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TITLE: Combined Effects of Primary and Tertiary Blast on Rat Brain: Characterization
of a Model of Blast-induced Mild Traumatic Brain Injury

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14. ABSTRACT We hypothesize that the biomechanical perturbations of the brain that yield blast-induced mTBI in injured warfighters can be recreated with reasonable fidelity in rats under carefully controlled experimental conditions, and that several of the characteristic sequelae of blast-induced mTBI observed clinically can be reproduced in a rodent injury model. In many, if not most circumstances yielding blast mTBI, brain injury results from a combination of blast overpressure (BOP) (i.e. primary blast) and head acceleration and/or impact (i.e. tertiary blast). The mTBI resulting from these combined insults may be fundamentally different from that seen from either insult alone.					
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INTRODUCTION

Many warfighters who sustain blast-induced TBI in combat are exposed to a brain insult resulting from a combination of both a shock wave and biomechanical perturbation related to rapid acceleration and/or impact with a solid object (MacDonald et al., 2011). The TBI resulting from these combined insults is likely to be fundamentally different from that seen from either insult alone. We hypothesize that the combined biomechanical perturbations of the brain that yield blast-induced mild TBI in injured warfighters can be recreated with reasonable fidelity in rats under carefully controlled experimental conditions, and that several of the characteristic sequelae of blast-induced mild TBI observed clinically can be reproduced in an established rodent injury model. We anticipate that this model can provide a valuable experimental tool to assist ongoing efforts to mitigate the risks and consequences of blast-induced mTBI in warfighters.

BODY

Research accomplishments associated with each task outlined in the approved Statement of Work are described below.

Task 1

Manipulate and monitor blast exposure conditions (i.e. incident flow conditions) in the compression-driven shock tube and recreate with reasonable fidelity the biomechanical loading conditions estimated to underlie primary blast-induced mild TBI in warfighters. Establish a mild injury severity based upon loss of consciousness (LOC), histopathology, and neurological and neurobehavioral outcomes.

Task 1 Progress

Blast exposure conditions in the shock tube have been generally standardized. Rats are consistently secured 2.5ft within the mouth of the tube in tautly drawn coarse mesh netting (reducing variability in potential acceleration contributions to injury that may occur with less taut netting). Pressure gauges in the rat holder record ambient flow conditions at this position. We have similarly down-selected and standardized several sensitive neurobehavioral assessments that appear to fairly consistently discern functional impairments in rats.

It is important to note that, at this stage, BOP alone does not produce robust neurobehavioral disruptions. These procedures, which include the rotary pole, open field and Morris water maze tests, reveal disruptions resulting from weight drop alone or when combined with BOP. Disruptions resulting from BOP exposure alone are generally mild and transient. Although mortality following combined injuries has provided significant challenges, progressive refinements of BOP exposure conditions have generally reduced BOP-induced mortality as well as brain injuries.

We also evaluated olfactory discrimination and visual discrimination as sensory-driven tasks that, based upon reports of anosmia and eye injuries in injured warfighters exposed to blast, we thought would provide relevant preclinical correlates. Olfactory discrimination proved to be very resistant to disruption in injured rats, and we eventually abandoned it as a useful outcome measure. In contrast, visual discrimination procedures, in which rats are trained to distinguish and respond to visual cues by pressing left or right levers for delivery of food pellets, have been much more promising. Through progressive revisions in testing procedures, we have established parameters that incorporate total responses, accuracy and reaction time descriptions of visual discrimination performance. By manipulating the

complexity of the task, these visual discrimination procedures can be used to probe rats' capacities for learning and memory in addition to their visual acuity.

As noted in the quarterly reports, with improved positioning and securing of rats in the shock tube during BOP exposure, the neuropathological features produced by BOP alone have diminished and brain injuries are typically mild, even at the upper limits of survivable BOP exposures (21psi). During this past year, we initiated a collaboration with investigators at the Center for In Vivo Microscopy at Duke University Medical Center to complement conventional light microscopic histopathological assessments with diffusion tensor imaging. In addition to corroborating histopathological findings, the DTI capabilities of our collaborators provide extremely high resolution images of the entire brain that can be quantitatively analyzed and also followed longitudinally (i.e. images from the same subject compared at different stages post-injury). Brains of rats exposed to a single BOP, 2 closely coupled BOPs, or sham handling have been analyzed and, to date, these imaged brains revealed significant quantitative changes following closely coupled repeated BOP exposures, but not single exposures. In addition to corroborating histopathological findings, these DTI images provide a powerful comprehensive, quantitative analysis of sometimes subtle neuropathological changes throughout the entire brain, which is not possible with standard histopathological techniques.

Task 2

Establish conditions yielding a mild injury severity with a surgery-free adaptation of the weight drop brain injury model (or alternative) to create tertiary blast brain injury based upon LOC, histopathology, and neurological and neurobehavioral outcomes.

Task 2 Progress

Experiments with the instrumented weight drop device and removable instrumented helmets manufactured by ORA failed to generate consistently discernible brain injuries in rats, which we interpret to be due to protection against direct skull impact provided by the weight drop helmet. Since the head accelerations resulting from these weight drops were not vastly different than that generated with the technique described by Marmarou (in which a disc directly affixed to the exposed skull is impacted by the dropped weight), we interpret that impact, rather than acceleration, likely accounts for the lion's share of injury produced with the Marmarou technique.

We next evaluated rats in which weight drop was achieved by dropping a 500g weight through a cylinder onto a 10mm stainless steel disc that is positioned on the rat's head using a light weight Mylar headpiece. For this approach, the scalp is intact and the weight drop is performed within 45 seconds of blast exposure. Patterns of injury associated with different height-weight combinations were again less substantial and more variable than were observed in rats in which the dropped weight impacted the disc directly affixed to the skull.

Consequently, we pursued a fourth approach in which discs were affixed to the skull before BOP exposure, allowing weight drop to again be performed within 30-45 seconds of BOP exposure. Injuries and neurobehavioral disruptions produced with this approach appear to be greater and more consistent than were seen with the removable headpiece in which the scalp remained intact. Also, insofar as we could detect, affixing the disc to the skull before BOP exposure did not appear to alter the effects of BOP relative to those seen with BOP exposures in the absence of the disc. Although not perfect, we concluded that this fourth approach is the best means to combine weight drop with BOP with a minimal time separation (Task 3).

Task 3

Combine BOP and the selected impact acceleration insult at multiple combined severities, and evaluate the histopathological, physiological, and neurobehavioral outcomes relative to those seen following each insult alone. Establish combined injury conditions to produce mTBI.

Task 3 progress

Combinations of BOP and weight drops of varied heights were completed using the ORA weight drop device as well as the Marmarou approach, using a) discs affixed to the skull after BOP exposure, b) discs secured on the head immediately after BOP exposure using a flexible headpiece resting over an intact scalp, and c) discs affixed to the skull before BOP exposure. In general, across all 3 weight drop approaches employing the stainless steel disc after BOP exposure, there appeared to be persistent neurobehavioral deficits on the rotating pole that were not observed in rats subjected to either insult alone, or to BOP combined with weight drops from a lesser height. These findings are consistent with the primary hypothesis of the project. Namely, that the TBI resulting from these combined insults is fundamentally different from that seen from either insult alone.

Histopathological evaluations have revealed several generally consistent neuroanatomical features. Notably, the combined insults (BOP and weight drop) yield fiber degeneration that is most prominent in cerebellum, optic tract, corpus callosum, and internal and external capsules. In addition to neurobehavioral and histopathological assessments, we also conducted olfactory and visual discrimination evaluations and analyzed EEG recordings following BOP, weight drop, or combined insults. Measurements of neurobiological mediators (e.g. reactive oxygen species, DNA fragmentation, and various inflammatory mediators) across these 3 injury conditions were also performed, and revealed patterns of changes following combined insults that were not apparent (or as large) following either insult alone.

Task 4

Using a mach stem wedge equipped with a high velocity piston impactor, instantaneously combine impact acceleration with BOP within the shock tube to produce and evaluate the concomitant combined effects of primary and tertiary blast relative to those seen following each insult alone. Establish a mild injury severity based upon loss of consciousness (LOC), histopathology, and neurological and neurobehavioral outcomes.

Task 4 progress

A new shock tube (advanced blast simulator) was designed and ordered to achieve task 4 objectives. A module incorporating a pneumatically driven piston will enable near instantaneous blast and impact to be combined for improved fidelity of combined insults and with a 2ft internal width, the rat and holder will present less flow obstruction than exists with our current 1ft diameter tube. We await delivery to embark on these objectives.

KEY RESEARCH ACCOMPLISHMENTS

- Shock tube BOP exposure conditions have been more fully characterized and refined to yield a reproducible high fidelity simulation of blast TBI.
- Neurobehavioral, neuropathological, and neurochemical consequences of shock tube BOP exposures of varied intensities have been comprehensively evaluated.
- Neurobehavioral, neuropathological, and neurochemical consequences of weight drop-induced impact acceleration of varied intensities, alone and in combination with shock tube BOP exposures, have been comprehensively evaluated using a progression of weight drop approaches.
- EEG recordings have been performed to distinguish electrophysiological consequences of individual and combined blast- and weight drop-induced brain insults.

REPORTABLE OUTCOMES

Manuscripts:

Valiyaveetil M, Alamneh Y, Wang Y, Arun P, Oguntayo S, Wei Y, Long JB, Nambiar MP. Contribution of systemic factors in the pathophysiology of repeated blast-induced neurotrauma. *Neurosci Lett*. 2013 Feb 28;539:1-6. doi: 10.1016/j.neulet.2013.01.028. Epub 2013 Jan 28.

M. Valiyaveetil, Y.A. Alamneh YA, S.A. Miller, R. Hammamieh, P. Arun, Y. Wang, Y. Wei, S. Oguntayo, J.B. Long, M.P. Nambiar. Modulation of cholinergic pathways and inflammatory mediators in blast-induced traumatic brain injury. *Chem Biol Interact*, 2012, doi:pii: S0009-2797(12)00229-3.

M. Valiyaveetil, Y. Alamneh, S. Miller, R. Hammamieh, Y. Wang, P. Arun, Y. Wei, S. Oguntayo, M.P. Nambiar, Preliminary studies on differential expression of auditory functional genes in the brain after repeated blast exposures, *J Rehabil. Res. Dev.*, 2012; 49: 1153-1162.

P. Arun, S. Oguntayo, Y. Alamneh, C. Honnold, Y. Wang, M. Valiyaveetil, J.B. Long and M.P. Nambiar. Biological blast dosimeters, *Global Medical Discovery Series*, 2012 *Online*.

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Kamnaksh A, Kwon SK, Kovesdi E, Ahmed F, Barry ES, Grunberg NE, Long J, Agoston D. Neurobehavioral, cellular, and molecular consequences of single and multiple mild blast exposure. *Electrophoresis*. 2012 Dec;33(24):3680-92. doi: 10.1002/elps.201200319. Epub 2012 Nov 26.

Kovesdi E, Kamnaksh A, Wingo D, Ahmed F, Grunberg NE, Long JB, Kasper CE, Agoston DV. Acute minocycline treatment mitigates the symptoms of mild blast-induced traumatic brain injury. *Front Neurol.* 2012;3:111. doi: 10.3389/fneur.2012.00111. Epub 2012 Jul 16.

Abstracts and Meeting Presentations:

P. Arun, R. Abu-Taleb, M. Valiyaveetil, Y. Wang, J. Long and M. P. Nambiar. Cell membrane stabilizers protect against blast-induced Neurotrauma. Advanced Technology Applications for Combat Casualty Care / Military Health Sciences Research Symposium (ATACCC / MHSRS), Fort Lauderdale, FL, 8/13 - 8/16/12.

P. Arun, R. Abu-Taleb, S. Oguntayo, Y. Wang, M. Valiyaveetil, J. Long and M. P. Nambiar. Compromised cell membrane integrity and unusual changes in brain injury biomarkers after blast exposure. Advanced Technology Applications for Combat Casualty Care / Military Health Sciences Research Symposium (ATACCC / MHSRS), Fort Lauderdale, FL, 8/13 - 8/16/12.

P. Arun, R. Abu-Taleb, M. Valiyaveetil, Y. Wang, J. Long and M. P. Nambiar. Cellular mechanisms of blast induced traumatic brain injury: transient disruption of cell membrane and pharmacological interventions for protection. National Capital Region Traumatic Brain Injury Research Symposium held at National Institutes of Health, MD - 2012

P. Arun, R. Abu-Taleb, S. Oguntayo, Y. Wang, M. Valiyaveetil, J. Long and M. P. Nambiar. Atypical changes in biomarkers of traumatic brain injury after blast exposure: Role of compromised cell membrane integrity. CNRM annual meeting held at National Institutes of Health, MD - 2012

Wang Y, Wei Y, Oguntayo S, Valiyaveetil M, Arun P, Long J, Nambiar M. Blast exposure induces brain DNA damage. Advanced Technology Applications for Combat Casualty Care / Military Health Sciences Research Symposium (ATACCC / MHSRS), Fort Lauderdale, FL, 8/13 - 8/16/12.

DeMar JC, Tong LC, Hill MI, Dalmolin AR, Gharavi RB, Edwards AA, Riccio CA, Byzek SA, Oliver TG, and Long JB (2012). Assessment of Ocular Injuries to the Retina in Rats Exposed to Blast Over Pressure. Advanced Technology Applications for Combat Casualty Care / Military Health Sciences Research Symposium (ATACCC / MHSRS), Fort Lauderdale, FL, 8/13 - 8/16/12.

Riccio C, Van Albert S, Tong L, Edwards A, Long J. Combined effects of primary and tertiary blast of rat brain: preliminary characterization of a model of blast-induced mild traumatic brain injury. Advanced Technology Applications for Combat Casualty Care / Military Health Sciences Research Symposium (ATACCC / MHSRS), Fort Lauderdale, FL, 8/13 - 8/16/12.

Andrist J, Edwards A, Riccio C, Wilder D, Van Albert S, Long J. Neurobehavioral evaluation of rats using operant testing chambers after primary and tertiary blast exposure. Advanced Technology Applications for Combat Casualty Care / Military Health Sciences Research Symposium (ATACCC / MHSRS), Fort Lauderdale, FL, 8/13 - 8/16/12.

