparison to the acute (2 days) hearing loss (Fig. 4b), although they were higher than their baselines. Compared to lower frequency hearing, ABR threshold to higher frequency (40 KHz) stimuli did not show recovery at 28 days post-injury. DPOAE data indicated that hearing loss occurred immediate after blast exposure and extend out through 14 days post-injury (Fig. 6a and 6b). During this reporting period, a group of mice has been used for Rotarod testing. The result needs to be validated.

During this reporting period, we:

- Interviewed, hired and trained 2 research technicians who are now skilled in the animal handling, ABR testing, DPOAE testing, Rotarod testing and experimental data collection procedures required for this study.
- The ABS-induced auditory injuries in mouse models have been established after the pilot study.
- The Anesthesia issue for auditory functional assessments in the mouse has been successfully resolved.
- ABR and DPOAE testing in mouse are established. Data collection for long-term investigation is ongoing.

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

There is nothing to report

6. Products:

Other publications, abstracts, conference papers and presentations

The abstract ‘Characterization of Auditory Injury in Mice Exposed to Blast Overpressure in an Advanced Blast Simulator’ has been accepted by MHSRS 2016 and will be presented at the poster session in November at San Diego.

Ying Wang, MD[1], Yanling Wei, MD[1], Stephen Van Albert, BE[1], Tracy Fitzgerald PhD[2], Amy Northrop, BS[2], Rodrigo Uriste, BS[1], Peethambaran Arun, PhD[1], Donna Wilder,

BS[1], Sajja Venkatasivasaisujith, PhD[1], Irene D. Gist, BA[1] Stephen McInturff, BS[2], Weise Chang PhD[2], Matthew Kelley, PhD[2] and Joseph Long, PhD[1].

1(Blast-Induced Neurotrauma Branch, Center for Military Psychiatry and Neuroscience, WRAIR, Silver Spring, MD)

2(Porter Neuroscience Research Center, NIDCD/NIH, Bethesda, MD)

The abstract 'Auditory functional deficits and structures changes following blast shockwave exposure in mice' has been accepted by Society for Neuroscience 2016 and will be presented at the poster session in August at Florida.

Wang Y[1], Wei Y[1], Van Albert S[1], Northrop A[2], Uriste R[1], Arun P[1], Wilder D[1], Venkatasivasaisujith S[1], Gist I[1], McInturff S[2], Chang W[2], Fitzgerald T[2], Kelley M[2] and Long J[1].

1. WRAIR, Silver Spring, MD; 2. NIDCD/NIH, Bethesda, MD.

7. Participants & Other Collaborating Organizations

Name	Project Role	Percent Effort	Organization
Dr. Joseph Long	PI	10%	WRAIR
Ying Wang	Co-PI	70%	WRAIR
Yanling Wei	Research Associate	50%	Geneva
Donna Wilder	Lab Manager	50%	Geneva

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

8. Special Reporting Requirements

A Quad Chart is attached.

9. APPENDICES

Two abstracts are attached.

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

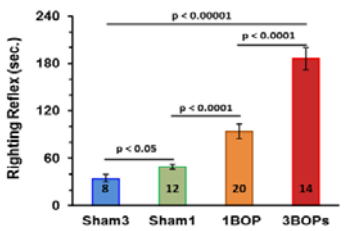
Blast injury model using well-characterized shock tube exposures of mice to repetitive blast overpressure

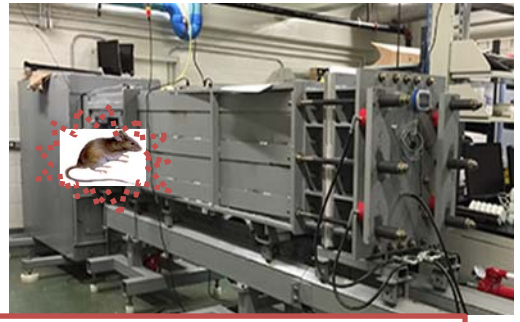
Combined functional, morphological, and neurobiological assessments

Functional testing using ABR, DPOAE, Rotarod test and VEPs

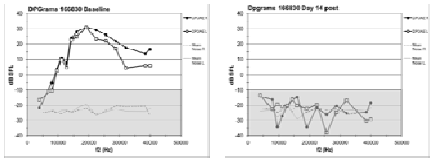
Morphological changes identified using immunohistochemistry, histological sections, TEM and SEM