Research Proposals:

Based upon work supported by this award, funding was sought through 8 research pre-proposals and proposals submitted to the CDMRP and DMRP during this reporting period.

CONCLUSION

Results to date are consistent with the hypothesis that BOP generates a closely-associated insult to the brain (and other organs as well), interactively compromises the brain's resilience and exacerbates the pathophysiological effects of other injury modalities, such as impact acceleration (i.e. tertiary injury). With continued refinement, under carefully controlled experimental conditions, the combined biomechanical perturbations of the brain that yield blast-induced mild TBI in injured warfighters can be recreated with reasonable fidelity to reproduce characteristic sequelae of blast-induced mild TBI. The end product model will provide an invaluable tool to define underlying neurobiological mechanisms and rationally establish effective countermeasures to lessen short-term impairments (e.g. return-to-duty) as well as chronic debilitation (e.g. chronic traumatic encephalopathy).

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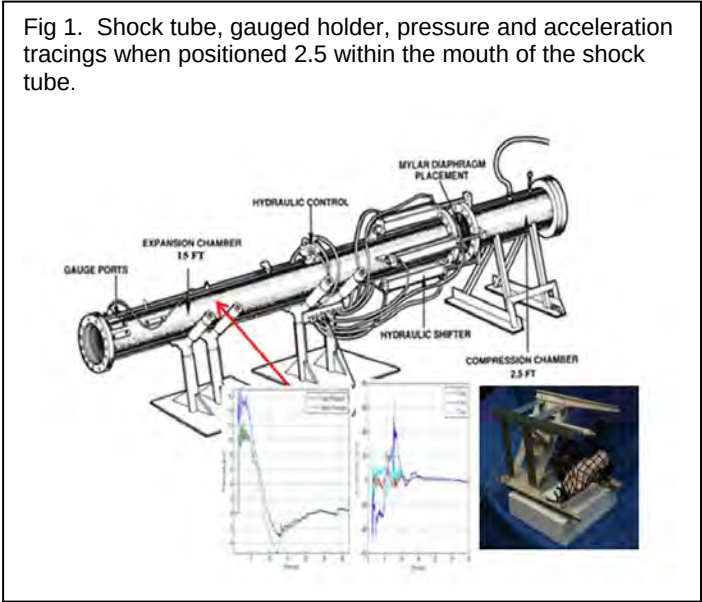
Marmarou A, Foda MA, van den Brink W, Campbell J, Kita H, Demetriadou K. A new model of diffuse brain injury in rats. Part I: Pathophysiology and biomechanics. *J Neurosurg.* 1994 Feb;80(2):291-300.

APPENDICES

None

SUPPORTING DATA

BOP exposure of isoflurane-anesthetized rats occurs in a shock tube (Fig. 1). Rats are suspended in tautly drawn, coarse mesh netting in a gauged holder that records incident and side-on pressures. Under these carefully controlled experimental conditions, blast-induced biomechanical perturbations can be recreated with reasonable fidelity; BOP pressures are determined by Mylar membrane thickness (Fig. 2). For impact-acceleration either alone (Task 2) or immediately after BOP exposure (Task 3), rats are subjected to weight drop head injury produced by dropping a 500g weight 100-200cm through a cylinder onto a 10mm stainless steel disc affixed to the skull, as described by Marmarou et al. (1994), or onto a stainless steel disc secured over the scalp by a light weight Mylar headpiece for a surgery-free weight drop that minimizes time constraints on dual injuries.



(Fig. 3). Since injuries resulting from different height-weight combinations using the headpiece have been less substantial and more variable than were observed in rats in which the dropped weight impacts the disc directly affixed to the skull, we have employed a fourth approach in which discs are affixed to the skull before BOP exposure, which for combination injuries allows weight drop to be performed within 30-45 seconds of BOP exposure.

Based upon observations of anosmia in pigs exposed to explosive blast (Bauman, personal communication), we explored olfactory discrimination in rats exposed to BOP in a shock tube using the procedure and device described by Slotnick and coworkers (Lu and Slotnick, 1997) pictured in Fig. 3. A positive or negative stimulus was randomly presented and the experimental subject was rewarded with water for responding (i.e. licking) in response to the positive stimulus. Rats were trained and tested in 8 blocks of 40 trials each, and $\geq 80\%$ accuracy is considered learned. The positive stimulus was 0.05% Amyl Acetate in water, which was tested with presentation concentrations ranging from 2ppm – 0.25ppm. The negative stimulus was water. Post-injury tests failed to reveal loss of olfactory discrimination, and rats continued to perform at $>80\%$ discrimination accuracy (Fig. 4).

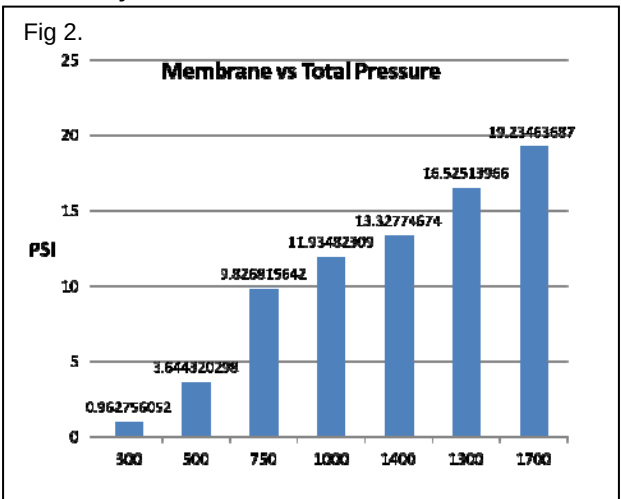
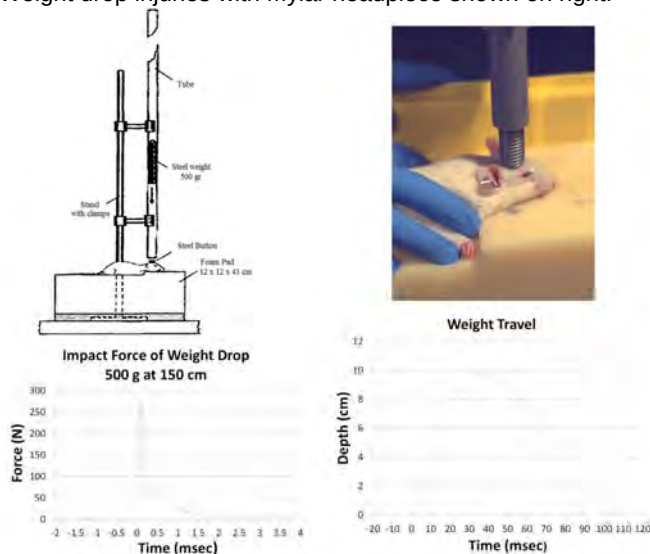


Fig 3. Weight drop injuries with Mylar headpiece shown on right.



Visual discrimination tasks provide another sensory based neurobehavioral test. In these procedures, rats are trained to distinguish and respond to visual cues by pressing left or right levers for delivery of food pellets. Through progressive revisions in testing procedures, we have established parameters that incorporate total responses, accuracy, and reaction time descriptions of visual discrimination performance (Figs. 6-8). By manipulating the complexity of the task, these visual discrimination procedures can be used to probe rats' capacities for learning and memory in addition to their visual acuity. Results to date point to

Fig 4. Olfactometer

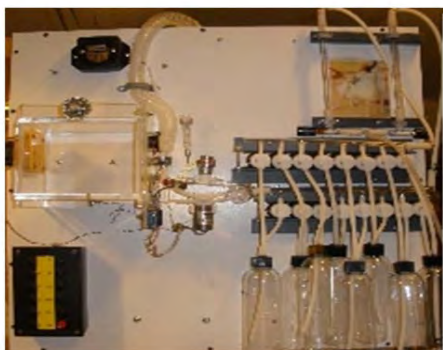


Fig. 5. Olfactory discrimination accuracy following combined insults (BOP & weight drop). N=12 pre- and 6,6, & 3 post-injury.

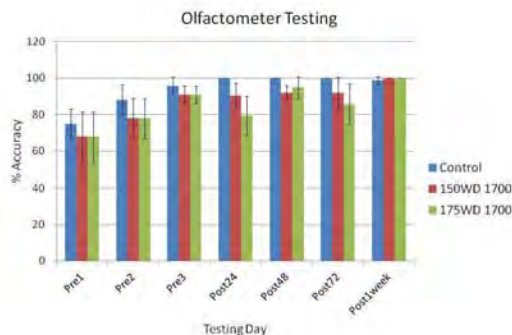
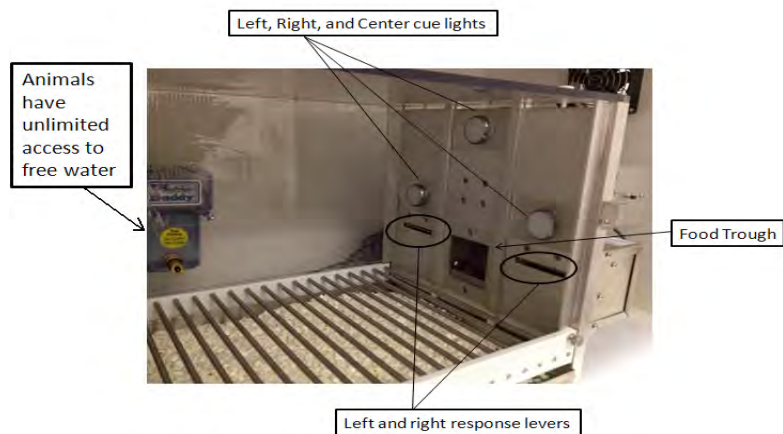


Fig 6. Visual discrimination chamber.



these tests as highly sensitive indicators of blast TBI.

Fig 7. Visual discrimination accuracy relative to preinjury baseline (N=5 rats/gp).

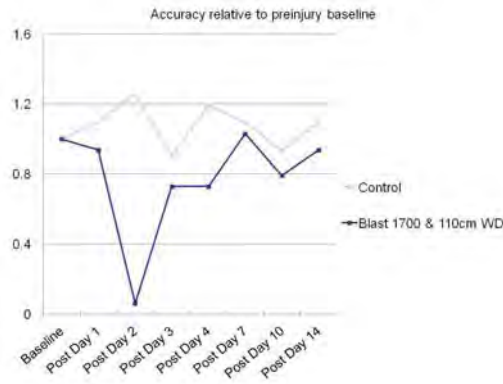
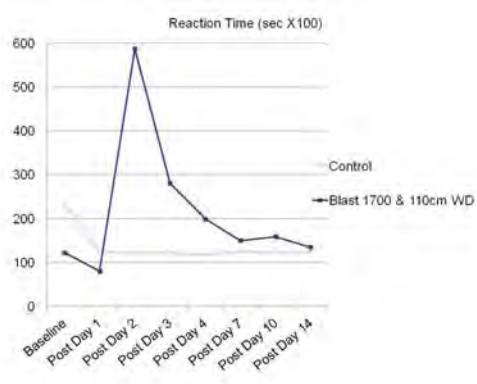
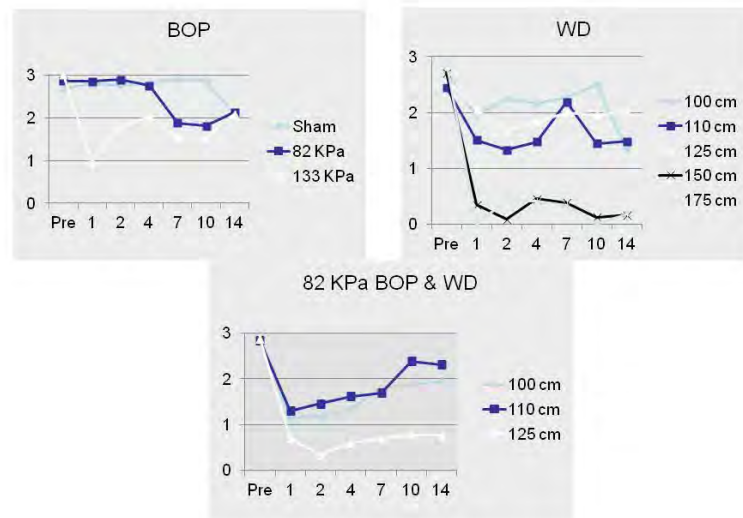


Fig 8. Visual discrimination reaction time (sec X100, N=5 rats/gp).



Among the other functional neurobehavioral assessments that are used to evaluate these rats (e.g. rotary pole, Morris water maze, open field), the level of performance disruption varied greatly depending upon how weight drop was performed (i.e. ORA helmet, Mylar headpiece over intact scalp, or disc affixed directly to the skull). Although the injury conditions have varied, generally when BOP and weight drop are combined, we have established that the combined insults produce lasting deficits that are not seen following either BOP or weight drop alone (Figs. 9-11). For the rotary pole test, at varied times post-injury, pre-trained rats traverse a rotating pole and are graded using a scoring scheme that incorporates balance (1 point for not falling; 0 points for a fall), velocity (distance on the pole covered/time) and distance completed (1 point for a complete run, 0.75 for a fall at the $\frac{3}{4}$ mark, 0.5 for a fall in the middle and 0 points for a fall at the beginning). On each test day, each rat is given three trials. The two highest score runs are averaged as the score for that rat on that day.

Fig 9. Rotary pole performance following individual or combined injuries (Disc on skull with weight drop 30 sec post BOP, N = 8-11 rats/gp).



The Morris water maze (MWM) is used to evaluate spatial learning and navigation during the first week after injury as rats locate a submerged platform in a 170 cm diameter water-filled pool placed in a room with unchanging extra-maze cues. Video-tracking software records time spent and distance swum in each quadrant, the time spent locating the platform, the total distance swum, and the velocity of swimming. In the open field test, a video system monitors individual rats as they freely wander within an enclosed circle (6ft diameter) which is divided by a video template into 21 sectors. This test provides a quantitative measure of locomotor and exploratory activity as a partial indicator of stress/anxiety levels.

These combinations of BOP and weight drop insults also produce a variety of neurobiological changes (e.g. DNA fragmentation, reactive oxygen species production, proteomic responses) that are not seen following either insult alone (e.g. Fig. 12).

Fig 10. MWM and rotary pole after BOP and weight drop (N = 8-9 rats/gp).

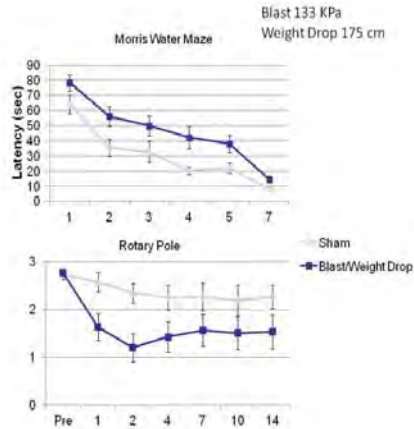


Fig 11. Open field exploration after BOP (11 psi) and weight drop (disc on skull with weight drop 30 sec post BOP, N=6-7 rats/gp)

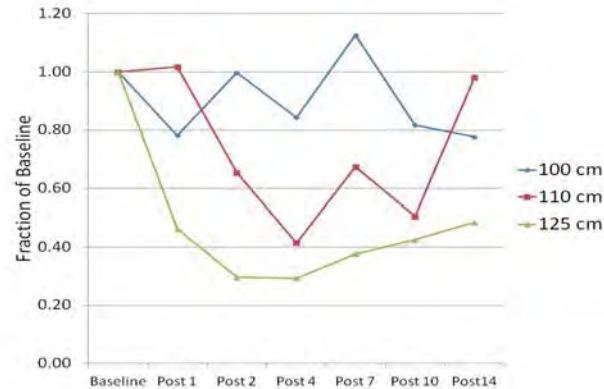
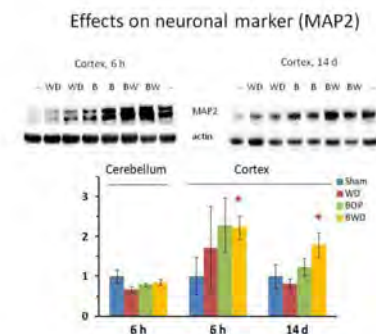


Fig 12. Changes in the neuron-specific cytoskeletal protein Microtubule-associated protein 2 (MAP2). N=6 rats/gp.



The hallmark neuropathological feature in rat brains following BOP exposure is widespread fiber degeneration that is most prominent in cerebellum, optic tracts and external capsule. Similar injuries are evident in weight drop-injured brains and are accompanied by additional degeneration in the pyramidal decussation, paraolivary nucleus, and cerebral cortex (Figs. 12, 13). Neuropathological scoring completed to date suggests higher levels of injury following combined insults (Fig. 14). Similarly, immunohistochemical assessments reveal greater

Fig 13. Weight drop-induced cerebellar degeneration evident 14 days postinjury in silver impregnated sections.

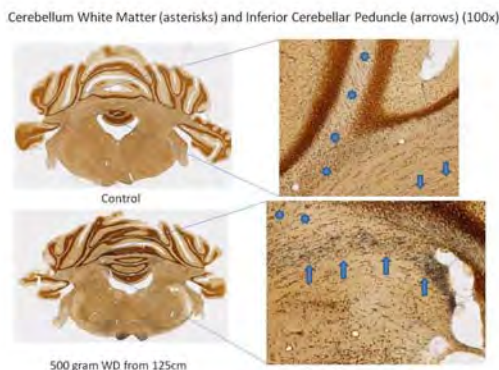
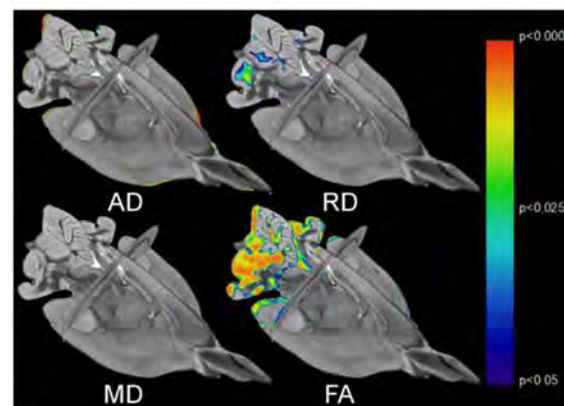
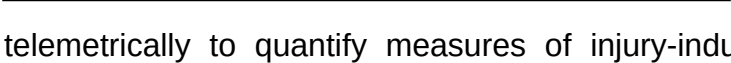


Fig 14. DTI images from rats subjected to closely coupled repeated BOP exposures (21 psi). Brains were removed 48 h post-blast, fixed, and imaged. N=8 rats/gp (sham, single BOP, and repeated BOP).



glial fibrillary acidic protein (GFAP) and IBa1 immunoreactivity in rats subjected to combined injuries than are seen following either injury alone. Working with collaborators at the Center for In Vivo Microscopy at Duke University, ex vivo DTI MRI comparisons of sham- and BOP-exposed brains provided high resolution quantitative corroboration of light microscopic neuropathological findings. After tensor estimation and computation of DTI parametric maps (i.e. fractional anisotropy [FA], radial diffusivity [RD], axial diffusivity [AD], mean diffusivity [MD]) were carried out, image data were spatially normalized using non-linear, diffeomorphic image registration to create an average

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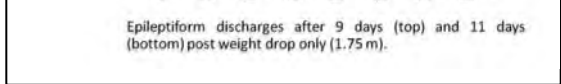
recordings. The algorithms use short-time Fourier transforms to compute the power spectrum of the signal for 2 second intervals. The spectrum is then divided into the delta, theta, alpha and beta frequency bands. A linear fit to the power spectrum is used to distinguish normal EEG activity from artifacts and high amplitude spike wave activity. A graphical user interface has been created that simultaneously plots the raw EEG in the time domain transform. Motor activity and temperature are associated with the EEG. The accuracy of this algorithm is also verified against EEG recordings taken 1 to 3 days after exposure.

brain template for voxelwise comparisons. Voxelwise analysis of DTI parameter changes were performed using SurfStat MATLAB tools and generated statistical significance maps using false discovery rate (FDR) to correct for multiple comparisons.

Prolonged telemetric EEG recordings allow detection of epileptiform electrocorticography events and other EEG anomalies that can occur several days post-insult. These discharges can precede the appearance of spontaneous recurrent seizures and are not always associated with behavioral abnormalities. Cortical EEG is recorded during seizure activity. Baseline EEG is recorded continuously for 2 days pre-injury, and is continued through 2 weeks after P and/or weight drop injuries. A set of automatic MATLAB algorithms have been developed to remove artifacts and measure characteristics of long-term EEG

Fig 17.

Epileptiform discharges after 9 days (top) and 11 days (bottom) post weight drop only (1.75 m).





Acute minocycline treatment mitigates the symptoms of mild blast-induced traumatic brain injury

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Mild traumatic brain injury (mTBI) represents a significant challenge for the civilian and military health care systems due to its high prevalence and overall complexity. Our earlier works showed evidence of neuroinflammation, a late onset of neurobehavioral changes, and lasting memory impairment in a rat model of mild blast-induced TBI (mbTBI). The aim of our present study was to determine whether acute treatment with the non-steroidal anti-inflammatory drug minocycline (Minocin®) can mitigate the neurobehavioral abnormalities associated with mbTBI. Furthermore, we aimed to assess the effects of the treatment on select inflammatory, vascular, neuronal, and glial markers in sera and in brain regions associated with anxiety and memory (amygdala, prefrontal cortex, ventral, and dorsal hippocampus) following the termination (51 days post-injury) of the experiment. Four hours after a single exposure to mild blast overpressure or sham conditions, we treated animals with a daily dose of minocycline (50 mg/kg) or physiological saline (vehicle) for four consecutive days. At 8 and 45 days post-injury, we tested animals for locomotion, anxiety, and spatial memory. Injured animals exhibited significantly impaired memory and increased anxiety especially at the later testing time point. Conversely, injured and minocycline treated rats' performance was practically identical to control (sham) animals in the open field, elevated plus maze, and Barnes maze. Protein analyses of sera and brain regions showed significantly elevated levels of all of the measured biomarkers (except VEGF) in injured and untreated rats. Importantly, minocycline treatment normalized serum and tissue levels of the majority of the selected inflammatory, vascular, neuronal, and glial markers. In summary, acute minocycline treatment appears to prevent the development of neurobehavioral abnormalities likely through mitigating the molecular pathologies of the injury in an experimental model of mbTBI.

Keywords: TBI, anti-inflammatory, treatment, neurobehavior, proteomics

INTRODUCTION

Traumatic brain injury (TBI) is a prominent health concern worldwide as it is one of the major causes of death and chronic disability (Hyder et al., 2007). The mild form of traumatic brain injury (mTBI) has become an especially significant challenge for the civilian (Thurman et al., 1999) and the military healthcare systems (Hoge et al., 2008; Tanielian and Jaycox, 2008) due to its high prevalence and the absence of serious acute symptoms following injury. Blast-induced mTBI (mbTBI) was the most frequent form of mTBIs sustained during recent military conflicts (Warden, 2006; Terrio et al., 2009). There is currently no objective diagnosis for mbTBI, a minimal understanding of its underlying pathologies, and consequently a lack of specific, evidence based treatments.

Symptoms of blast-induced TBI (bTBI) include increased anxiety as well as memory impairment that may not be detectable for weeks or months after the exposure (Ryan and Warden, 2003; Okie, 2005; Nelson et al., 2009; Terrio et al., 2009; Cernak and

Noble-Haeusslein, 2010; Hoffer et al., 2010). The delayed onset of neurobehavioral impairments suggests a lasting secondary injury process involving distinct brain regions (Moser and Moser, 1998). The ventral hippocampus (VHC) along with the prefrontal cortex (PFC) and the amygdala (AD) are involved in mediating anxiety, while the dorsal hippocampus (DHC) is involved in mediating spatial learning and memory (Henke, 1990; Moser and Moser, 1998; Bremner, 2005, 2007). Using a rat model of bTBI, we found that a single mild blast overpressure exposure results in increased anxiety and memory impairment (Kovcsdi et al., 2011; Kwon et al., 2011). Importantly, the memory impairment was not detectable within the first week of the exposure; it became significant 2 weeks post-injury and persisted for at least 2 months after (Kovcsdi et al., 2011; Kwon et al., 2011).

Our immunohistochemical and proteomics analyses of these animals showed evidence of neuronal and glial cell loss, gliosis, and neuroinflammation at 2 months post-injury. In addition to

an increased presence of microglia in the DHC and the VHC of injured animals as well as increased tissue levels of interleukin-6 (IL-6) and interferon-gamma (IFN γ) in these brain regions. Neuroinflammation can adversely affect neuronal function by directly causing neuronal cell death as well as increasing neuron vulnerability to noxious factors like excitotoxins, which are also elevated after injury (Arvin et al., 1996; Morganti-Kossmann et al., 2002; Cacci et al., 2005; Floyd and Lyeth, 2007; Kochanek et al., 2008; Agoston et al., 2009; Agostinho et al., 2010; Czlonkowska and Kurkowska-Jastrzebska, 2011; Robel et al., 2011). Based on our previous evidence linking neuroinflammation to neurobehavioral abnormalities (Kovesdi et al., 2011), we hypothesized that anti-inflammatory treatment may improve the functional outcome in mbTBI.

To test our hypothesis, we selected the anti-inflammatory drug minocycline for several reasons. Minocycline hydrochloride easily crosses the blood brain barrier (BBB), is well characterized, safe, FDA approved, and has been used experimentally and clinically (Macdonald et al., 1973; Saivin and Houin, 1988). Similar to its tetracycline analogs, the side effects of minocycline treatment are mild and include discoloration of the teeth, gastrointestinal irritability, and candidiasis (Fanning et al., 1977; Gump et al., 1977). In humans, long-term treatment is generally safe and is well tolerated up to 200 mg/day. In animals, the lethal dose of minocycline is very high at 3600 mg/kg (Blum et al., 2004); the “therapeutic” dosage utilized in animal experiments ranges between 10 and 90 mg/kg with an average of 50 mg/kg for daily treatments (e.g., Wells et al., 2003; Stirling et al., 2004; Festoff et al., 2006; Li and McCullough, 2009; Abdel Baki et al., 2010; Lee et al., 2010; Siopi et al., 2011; Wixey et al., 2011; Ng et al., 2012).

Minocycline has been successfully used in various animal models of brain and spinal cord injuries as well as neurodegenerative diseases like Huntington’s (Blum et al., 2004), where it was shown to reduce tissue damage and inflammation, and improve neurological outcome (Yrjanheikki et al., 1999; Chen et al., 2000; Kriz et al., 2002; Wu et al., 2002; Wells et al., 2003; Xu et al., 2004; Zemke and Majid, 2004; Festoff et al., 2006; Marchand et al., 2009). Using a rat model of mbTBI, we report that acute treatment with minocycline mitigates the inflammatory response to injury and results in normalized neurobehavior.

MATERIALS AND METHODS

EXPERIMENTAL GROUPS AND HOUSING CONDITIONS

Thirty-two male Sprague Dawley rats (Charles River Laboratories, Wilmington, MA, USA) were used, weighing 245–265 g at the beginning of the experiment. All animals were kept under normal housing conditions (two rats/cage) in a reverse 12–12 h light-dark cycle and provided with food and water *ad libitum* for the entire length of the study. Following baseline behavioral testing (described below), animals were assigned to one of the following experimental groups: (1) sham saline treated (*sham-vehicle*; $n = 8$) and (2) sham minocycline treated (*sham-mino*; $n = 8$), which served as controls for (3) blast injured saline treated (*injured-vehicle*; $n = 8$) and (4) blast injured-minocycline treated (*injured-mino*; $n = 8$), respectively. All animals were handled according to protocol approved by the Institutional Animal

Care and Use Committee (IACUC) at the Uniformed Services University (USU).