Biochemical alterations evaluated by qPCR, and in situ hybridization analysis

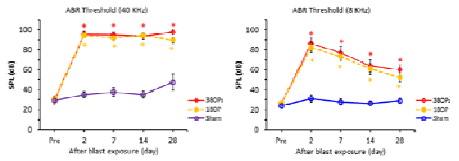




Auditory functional assessments showed hearing loss at 28 days post-injury



Change in DPOAE



Changes in ABR threshold

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**	

Updated: (06/29/16)

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: $494,013

Actual Expenditure: $324,954

Characterization of Auditory Injury in Mice Exposed to Blast Overpressure in an Advanced Blast Simulator

Ying Wang, MD[1], Yanling Wei, MD[1], Stephen Van Albert, BE[1], Tracy Fitzgerald PhD[2], Amy Northrop, BS[2], Rodrigo Uriste, BS[1], Peethambaran Arun, PhD[1], Donna Wilder, BS[1], Sajja Venkatasivasaisujith, PhD[1], Irene D. Gist, BA[1] Stephen McInturff, BS[2], Weise Chang PhD[2], Matthew Kelley, PhD[2] and Joseph Long, PhD[1].

[1](Blast-Induced Neurotrauma Branch, Center for Military Psychiatry and Neuroscience, WRAIR, Silver Spring, MD)
[2](Porter Neuroscience Research Center, NIDCD/NIH, Bethesda, MD)

Background: Blast exposure is the most common cause of traumatic brain injury (TBI) in Warfighters. 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. Using this device, the present research is aimed at producing a comprehensive characterization of auditory functional deficits and associated pathological changes in the central and peripheral auditory signal processing regions disrupted by exposure to blast shockwaves.

Methods: Isofluorane anesthetized CBA mice (male, 23 - 28 g) were exposed to blast overpressure (peak static pressure of 19 psi and 4 msec positive phase duration) generated by the ABS which 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. 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. At 14 days post-injury, the brains and inner ears of mice were fixed in 4%PFA solution and sectioned for pathological evaluation using silver staining, hematoxylin and eosin staining, and immunohistochemistry.

Results: Audiometry data revealed that blast exposure caused significant elevations of ABR threshold, increased ABR wave latency, and reductions in ABR wave amplitude immediately following insult. These changes were observed over the entire acoustic frequency spectrum and persisted over 14 days. DPOAE signals were undetectable immediately after blast exposure and their disappearance persisted over 14 days, suggesting significant damage to the inner ear. Silver staining of brain sections showed significant axonal degeneration in auditory and vestibular signal processing regions in brainstem and cerebellum. Immunohistochemical evaluations revealed a significant increase in the expression of GFAP and Iba1 in the brainstem and cerebellum. Immunostaining of Myo7a and Phalloidin in wholemount cochlea revealed appreciable damage to outer hair cells, inner hair cells, as well as to other structures in the inner ear. Discussion: 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 mechanism of hearing impairment after blast exposure and for evaluating potential strategies for prevention and cure.

Auditory functional deficits and structures changes following blast shockwave exposure in mice

Wang Y[1], Wei Y[1], Van Albert S[1], Northrop A[2], Uriste R[1], Arun P[1], Wilder D[1], Venkatasivasaisujith S[1], Gist I[1], McInturff S[2], Chang W[2], Fitzgerald T[2], Kelley M[2] and Long J[1].

(1) WRAIR, Silver Spring, MD; (2) NIDCD/NIH, Bethesda, MD.

A high fidelity animal model is critical to define the mechanism(s) of blast-induced auditory injury and to develop therapeutic strategies. 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. Using this device, the present research is aimed at producing a comprehensive characterization of auditory functional deficits and associated pathological changes in the central and peripheral auditory signal processing regions disrupted by exposure to blast shockwaves. isofluorane anesthetized CBA mice (male, 23 - 28 g) were exposed to blast overpressure (peak static pressure of 19 psi and 4 msec positive phase duration) generated by the ABS which 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. A time-course of blast effects on auditory function was assessed by analyzing auditory brainstem response (ABR) and distortion product otoacoustic emission (DPOAE) under anesthesia. Data revealed that 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. DPOAE signals were undetectable immediately after blast exposure and their disappearance persisted over 14 days, suggesting significant damage to the inner ear. Immunostaining of Myo7a and Phalloidin in wholemount cochlea revealed appreciable damage to outer hair cells, inner hair cells, as well as to other structures in the inner ear. Silver staining of brain sections showed significant axonal degeneration in auditory and vestibular signal processing regions in brainstem and cerebellum. 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.