BEHAVIORAL TESTS

Prior to injury, all rats underwent baseline behavioral assessments for general locomotor activity by the open field (OF) test, and for anxiety by the elevated plus maze (EPM). Rats were also trained for five consecutive days in the Barnes maze (BM) for spatial learning and memory. The baseline test results (data not shown) were used to create the aforementioned experimental groups with no statistical significance among them. Following injury or sham, two behavioral test sessions were conducted starting at 8 and 45 days. The experimental schedule is illustrated in **Figure A1** in Appendix. Within each testing session, the behavioral tests were performed on separate days in the following order: OF (day 1), EPM (day 2), and BM (days 3–7). All behavioral tests were performed during animals’ dark cycle.

Open field

Tests were performed using AccuScan’s infrared light beams OF system (AccuScan Instruments, Inc.) at baseline and 1, 8, and 45 days post-injury. The OF system is a 16.5 × 16.5 × 13 (L × W × H) inches clear Plexiglas arena with a perforated lid. The system uses 16 × 16 grid light beam arrays in the X and Y axes to measure locomotor activity. The system detects beam breaks by the animal and determines the location of the rat within the cage. During the 60 min testing period, horizontal activity (number of beam breaks) and resting time (time spent with inactivity greater than or equal to 1 s) were measured. Data for each animal were recorded and analyzed automatically with Fusion 3.4 software (AccuScan Instruments, Inc.). The horizontal activity and resting time are presented as the average performance of all animals in each experimental group ± standard error of the mean (SEM) at each of the individual time points.

Elevated plus maze

The EPM is an ethologically relevant assessment of anxiety levels in rodents (Carobrez and Bertoglio, 2005; Salzberg et al., 2007; Wolf and Frye, 2007). Tests were carried out prior to injury and at 9 and 46 days post-injury as described earlier in details (Kovesdi et al., 2011). Briefly, rats were placed one by one in the center of the maze facing one of the open arms. During the 5 min testing session, each animal was allowed to explore the maze freely while its movement was video-tracked. Time spent in the open and the closed arms (seconds) was recorded for each animal using ANY-maze 4.2 Software (Stoelting Company, Wood Dale, IL, USA). The maze was cleaned with a 30% ethanol solution between each trial. Data are presented as the average time (in seconds) spent in the open vs. the closed arms of the maze in each experimental group ± SEM.

Barnes maze

Barnes maze represents a widely used and less stressful alternative to the Morris water maze for assessing spatial memory in rodents (Barnes, 1979; Maegele et al., 2005; Doll et al., 2009; Harrison et al., 2009). Tests were carried out prior to injury (training session), and at 10 and 47 days post-injury (Test Session I and II,

respectively; Kovesdi et al., 2011). The maze is a circular platform (1.2 m in diameter) that contains 18 evenly spaced holes around the periphery. One of the holes is the entrance to a darkened escape box that is not visible from the surface of the board. The position of the escape chamber relative to the other holes and the testing room remains fixed during all BM trials. On the first day of the training session, each rat was placed in the escape box and covered for 30 s. The escape box was then removed with the animal inside and moved to the center of the maze. The rat was allowed to explore the maze for a few seconds after which it was returned to its home cage. In the second and third trial (only day 1 of the BM training session has three trials), the same rat was placed under a start box in the center of the maze for 30 s. The start box was removed and the rat was allowed to explore freely to find the escape box. Training sessions ended after the animal had entered the escape box or when a pre-determined time (240 s) had elapsed. If the animal had not found the escape box during the given time period, it was placed in the escape box for 1 min at the end of the trial. During the baseline BM session, animals were trained until their daily latency time averaged 10 s. The two post-injury BM test sessions were run for five consecutive days; every rat was tested twice per day as described above. In each trial, the latency to enter the escape box was measured and recorded using ANY-maze 4.2 Software (Stoelting Company, Wood Dale, IL, USA). The escape box and the maze were cleaned with a 30% ethanol solution between each trial and animal. Data are presented as the average latency times of two daily trials per animal per experimental group $\pm$ SEM.

MILD BLAST INJURY

On the day of the injury all rats (average weight $\sim$ 300 g) were transferred to Walter Reed Army Institute of Research (Silver Spring, MD, USA) as described in detail (Kamnaksh et al., 2011). Sixteen rats were exposed to whole body mbTBI as described earlier (Long et al., 2009; Kovesdi et al., 2011; Kwon et al., 2011). Briefly, rats were anesthetized with 4% Isoflurane for 6 min in an induction chamber (Forane, Baxter Healthcare Corporation, Deerfield, IL, USA), placed in an animal holder within the shock tube in a transverse prone position, and exposed to whole body blast overpressure (20.6 ± 3 psi) while wearing chest protection. The other 16 rats were similarly anesthetized, placed in the shock tube, but were not exposed to blast overpressure (sham). Following blast injury or sham, rats were moved back to their home cages and transported back to the USU animal facility.

PHARMACOLOGICAL TREATMENT

Four hours after injury or sham, rats received a total volume of 0.25 ml/100 g body weight of either physiological saline alone (*vehicle*) or 50 mg/kg of clinical grade minocycline (Minocin®, Triax Pharmaceuticals, Italy) dissolved in saline (*mino*) intraperitoneally (i.p.). Animals received minocycline or saline for four consecutive days at identical times each day. Our minocycline dosage and treatment paradigm was based on previous studies using rodent models of various neurological conditions where minocycline was administered i.p. at an average dose of 50 mg/kg (see Table A1 in Appendix).

TISSUE COLLECTION AND PROCESSING

At the completion of the last behavioral test session (51 days post-injury or sham), animals were placed inside an induction chamber saturated with Isoflurane and deeply anesthetized until a tail pinch produced no reflex movement. Anesthesia was maintained using a mask/nose cone attached to the anesthetic vaporizer and blood was collected (1.5 ml) from a tail vein; serum was prepared as described earlier (Kwon et al., 2011). For measuring tissue levels of protein markers, rats were decapitated and brains were immediately removed and placed on ice. The amygdala (AD), PFC, VHC, and DHC were dissected, frozen, and stored at -80°C until use as described earlier (Kwon et al., 2011).

Protein measurements

Sample preparation, printing, scanning, and data analysis of serum and brain regions were performed using Reverse Phase Protein Microarray (RPPM) as described earlier (Kovesdi et al., 2011; Kwon et al., 2011). Briefly, frozen brain tissues were pulverized in liquid nitrogen, the powder was transferred into a lysis buffer (Thermo Fisher, Waltham, MA, USA) with protease and phosphatase inhibitors (Thermo Fisher), sonicated, centrifuged, and the supernatants aliquoted and stored at -80°C . Protein concentrations were measured by BCA assay (Thermo Fisher). Blood samples were centrifuged at $10,000 \times g$ for 15 min at 4°C ; supernatants were aliquoted, flash-frozen, and stored at -80°C .

Tissue samples were diluted in print buffer and then subjected to an 11-point serial 1:2 dilution and transferred into Genetix 384-well plates (X7022, Fisher Scientific, Pittsburg, PA, USA) using a JANUS Varispan Integrator and Expanded Platform Workstation (PerkinElmer, Waltham, MA, USA). Plates were transferred into an Aushon 2470 Arrayer (Aushon Biosystem, Billerica, MA, USA) to be printed on ONCYTE Avid (brain samples) or ONCYTE Nova (serum samples) single-pad nitrocellulose coated glass slides (Grace Bio-Labs, Bend, OR, USA; Gyorgy et al., 2010).

Primary antibodies (Table A2 in Appendix) were diluted to $10\times$ the optimal Western analysis concentration in antibody incubation buffer as described earlier (Gyorgy et al., 2010). The primary antibody solution was incubated overnight at 4°C with a cover slip. The following day slides were washed and then incubated with an Alexa Fluor® 635 goat anti-mouse (Cat# A-31574), goat anti-rabbit (Cat# A-31576), or rabbit anti-goat IgG (H + L; Cat# A-21086) secondary antibodies from Invitrogen at 1:6000 dilution in antibody incubation buffer for 1 h at room temperature. After washing and drying, fluorescent signals were measured by a Scan Array Express HT microarray scanner (Perkin Elmer, Waltham, MA, USA) using a 633 nm wavelength laser and a 647 nm filter.

Data from the scanned images were imported into a Microsoft Excel-based bioinformatics program developed in-house for analysis (Gyorgy et al., 2010). The linear regression of the log-log data was calculated after the removal of flagged data, which include signal to noise ratios of less than 2, spot intensities in the saturation range or noise range, or high variability between duplicate spots ($>10\text{--}15\%$). The total amount of antigen is determined by the y -axis intercept (Y -cept; Gyorgy et al., 2010). Data is reported as the mean Y -cept $\pm$ SEM.

Corticosterone assay

Serum corticosterone (CORT) levels were measured with Cayman's Corticosterone EIA Kit according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI, USA). Each sample was diluted 1:500 and measured in triplicate (Kwon et al., 2011). Data is reported as the mean concentration (in pg/mg) $\pm$ SEM.

STATISTICAL ANALYSIS

All data were analyzed using Graph Pad InStat software (GraphPad Software, Inc., La Jolla, CA, USA). Statistical significance was verified by one-way analysis of variance (ANOVA), followed by Tukey *post hoc* test for multiple comparison. Differences with a *p* value of <0.05 were considered significant.

RESULTS

BEHAVIORAL TESTS

One day following blast exposure, injured rats showed reduced horizontal activity and slightly increased resting time in the OF compared to sham animals, but the differences were not statistically significant (Figure 1A). At 8 days post-injury, the horizontal activity of injured-vehicle animals further decreased. On the other hand, injured-mino rats had a similar horizontal activity to animals in the two sham groups. The horizontal activity of animals

in all groups was the lowest at 45 days after injury. Similarly, animals in all experimental groups spent more time resting with injured-vehicle animals spending significantly more time resting than animals in the other three groups (Figure 1B).

During the first EPM testing performed 9 days after exposure, injured-vehicle animals spent less time in the open arms and more time in the closed arms of the maze than animals in the other three groups (Figures 2A,B). However, the difference at this time point was not statistically significant. At 46 days after injury, the differences in the time spent in the open and closed arms of the maze became significant between injured-vehicle and injured-mino animals. At this later time point, injured-vehicle animals barely spent any time in the open arms of the maze and practically spent all of their time in the closed arms of the maze (Figures 2A,B). By contrast, injured-mino animals spent a comparable amount of time to animals in the two other groups in the open and closed arms of the maze.

In order to assess time-dependent changes in spatial memory, we performed two tests in the BM at two different time points. Test Session I started at 10 days after injury and lasted for 5 days. Injured-vehicle animals performed poorly during the first 2 days of the test (Figure 3A). They required approximately twice as much time as animals in the other experimental groups to find the escape

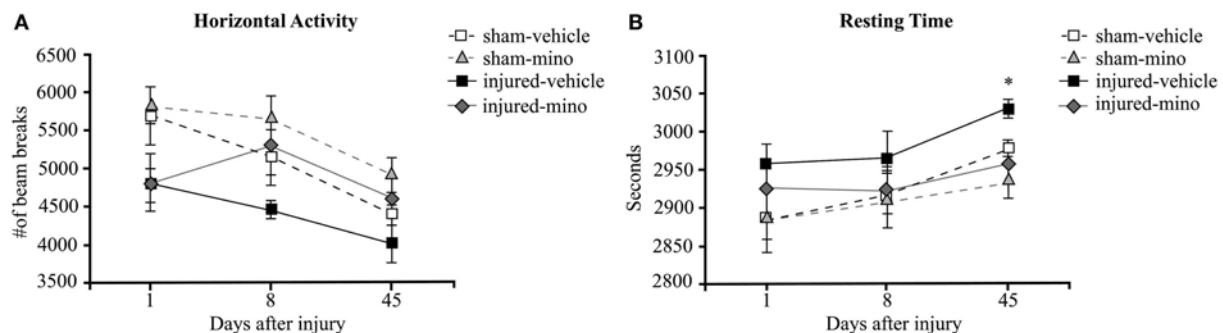


FIGURE 1 | The effect of injury and minocycline treatment on basic locomotor activities at different time points after mbTBI. (A) Horizontal activity (number of beam breaks), and **(B)** Resting time (seconds) were measured in Open field. Data are presented as mean $\pm$ SEM. **p* < 0.05 for injured-vehicle vs. sham-mino rats.

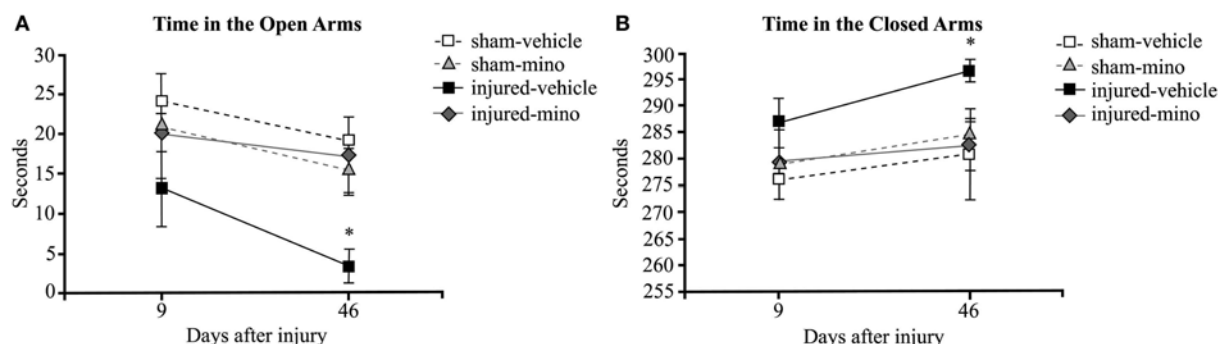


FIGURE 2 | The effect of injury and minocycline treatment on anxiety levels at different time points after mbTBI. (A) Time spent in the open arms (seconds), and **(B)** time spent in the closed arms (seconds) were measured for all animals in the elevated plus maze. Data are presented as mean $\pm$ SEM. **p* < 0.05 for injured-vehicle vs. sham-vehicle rats.

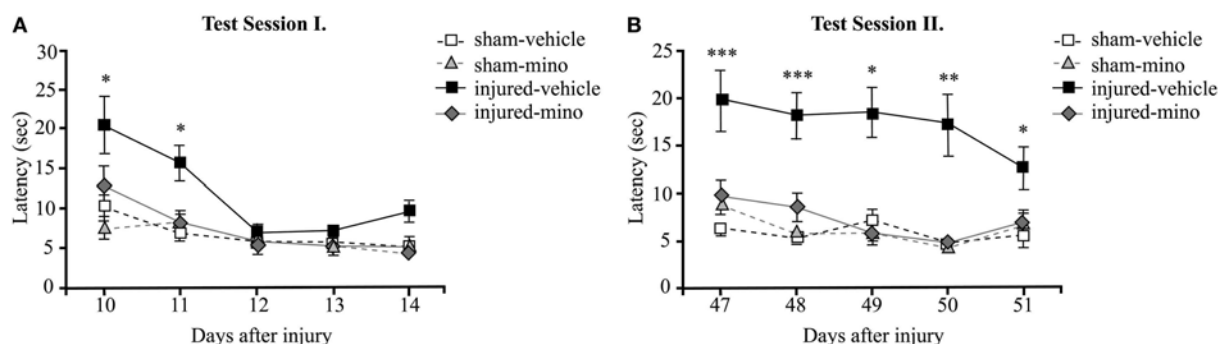


FIGURE 3 | The effect of injury and minocycline treatment on spatial memory at different time points after mbTBI. Latency (seconds) to find and enter the escape box was measured for five consecutive days in the Barnes maze starting at (A) 10 days, and (B)

47 days after injury or sham. Data are presented as the average of the 2 daily trials per animal in each experimental group $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ for injured-vehicle vs. sham-vehicle rats.

box. While their performance improved slightly on the second day of testing, injured-vehicle animals still required significantly more time to find the escape box compared to their sham group. On the third day of testing, their performance became roughly similar to animals in the other experimental groups. By contrast, the performance of injured-mino animals was very similar to uninjured (sham) animals; their measured latency times to locate and enter the escape box were almost identical on days 11 through 14. They found the escape box with slightly improved efficiency every day.

During Test Session II (beginning at 47 days post-injury), the performance of injured-vehicle animals was significantly worse than sham-vehicle animals on all five testing days (Figure 3B). While their performance slightly improved on each subsequent testing day, injured-vehicle rats still needed significantly more time to find the escape box, even on the last day of testing. Conversely, injured-mino animals performed similar to animals in the two control groups (sham-vehicle and sham-mino). Their performance during Test Session II was similar to that in Test Session I; they required about the same time to find the escape box on each testing day.

PROTEIN ANALYSES

Select protein marker levels were measured in the serum and dissected brain regions of animals in all four experimental groups. Injury without minocycline treatment caused a significant increase in the serum levels of all biomarkers measured (Figure 4). Both inflammatory markers, CRP and MCP-1, were significantly elevated in injured-vehicle animals; minocycline treatment resulted in normal or near normal (i.e., sham) sera levels in the injured-mino group. Claudin 5 levels were also elevated following blast injury in the vehicle-treated group, but were reduced to sham levels in injured-mino animals. Similarly, neuronal and glial loss and/or damage markers like NSE, NF-H, Tau, S100 β , and GFAP were all significantly elevated in the sera of injured-vehicle animals. Minocycline treatment resulted in a significant reduction in serum levels of all of the markers except for GFAP. Lastly, serum CORT levels were also significantly increased in injured-vehicle rats, but minocycline treatment resulted in significantly lower serum CORT levels in injured-mino animals.

Tissue levels of 13 selected protein biomarkers (Figure 5; Table A3 in Appendix) were determined in the AD, PFC, VHC, and DHC of animals in the various experimental groups. We found significantly elevated levels of all three inflammatory markers (CRP, MCP-1, and TLR9) in the brains of injured-vehicle animals (Figure 5). Importantly, minocycline treatment of injured animals resulted in normal or near normal levels of these inflammatory markers; tissue levels of these markers in all four brain regions of injured-mino rats were not statistically different from those of sham-vehicle or sham-mino animals. NSE, S100 β , and GFAP similarly showed injury-induced increases in all four brain regions. Minocycline treatment normalized their tissue levels with the exception of GFAP in the PFC, where GFAP levels of injured-vehicle and injured-mino animals were practically the same.

Some of the protein biomarkers that were analyzed showed brain region-dependent increases in response to injury. Of the vascular markers, tissue levels of FLK-1 (Figure 5), Claudin 5 and AQP4 (Table A3 in Appendix) were significantly elevated in the VHC following injury; FLK-1 and AQP4 levels were also elevated in the DHC and the AD, respectively. Similarly, neuronal and glial markers showed brain region-specific increases to injury. For instance, all three markers (NF-H, Tau, and MBP) showed injury-induced increases in the VHC but not in the PFC. Minocycline treatment of injured animals significantly reduced the tissue levels of all of the markers with the exception of Tau, which was not significantly reduced in the AD. Interestingly, VEGF did not show any significant changes in response to injury in any of the analyzed brain regions.

DISCUSSION

Minocycline is an FDA approved, semisynthetic, second-generation tetracycline drug that exhibits anti-inflammatory and/or neuroprotective effects in various experimental models of CNS disorders. These include focal and cerebral ischemia (Yrjanheikki et al., 1998; Xu et al., 2004), TBI (Sanchez Mejia et al., 2001), amyotrophic lateral sclerosis (Zhu et al., 2002), Parkinson's disease (Wu et al., 2002), kainic acid treatment (Heo et al., 2006), Huntington's disease (Chen et al., 2000; Du et al., 2001; Wu et al.,

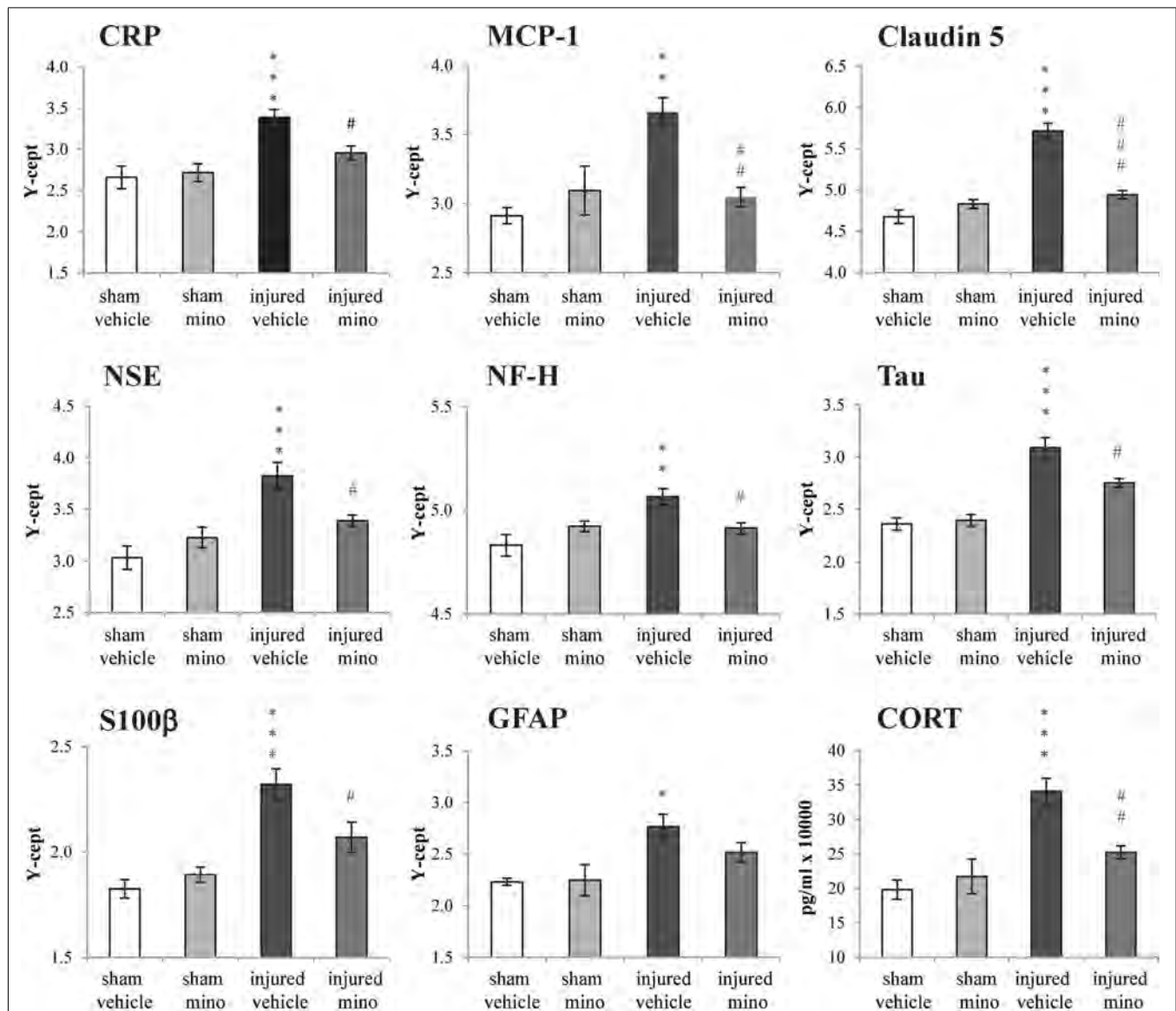


FIGURE 4 | The effect of injury and minocycline treatment on serum levels of selected markers in the different experimental groups. Serum levels of 8 protein markers were assayed by RPPM; CORT levels were assayed by ELISA. Protein values are expressed as y-axis intercept (Y-sept)

and CORT values are expressed as pg/ml. Data are presented as mean $\pm$ SEM. * p < 0.05, ** p < 0.01, and *** p < 0.001 for injured-vehicle vs. sham-vehicle rats. # p < 0.05, ## p < 0.01, and ### p < 0.001 for injured-vehicle vs. injured-mino rats.

2002; Wang et al., 2003), multiple sclerosis (Brundula et al., 2002; Popovic et al., 2002), Alzheimer's disease (Choi et al., 2007), and spinal cord injury (Wells et al., 2003; Stirling et al., 2004; Festoff et al., 2006; **Table A1** in Appendix). Minocycline's ability to improve outcome in distinct types of CNS disease models may stem from its ability to find multiple targets in different biochemical cascades that play a role in the development of the above-mentioned diseases. Previous studies indicated that minocycline acts as a pleiotropic molecule; it can reduce the release of various chemokines and cytokines (Sanchez Mejia et al., 2001; Bye et al., 2007), lipid mediators of inflammation, matrix metalloproteinases (MMPs), and nitric oxide (NO; Stirling et al., 2005). Minocycline can also inhibit microglia activation (Yrjanheikki et al., 1998,

1999; Tikka and Koistinaho, 2001). The inhibition of microglial inflammatory responses has been reported in various neurodegenerative diseases (Yrjanheikki et al., 1999) including Huntington's (Chen et al., 2000; Popovic et al., 2002; Wu et al., 2002); additional anti-inflammatory actions may be through the impediment of molecules like cyclooxygenase-2 (Patel et al., 1999; Yrjanheikki et al., 1999). Minocycline exerts its neuroprotective effects (Kriz et al., 2002; Wells et al., 2003; Stirling et al., 2004; Zemke and Majid, 2004; Marchand et al., 2009) through the repression of poly (ADP-ribose) polymerase-1 activity (Alano et al., 2006), which plays a central role in caspase-independent apoptosis (Susin et al., 1999; Zhang et al., 2002; Cao et al., 2003; Du et al., 2003), and the suppression of caspase-1 and caspase-3 expression (Chen et al., 2000)

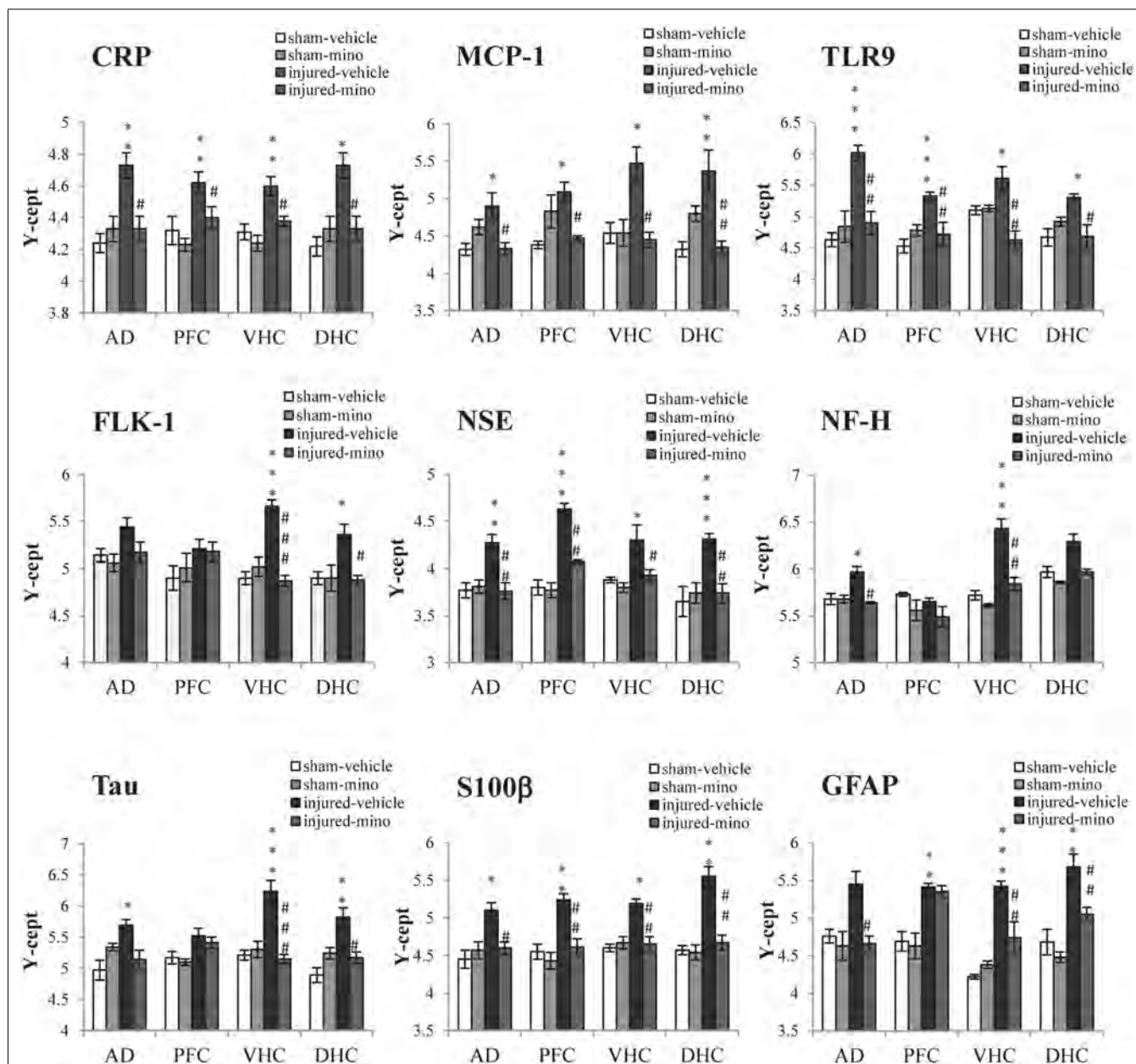


FIGURE 5 | The effect of injury and minocycline treatment on the levels of protein markers in various brain regions in the different experimental groups. Tissue levels of 9 protein markers were measured in the AD, PFC, VHC, and DHC of rats by RPPM. Protein values are

expressed as y-axis intercept (Y-sept) and data are presented as mean $\pm$ SEM. * p < 0.05, ** p < 0.01, and *** p < 0.001 for injured-vehicle vs. sham-vehicle rats. # p < 0.05 and ## p < 0.01 for injured-vehicle vs. injured-mino rats.

and cytochrome c release from the mitochondria (Zhu et al., 2002). Moreover, minocycline has been shown to sequester excess Ca^{2+} released after injury (Antonenko et al., 2010), and block the injury-induced decrease of soluble alpha amyloid precursor protein in the attenuation of diffuse axonal injury (Siopi et al., 2011). Based on all of these findings, we were compelled to test the effects of minocycline in our rat model of mTBI.

During our pilot studies we followed a reported treatment schedule of 90 mg/kg of minocycline administered i.p. twice on the first day, 50 mg/kg twice per day for 2 subsequent days, and

50 mg/kg once per day for three additional days (Lee et al., 2003; Teng et al., 2004; Festoff et al., 2006; Yune et al., 2007). However, we found that this treatment caused substantial weight loss likely due to gastrointestinal problems (i.e., diarrhea). Based on these preliminary findings, we decided to modify the treatment paradigm by lowering the dose to 50 mg/kg once per day for four consecutive days. Our conservative treatment schedule caused light and transient diarrhea, and animals recovered and gained weight normally from the third day post-injury until the termination of the experiment on day 51 (data not shown).

Consistent with our previous findings, injured rats had reduced horizontal activity and a somewhat higher resting time than sham animals in the OF 1 day after injury (Kwon et al., 2011). Interestingly, all of the rats, independent of injury and treatment, showed gradually decreasing horizontal activities during the two subsequent OF sessions. There are two plausible explanations for this behavior. Rodents actively explore new areas, but inadvertently become less active on subsequent exposures to the same environment, a process called habituation (Pitkänen et al., 2006). We also observed on numerous occasions in other experiments that the horizontal activity of naïve rats in the OF at baseline is higher than it is 24 h later. We believe that since the OF represents a novel environment for the rats, they actively explore it (Bolivar et al., 2000; Daenen et al., 2001). However, repeated testing may cause the animals to habituate to the OF and in turn spend less time exploring and more time resting. Another possible explanation may be aging, especially during the last OF session, as young rodents have higher motor activity levels than more mature rodents (Sprott and Eleftheriou, 1974; Ingram et al., 1981; Gage et al., 1984; Lamberty and Gower, 1993). The effects of aging have also been observed as decreases in distance traveled in the EPM over time in both, sham and blast injured animals (Kovesdi et al., 2011).

Epidemiological studies have indicated that soldiers frequently develop neurobehavioral abnormalities like increased anxiety and memory impairments in mbTBI (Belanger et al., 2007; Brenner et al., 2009). Anxiety affects rehabilitation, psychosocial adjustment, and cognition in humans (Kersel et al., 2001; Rapoport et al., 2005). The EPM is a simple behavioral assay for evaluating the anxiety responses of rodents (Pellow et al., 1985) and studying the brain sites (limbic regions, hippocampus, amygdala; Silveira et al., 1993; Gonzalez and File, 1997) and the mechanisms underlying anxiolytic behavior (GABA, glutamate, serotonin, hypothalamic–pituitary–adrenal axis neuromodulators; Handley and Mithani, 1984; Pellow et al., 1985; Rodgers et al., 1992; Silva and Brandao, 2000; Korte and De Boer, 2003; Overstreet et al., 2003; Cortese and Phan, 2005). Rodents naturally prefer dark, enclosed spaces, and demonstrate an aversion to open spaces and a fear of heights (Barnett, 1975). Despite these natural inclinations, non-anxious rodents possess exploratory behaviors that cause them to investigate the open arms of the maze while more anxious rats remain in the closed arms of the maze for longer periods of time.

We previously found increased anxiety in our rodent model of mbTBI (Kovesdi et al., 2011). As our current EPM data illustrates, acute minocycline treatment prevented the increase in anxiety following blast overpressure. The time spent in the closed arms of the maze by injured-mino rats was indistinguishable from that of the two sham groups at both testing time points. Conversely, injured-vehicle animals showed signs of increased anxiety early on; they spent less time on the open arms of the maze than animals in the other three experimental groups. While the difference was not statistically significant at this early time point, injured-vehicle animals barely spent any time outside of the closed arms of the maze 46 days after the injury. Even though there is very little information available about the effects of minocycline on anxiety, especially in brain injury, minocycline treatment reduced anxiety in the EPM in models of cardiac arrest/cardiopulmonary

resuscitation and fragile X syndrome (Bilousova et al., 2009; Neigh et al., 2009).

Current treatments of increased anxiety are mostly symptomatic (Tenovuo, 2006; Silver et al., 2009), and patients frequently experience side effects from the use of drugs like benzodiazepines (Rickels et al., 1991; Baldwin et al., 2005). Acute minocycline treatment may provide an alternative to the use of these drugs. Interestingly, injured-mino animals also had lower serum CORT levels than injured-vehicle animals at 51 days after the injury. While serum CORT levels have been used as indicators of stress (Dunn et al., 2004), the correlation between serum CORT levels and anxiety is rather complex and likely involve multiple regulatory pathways.

Consistent with available epidemiological data and our previous studies, the memory impairment associated with mbTBI develops over several weeks after the insult (Kovesdi et al., 2011; Kwon et al., 2011). Importantly, the deficit persists for at least 2 months post-injury (Kovesdi et al.). Given that 2 months in the lifespan of a rat roughly translates into several human years (Quinn, 2005), the observed memory impairment mirrors the chronic condition that manifests in humans reasonably well. The BM has been extensively used to study spatial learning and memory in rats (Barnes, 1979), and is considered a less anxiogenic alternative to the Morris water maze since it does not involve swimming (Pompl et al., 1999; Miyakawa et al., 2001; Deacon and Rawlins, 2002; Holmes et al., 2002). BM has been applied to studies of TBI; rodents with hippocampal damage show impaired performance in the maze, supporting the spatial nature of the task (Fox et al., 1998; Paylor et al., 2001; Deacon and Rawlins, 2002; Raber et al., 2004). In BM animals are presumed to learn the location of an escape hole using spatial reference points that are either fixed in relation to the maze (extra-maze cues) or are fixed on the maze itself in relation to the escape hole (proximal cues). It is important to note that during our acclimation and baseline behavioral testing, all animals were exposed to the maze and were trained to “learn” the task of locating and entering the escape box.

Early signs of the memory deficit were detected in the first testing session. Injured-vehicle animals required approximately twice as long to locate the escape box on the first day of testing, while injured-mino animals performed similar to the uninjured shams. On the second day of testing, injured-vehicle rats still needed more time than the other groups. During the last 3 days of testing, injured-vehicle rats relearned and remembered the task, requiring about the same amount of time as the other groups. However, during the second testing session, injured-vehicle rats performed poorly on all five testing days with only minor improvements in their speed from day to day. Conversely, injured-mino rats performed as well as sham animals did throughout. A similar effect was found in a study by Siopi et al. (2011) where acute treatment with minocycline significantly improved recognition memory; the effects lasted for up to 13 weeks in a mouse closed head injury model. There are currently no effective treatments in clinical use for memory impairment. Existing therapies predominantly target symptoms associated with mood disorders (e.g., depression) that can also improve memory performance (Tenovuo, 2006; Silver et al., 2009). Therefore, acute minocycline treatment has the potential to offer a potentially effective alternative.

The observed neurobehavioral impairments implicate the AD, PFC, VHC, and DHC due to their involvement in mediating anxiety and memory (Henke, 1990; Moser and Moser, 1998). In our earlier works we found indications of inflammation, axonal, glial, and neuronal damage in these brain regions (Kovesdi et al., 2011; Kwon et al., 2011). The neuroinflammatory response to various brain insults has been suggested as a potential link between injury and altered behavior, including increased anxiety. As reported earlier, blast can trigger a systemic inflammatory process even when the body is fully protected and only the head is exposed (Cernak et al., 2011). It is crucial to note that the similarities and the dissimilarities between mbTBI and other better-characterized forms of closed head injuries are currently not known with regards to their primary and secondary injury mechanisms. Nevertheless, it has been hypothesized that the different types of TBIs may share pathological components like neuroinflammation, neuronal and glial cell loss, and axonal injuries (Agoston et al., 2009).

In our current study, we found that minocycline treatment normalized significantly elevated sera levels of the inflammatory markers CRP and MCP-1 following exposure to mild blast. CRP and MCP-1 levels are routinely monitored in clinical settings and are used as an indicator of inflammation (Berman et al., 1996; Glabinski et al., 1996; Du Clos, 2000; Lobo et al., 2003). CRP is a component of the acute phase response to injury (Du Clos, 2000) and its expression is stimulated by the release of cytokines (Okamura et al., 1990); elevated CRP serum levels may reflect a combination of systemic as well as neuronal inflammation. Increased levels of MCP-1 are associated with neurological dysfunction after traumatic axonal injury in rats (Rancan et al., 2001), and are detected in the cerebrospinal fluid in diseases related to neuroinflammation such as stroke, meningitis, and multiple sclerosis (Mastroianni et al., 1998; Losy and Zaremba, 2001; Sindern et al., 2001; Chen et al., 2003; Sorensen et al., 2004). MCP-1 has also been suggested to regulate vascular permeability during CNS inflammation (Tekstra et al., 1999; Stamatovic et al., 2003, 2006).

While tissue levels of Claudin 5 did not significantly change except in the VHC, serum levels were significantly increased in injured-vehicle animals. Claudin 5 is a part of the tight junction complex in brain endothelial cells that contribute to the formation of the BBB (Morita et al., 1999; Liebner et al., 2000); increased serum levels suggest that there may be vascular damage in mbTBI that results in the release of Claudin 5 into systemic blood. Importantly, minocycline treatment normalized Claudin 5 sera levels indicating that vascular changes may be secondary to the inflammatory process or that minocycline possesses cytoprotective effects that also extend to endothelial cells.

Elevated serum levels of neuron- and glia-specific proteins have been found clinically as well as experimentally in various forms of TBI (Povlishock and Christman, 1995; Povlishock and Pettus, 1996; Buki and Povlishock, 2006). Increased serum levels of large neuron-specific molecules also point toward a vascular pathology; heightened BBB permeability is required for the release of large proteins like NF-H from the brain parenchyma and into systemic circulation. In a large animal model of blast TBI, the temporal pattern of serum NF-H levels correlated with clinical and

pathological outcomes (Gyorgy et al., 2011). In our current study, minocycline treatment significantly reduced sera levels of NSE, NF-H, Tau, and S100 β after injury, but not GFAP, an astroglia-specific intermediate filament (Missler et al., 1999) indicative of brain damage.

Consistent with our behavioral and serum data, we found that minocycline treatment prevented or mitigated injury-induced increases of the selected inflammatory markers CRP, MCP-1, and TLR9 in all four brain regions. TLR9 is member of the toll-like receptor family (Aderem and Ulevitch, 2000; Akira et al., 2001; Takeda and Akira, 2005; Mishra et al., 2006; O'Neill, 2006; Casanova et al., 2011) involved in the induction and the regulation of the inflammatory response in TBI (Hua et al., 2007, 2009) as well as other disorders involving neuroinflammation (Prat and Antel, 2005) and ischemic brain damage (Hua et al., 2007, 2009; Doyle et al., 2008; Gao et al., 2009; Marsh et al., 2009).

Of the vascular markers only FLK-1 and AQP4 tissue levels increased in response to the injury; minocycline treatment mitigated the effect of injury on FLK-1 levels but showed no effect on the tissue levels of AQP4. Increases in AQP4 were only detected in the AD and in the VHC while FLK-1 was in the VHC and the DHC. Elevations in AQP4 expression can contribute to the formation as well as the resolution of edema (Kimelberg, 1995; Papadopoulos et al., 2002; Amiry-Moghaddam and Ottersen, 2003; Neal et al., 2007). The pathology of severe bTBI includes the development of rapid and malignant brain edema (Ling et al., 2009; Ling and Ecklund, 2011) probably involving AQP4 (Neal et al., 2007). However, we currently have no information about water imbalance in mbTBI; if present, it is likely limited to the early phase following injury.

FLK-1 is a membrane-bound tyrosine kinase that mediates the effects of VEGF in the CNS (Sondell et al., 2000; Ogunshola et al., 2002; Rosenstein et al., 2003). Activation of FLK-1 stimulates various intracellular signal transduction pathways including the PI3K/Akt pathway that mediates the neuroprotective function of VEGF (Gerber et al., 1998; Wu et al., 2000; Kilic et al., 2006). VEGF/FLK-1 up-regulation following TBI seems to perform an important endogenous cytoprotective mechanism (Skold et al., 2006; Lee and Agoston, 2009). Interestingly, we did not detect changes in the abundance of VEGF in any of the analyzed brain regions following injury. A potential explanation for this negative finding is the relatively late testing time point (51 days post-injury). In a previous study using another model of TBI, we observed significant increases in VEGF tissue levels in the hippocampus (Lee and Agoston, 2009, 2010); the increases were limited to a few days after the injury.

The tissue levels of NSE, NF-H, Tau, S100 β , GFAP, and MBP similarly increased in response to the injury, however, increases were brain region-specific. We measured significant injury-induced increases in sera levels of these proteins indicative of neuronal and glial cell losses. Thus, the detected increases in the tissue levels of these proteins are likely compensatory in nature and can be a part of the repair mechanism (Fawcett, 2009). Importantly, in all cases where injury resulted in an increase in the tissue levels of these markers, minocycline treatment mitigated the effect and tissue levels of these markers were restored to levels measured in sham animals.

CONCLUSION

Our study demonstrates that acute minocycline treatment substantially improve the neurobehavioral outcome in a rodent model of mTBI likely through mitigating the neuroinflammatory response to injury. The strength of our study lies in combining neurobehavioral tests performed at two different time points after injury with determining changes in serum and brain tissue levels of protein biomarkers. The limitations of the current study are the limited types of neurobehavioral and a single terminal time point of proteomics analyses. Based on these promising results,

additional neurobehavioral testing shall be performed in future studies along with obtaining blood at several clinically relevant time points for protein assays. Nevertheless, our findings provide a rationale for exploring the viability of using acute minocycline treatment in mTBI.

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APPENDIX

Table A1 | List of animal models of various diseases, dose of minocycline treatment and the observed effects of the treatment.

Animal model of disease	Dose	Effect	Reference
Acute spinal cord injury (mouse)	1 and 24 h (50 mg/kg, i.p.), then 25 mg/kg dose every 24 h for the next 5 days	Improved both hindlimb function and strength after injury and reduced lesion size	Wells et al. (2003)
Amyotrophic lateral sclerosis (mouse)	1 g/kg in a custom made rodent diet	Delayed the onset of motor neuron degeneration, less activation of microglia was detected at early symptomatic stage (46 weeks) and at the end stage of disease in the spinal cord	Kriz et al. (2002)
Cervical spinal cord injury (rat)	1 h (90 mg/kg), then for 3 days after injury	Failed to improve functional and histological recovery.	Lee et al. (2010)
Closed head injury (mouse)	5 min (90 mg/kg, i.p.), and at 3 and 9 h (45 mg/kg) post-TBI	Attenuation of the decrease of post-TBI sAPP α 24 h post-injury. Corpus callosum and striatal atrophy, ventriculomegaly, astrogliosis, and microglial activation reduced 3 months post-injury	Siopi et al. (2011)
Closed head injury (mouse)	30 min (45 mg/kg, i.p.) and every 12 h (22.5 mg/kg, i.p.) for 1 week. Or twice-daily minocycline injections for 2 weeks (6 weeks surviving)	Reduced the activation of microglia/macrophages and improved neurological outcome, but any increase of neurogenesis	Ng et al. (2012)
Controlled contusion spinal cord injury (rat)	Multiple injections (30 mg/kg, i.p.) at 0.5, 1, and 24 h, or a single injection of 90 mg/kg at either 0.5, 1.0, or 24 h after injury	Improved functional recovery, reduced tissue damage, cavity size, apoptosis and activated caspase-3 signal	Festoff et al. (2006)
Controlled cortical impact (rat)	45 mg/kg, i.p. at 1 h, 24 and 48 h after injury	Improved active place avoidance following CCI	Abdel Baki et al. (2010)
Endothelin-1 (ET-1) model of focal ischemia (rat)	45 mg/kg, i.p. at 2 and 12 h following the last injection of ET-1, then 22.5 mg/kg every 12 h (5 $\times$)	Improved behavioral outcome. Reduced subcortical and whole hemisphere infarct volume	Hewlett and Corbett (2006)
Focal cerebral ischemia (rat)	45 mg/kg, i.p. twice a day for the first day; 22.5 mg/kg for the subsequent 2 days	Reduced cortical infarction volume, inhibited morphological activation of microglia in the area adjacent to the infarction, induction of IL-1 β -converting enzyme, and reduced cyclooxygenase-2 expression and prostaglandin E2 production	Yrjanheikki et al. (1999)
Huntington disease (mouse)	daily 5 mg/kg, i.p.	Inhibited caspase-1 and caspase-3 up-regulation	Chen et al. (2000)
Middle cerebral artery occlusion (MCAO; mice)	45 mg/kg two times in every 12 h starting at 30 min after the onset of MCAO	Neuroprotectant at males, but ineffective at reducing ischemic damage in females	Li and McCullough (2009)
Neonatal hypoxia-ischemia (HI; rat)	2 h after hypoxia (45 mg/kg, i.p.), then every 24 h from P4–P9 (22.5 mg/kg)	Prevention of HI induced changes in SERT, 5-HT and 5-HT positive dorsal raphe neurons. Lasting effect after 6 week of HI	Wixey et al. (2011)
Parkinson disease (mouse)	Daily twice (12 h apart) injections from 1.4 to 45 mg/kg (i.p.) starting 30 min after the first MPTP injection and continuing through four additional days after the last injection of MPTP	Inhibited microglial activation, mitigated both the demise of nigrostriatal dopaminergic neurons and the formation of nitrotyrosine. Prevented the formation of mature interleukin-1 β and the activation of NADPH-oxidase and inducible nitric oxide synthase (iNOS)	Wu et al. (2002)
Spinal cord injury (T13 hemisection of the spinal cord; rat)	30 min (40 mg/kg, i.p.) followed twice per day for 2 days post-injury	Reduced the development of pain behaviors at 1 and 2 weeks after SCI, reduced microglial OX-42 expression and decreased the expression of noxious stimulation-induced c-Fos	Marchand et al. (2009)

(Continued)

Table A1 | Continued

Animal model of disease	Dose	Effect	Reference
Spinal cord injury (rat)	Twice a day beginning 30 min after injury (50 mg/kg, i.p.) for 2 days	Reduced apoptotic oligodendrocytes and microglia in proximal and distal segments of the ascending sensory tract. Reduced microglial/macrophage density, attenuated axonal dieback and improved functional outcome	Stirling et al. (2004)
Temporary middle cerebral artery occlusion model (TMCAO; rat)	For 4 h post TMCAO protocol: 3 or 10 mg/kg i.v. at 4, 8, and 12 h; for the 5-h post TMCAO protocol: at 5, 9, and 13 h; and for the 6-h post TMCAO protocol at 6, 10, and 14 h	3 and 10 mg/kg i.v. were effective at reducing infarct size with a 5 hour therapeutic time window after TMCAO. 10 mg/kg extended the window time to ameliorate neurological deficits to 5 h	Xu et al. (2004)

Table A2 | List of antibodies and their respective classifications and dilutions used to measure protein biomarker levels in sera and brain tissues.

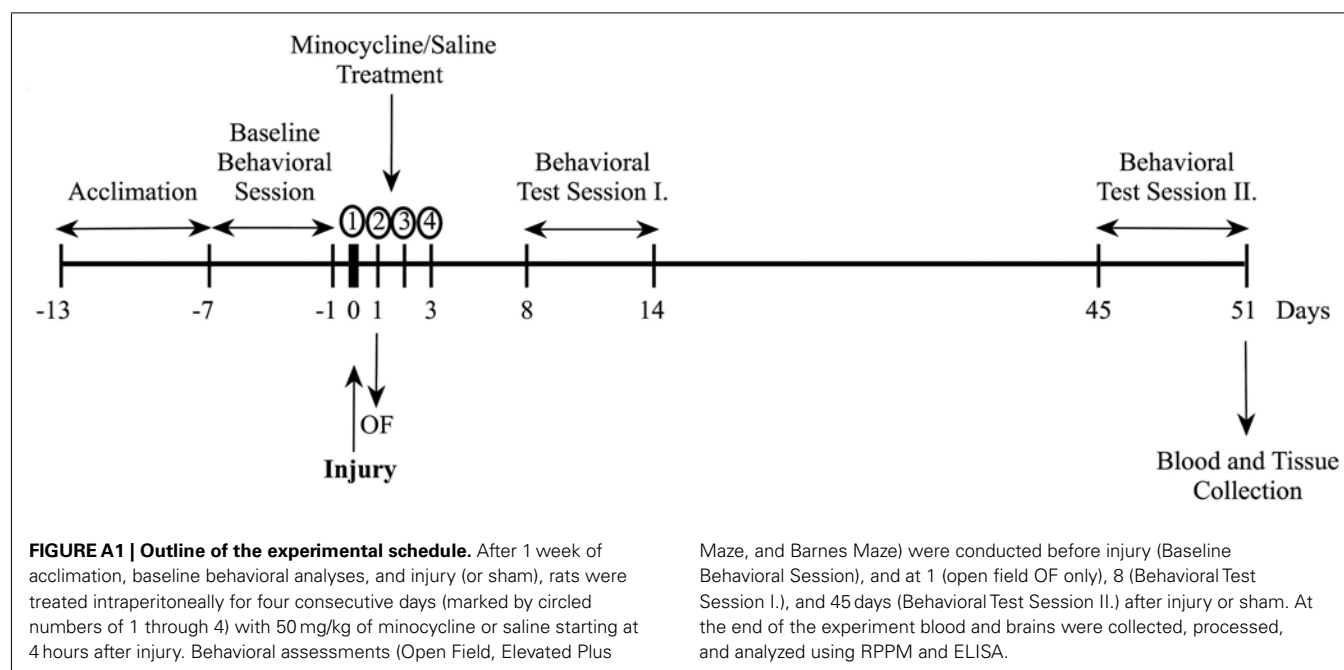
Antibody	Vendor	Catalog No.	Dilution in RPPM
INFLAMMATORY			
C-reactive protein (CRP)	Santa Cruz Biotechnology, Inc.	sc-30047	1:20
Monocyte chemoattractant protein (MCP-1)	Santa Cruz Biotechnology, Inc.	sc-1784	1:20
<i>Toll-like receptor 9 (TLR9)</i>	<i>Santa Cruz Biotechnology, Inc.</i>	<i>sc-13218</i>	<i>1:20</i>
VASCULAR			
Claudin 5	Santa Cruz Biotechnology, Inc.	sc-28670	1:20
<i>Vascular endothelial growth factor (VEGF)</i>	<i>Abcam</i>	<i>ab-53465</i>	<i>1:50</i>
<i>VEGF receptor 2 (FLK-1)</i>	<i>Santa Cruz Biotechnology, Inc.</i>	<i>sc-315</i>	<i>1:20</i>
<i>Aquaporin 4 (AQP4)</i>	<i>Abcam</i>	<i>ab-97414</i>	<i>1:50</i>
NEURONAL			
Neuron-specific enolase (NSE)	Abcam	ab-53025	1:20
Neurofilament heavy chain (NF-H)	Sigma Aldrich	N-4142	1:20
Tau protein	Santa Cruz Biotechnology, Inc.	sc-1995P	1:20
GLIAL			
S100 beta protein (S100 β)	Abcam	ab-41548	1:20
Glial fibrillary acidic protein (GFAP)	Abcam	ab-7260	1:50
<i>Myelin basic protein (MBP)</i>	<i>Santa Cruz Biotechnology, Inc.</i>	<i>sc-13914</i>	<i>1:20</i>

Biomarkers labeled with italics were only measured in the brain.

Table A3 | The effect of injury and minocycline treatment on tissue levels of the selected protein biomarkers in the different experimental groups.

Markers	Amygdala				Prefrontal cortex				Ventral hippocampus				Dorsal hippocampus			
	Sham vehicle	Sham mino	Injured vehicle	Injured mino	Sham vehicle	Sham mino	Injured vehicle	Injured mino	Sham vehicle	Sham mino	Injured vehicle	Injured mino	Sham vehicle	Sham mino	Injured vehicle	Injured mino
VASCULAR																
Claudin 5	4.49 ± 0.14	4.61 ± 0.09	4.90 ± 0.18	4.81 ± 0.14	4.25 ± 0.16	4.40 ± 0.15	4.63 ± 0.05	4.75 ± 0.08	4.10 ± 0.09	4.38 ± 0.14	4.78 ± 0.15	4.59 ± 0.08	4.65 ± 0.12	4.61 ± 0.12	4.64 ± 0.18	4.59 ± 0.06
VEGF	4.97 ± 0.06	5.15 ± 0.07	5.30 ± 0.11	4.97 ± 0.10	5.07 ± 0.05	4.83 ± 0.06	5.12 ± 0.07	4.97 ± 0.12	5.03 ± 0.04	4.90 ± 0.06	5.18 ± 0.07	5.10 ± 0.04	4.99 ± 0.08	5.01 ± 0.08	4.89 ± 0.16	5.00 ± 0.15
AQP4	4.36 ± 0.05	4.44 ± 0.08	4.78 ± 0.04	4.53 ± 0.07	4.36 ± 0.12	4.37 ± 0.11	4.58 ± 0.05	4.68 ± 0.13	4.25 ± 0.07	4.30 ± 0.10	4.80 ± 0.04	4.49 ± 0.07	4.32 ± 0.09	4.36 ± 0.07	4.41 ± 0.08	4.41 ± 0.12
GLIAL																
MBP	5.63 ± 0.14	5.80 ± 0.06	5.55 ± 0.08	5.64 ± 0.09	5.49 ± 0.06	5.54 ± 0.06	5.64 ± 0.04	5.93 ± 0.15	5.94 ± 0.08	6.00 ± 0.04	6.37 ± 0.11	6.05 ± 0.04	5.94 ± 0.07	6.04 ± 0.08	6.51 ± 0.04	5.95 ± 0.11

The levels of seven protein markers were measured by RPPM in the amygdala, prefrontal cortex, ventral hippocampus, and dorsal hippocampus. Measured protein levels are expressed as y-axis intercept (y-intercept) and are presented as mean ± SEM. Values listed in underline italics indicate $p < 0.05$ for injured-vehicle vs. sham-vehicle rats. **Bold-faced** values indicate $p < 0.05$ for injured-vehicle vs. injured-minorants.





Contribution of systemic factors in the pathophysiology of repeated blast-induced neurotrauma

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HIGHLIGHTS

- Repeated blast exposure increases the activation of platelets.
- Activation of leukocytes was observed after blast exposure.
- Vasoconstriction was evident in the cerebral cortex after blast exposure.
- Platelet/leukocyte targeted drugs to mitigate brain injury after blast exposure.

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ABSTRACT

Blast-induced traumatic brain injury is complex and involves multiple factors including systemic pathophysiological factors in addition to direct brain injuries. We hypothesize that systemic activation of platelets/leukocytes plays a major role in the development and exacerbation of brain injury after blast exposure. A mouse model of repeated blast exposure that results in significant neuropathology, neuro-behavioral changes and regional specific alterations in various biomolecules in the brain was used for the proposed study. Activation of platelets was evaluated by flow cytometry and serotonin content was analyzed by ELISA. Expression of myeloperoxidase was analyzed by Western blotting. Histopathology of the brain was used to assess blast-induced cerebral vasoconstriction. The data showed an increase in the activation of platelets at 4 h after repeated blast exposures, indicating changes in platelet phenotype in blast neurotrauma. Platelet serotonin concentration showed a significant decrease at 4 h after blast with a concurrent increase in the plasma serotonin levels, confirming the early onset of platelet activation after repeated blast exposures. Blood, plasma and brain myeloperoxidase enzyme activity and expression was increased in repeated blast exposed mice at multiple time points. Histopathological analysis of the brains of blast exposed mice showed constriction of blood vessels compared to the respective controls, a phenomenon similar to the reported cerebral vasoconstriction in blast affected victims. These results suggest that repeated blast exposure leads to acute activation of platelets/leukocytes which can augment the pathological effects of brain injury. Platelet/leukocyte targeted therapies can be evaluated as potential acute treatment strategies to mitigate blast-induced neurotrauma.

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1. Introduction

Blast-induced traumatic brain injuries (blast TBI) sharply increased in recent military conflicts due to asymmetric warfare and high use of improvised explosive devices and hand held

grenades by insurgents. Definition of how the blast shockwaves enter the brain and cause biochemical and molecular changes leading to TBI or trigger secondary pathological processes and long-term neurobehavioral deficits is still elusive. Proposed major mechanisms of blast-induced TBI involve direct transmission of the shockwaves through the skull, or through orifices of eyes, nose and ear, and transmission of transient pressure waves from torso to brain [5,8,18]. Clinical and animal studies show that blast exposure leads to blood–brain barrier (BBB) breakdown along with neuronal/axonal/glia damage which are thought to be linked to behavioral deficits [4,25,26].

Recently, it has been reported that the severity of blast neurotrauma was higher following blast exposure to the torsoes of subjects with head protection than was seen following head-only

Abbreviations: Blast TBI, blast-induced traumatic brain injury; PMNs, polymorphonuclear neutrophils; BBB, blood–brain barrier; MPO, myeloperoxidase; PRP, platelet rich plasma; PGE₁, prostaglandin E₁; BCA, bicinchoninic acid; ELISA, enzyme linked immunosorbent assay; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; H & E, hematoxylin and eosin; LPA, lysophosphatidic acid.

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exposures [4,16]. Furthermore, blast neurotrauma was significantly attenuated by torso protection but not by head protection, indicating that blast mediated systemic responses play a critical role in the development of blast neurotrauma [4,16]. Previous studies from our laboratory in rats using Kevlar vest, which protected the thorax and abdomen, also showed significantly reduced mortality rates and brain injury after blast exposure [20]. The nature of the systemic factors that induce blast TBI is still obscure. Hemostatic abnormalities, cerebral vasospasm, pseudoaneurysm and neuroinflammation have been identified as key pathophysiological features of blast neurotrauma [1,19]. It has been suggested that altered blood components are involved in the development of blast TBI/cerebral vasospasm [1]. For example, activated blood components can promote BBB breakdown, neuroinflammation, and cerebral vasospasm which can augment the brain injury process leading to blast TBI. The contribution of systemic response along with the proposed torso to brain hydraulic overpressure transmission in the development of blast neurotrauma and cerebral vasospasm warrants further investigation [4,23].

We established a tightly-coupled single and repeated blast injury model in mice which resulted in significant neuropathology, neurobehavioral changes and brain regional specific alterations in various biomolecules [27,29]. Utilizing this model, we have evaluated the changes in systemic responses focusing on platelets and polymorphonuclear neutrophils (PMNs) activation process, which is presumed to be involved in the development and exacerbation of brain injury to outline their role in blast neurotrauma.

2. Materials and methods

2.1. Materials

Tissue protein extraction reagent and bicinchoninic acid (BCA) protein assay kits were purchased from Pierce Chemical Co. (Rockford, IL); serotonin research enzyme linked immunosorbent assay (ELISA) kit was purchased from Rocky Mountain Diagnostics, Inc. (Colorado Springs, CO); myeloperoxidase (MPO) enzyme assay kit was purchased from Cayman Chemical Company (Ann Arbor, MI). Anti-mouse MPO antibody was purchased from R&D Systems, Inc. (Minneapolis, MN). Human α -thrombin was purchased from Enzyme Research Laboratories (South Bend, IN). Prostaglandin E_1 (PGE₁) and monoclonal antibody against β -actin were purchased from Sigma–Aldrich (St Louis, MO). PE-conjugated anti-mouse $\alpha_{IIb}\beta_3$ antibody was purchased from Emfret Analytics (Germany).

2.2. Repeated blast injury mouse model

Animal procedures were performed at the Walter Reed Army Institute of Research (WRAIR) in compliance 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 (National Research Council Publication, 1996 edition) with an approved Institutional Animal Care and Use Committee protocol. Briefly, groups ($n=4-6$) of anesthetized mice (male C57BL/6J mice, Jackson Laboratory, Bar Harbor, ME; 8–10 weeks old, 22–26 g weight) were exposed to 20.6 psi blast overpressure three times with 1–30 min intervals as reported earlier [29]. At 4, 24, and 72 h after the last blast exposure, blood and brain were collected from sham and blast exposed animals.

2.3. Flow cytometric analysis of platelet activation

Blood from the sham and blast exposed mice was collected in acidified citrate dextrose solution (1:10) containing 1 μ g/ml of

PGE₁. Platelet rich plasma (PRP) was separated by centrifugation at 800 rpm for 10 min at 22 °C. Platelets were pelleted from PRP by centrifugation at 2000 rpm for 10 min at 22 °C, washed twice with Tyrode buffer (137 mM NaCl, 12 mM NaHCO₃, 2.5 mM KCl pH7.2) and resuspended in Tyrode buffer containing 0.35% bovine serum albumin, 0.1% glucose, 2 mM CaCl₂ and 1 mM MgCl₂. The washed platelets (2×10^5 /ml) from sham and blast exposed animals were incubated with 0.1 U/ml of thrombin for 15 min at room temperature followed by incubation with PE-conjugated anti- $\alpha_{IIb}\beta_3$ antibody (1:100) for 15 min. Data from 5000 PE-positive platelets were acquired using a Guava Technologies flow cytometer (Millipore, Billerica, MA) and analyzed by the FlowJo software (Tree Star, Ashland, OR).

2.4. Estimation of serotonin

Serotonin content in the plasma, platelets and frontal cortex extracts was analyzed by highly specific competitive inhibition ELISA kits according to the manufacturer's protocols. Briefly, blood was collected from sham and blast exposed animals in acidified citrate dextrose solution (1:10) containing 1 μ g/ml PGE₁ and 0.1% ascorbic acid. The platelets and plasma were separated from other blood components as described earlier, and the platelets were lysed in deionized water containing 0.1% ascorbic acid. The frontal cortex samples from sham and blast exposed animals were homogenized with tissue protein extraction reagent containing 0.1% ascorbic acid. Diluted serotonin standards, plasma, platelet lysates and frontal cortex extracts were acylated using acylation reagents provided with the ELISA kit at room temperature for 30 min with shaking. Aliquots of acylated standards and samples were incubated with serotonin antiserum overnight at 4 °C in a serotonin antigen pre-coated microtiter plate. The unbound antibody was washed with washing buffer, followed by incubation of the microtiter plate with anti-rabbit IgG conjugated with peroxidase for 30 min at room temperature. The bound enzyme was quantified using tetramethylbenzidine substrate and the absorbance was measured at 450 nm using a SpectraMax M5 spectrophotometer (Molecular Devices, Sunnyvale, CA). The concentrations of serotonin in the plasma, platelets, and frontal cortex extracts were calculated from the serotonin standard curve. This ELISA kit is 100% specific for serotonin with minor cross-reactivity to few other amino acid derivatives like tryptamine (0.2%), melatonin (0.03%), 5-hydroxyindole acetic acid, phenylalanine, and histidine etc. (all <0.002%).

2.5. Immunoblotting of myeloperoxidase (MPO)

Blood samples and frontal cortices of sham and blast exposed animals were lysed/homogenized with tissue protein extraction reagent containing protease inhibitor cocktail. Equal amount of proteins (~30 μ g) from blood and frontal cortex samples of sham and blast exposed mice were separated on 4–20% gradient SDS-PAGE gels, transferred to polyvinylidene membranes and blocked with 4% non-fat dry milk for 1 h at room temperature. The blots were incubated with anti-mouse MPO monoclonal antibody (1:1000) overnight at 4 °C. The blots were washed with phosphate buffered saline containing 0.1% Tween-20, and incubated with anti-mouse secondary antibody conjugated to peroxidase (1:5000) for 1 h at room temperature. The blots were washed and the chemiluminescence was developed by using ECL detection reagent and photographed with Alpha Imager (Cell BioSciences, Santa Clara, CA). The resultant blots were stripped with stripping buffer and re-probed with anti- β actin antibody as the loading control. The images were quantified by using ImageJ software.

2.6. Myeloperoxidase activity assay

The activity of MPO in the whole blood and plasma was analyzed using commercially available assay kits according to manufacturer's protocol. Briefly, aliquots of whole blood (lysed with deionized water) and plasma were incubated with a working solution containing 5 mM H_2O_2 and 1 mM peroxidation substrate (10-acetyl-3,7-dihydroxyphenoxazine) for 15 min at room temperature and the formation of highly fluorescent product resorufin was measured at an excitation wavelength of 540 nm and emission wavelength of 595 nm using a SpectraMax M5 fluorescence spectrophotometer (Molecular Devices, Sunnyvale, CA). The MPO activity in the whole blood and plasma samples was calculated from the resorufin standard curve.

2.7. Brain histopathology

Control and blast exposed mice were euthanized, perfused and fixed with 4% paraformaldehyde. The brain samples were processed using routine histological procedures, sectioned and stained with hematoxylin and eosin (H & E) at FD NeuroTechnologies, Ellicott City, MD and used for evaluating cerebral vasoconstriction.

2.8. Data analysis

The data were tabulated by using GraphPad Prism software and statistical analysis was performed by using Mann–Whitney test. Probability (p) values less than or equal to 0.05 considered as significant.

3. Results

3.1. Acute activation of platelets after repeated blast exposure

Integrins are transmembrane heterodimeric receptors involved in series of cellular events including cell attachment and signaling. Integrin $\alpha_{\text{IIb}}\beta_3$ is exclusively present in a low affinity state in resting platelets. Upon activation, integrin $\alpha_{\text{IIb}}\beta_3$ will shift into

a high-affinity state, which is being used as a classical marker in platelet activation studies. Washed platelets collected from sham and repeated blast exposed mice were activated with thrombin agonist and the extent of activation was analyzed by using integrin $\alpha_{\text{IIb}}\beta_3$ surface expression with flow cytometer. As shown in Fig. 1A, platelets from repeated blast exposed mice showed a significant acute (4 h post-blast) increase in the expression of integrin $\alpha_{\text{IIb}}\beta_3$, indicating that blast exposure can activate the platelets to a pro-thrombotic phenotype. The acute increase in the activation of platelets after blast exposure returned to normal levels with time and was comparable to respective sham controls.

Levels of serotonin in the plasma, platelets, and frontal cortices of sham and blast exposed mice were analyzed by commercially available ELISA kits. As shown in Fig. 1B, blast exposure caused an acute significant increase in the concentration of serotonin in the plasma compared to sham controls, which tended to normalize to control values by 72 h post-blast. Meanwhile, a significant acute decrease in the concentration of serotonin was observed in the platelets of blast exposed mice compared to respective sham controls followed by a significant increase by 72 h after blast exposure (Fig. 1B). The concentration of serotonin in the frontal cortex of blast exposed mice also showed a significant decrease at multiple time points studied (4–72 h) compared to sham controls (Fig. 1B).

3.2. Myeloperoxidase activity and expression after repeated blast exposure

Myeloperoxidase is a peroxidase enzyme expressed abundantly on PMNs. Activated PMNs will release MPO which is considered as a potent inflammatory agent. Mice exposed to repeated blast exposures showed a significant increase in the plasma MPO activity at 4 h post-blast, which tend to normalize by 24–72 h post-blast compared to sham controls (Fig. 2A). MPO activity in the whole-blood of repeated blast exposed mice showed a significant increase at 4 and 24 h post-blast, with normal values by 72 h post-blast compared to respective sham controls (Fig. 2B). These results were confirmed by Western blotting which showed increased expression of MPO in the whole blood of blast exposed mice (Fig. 2C). The expression of

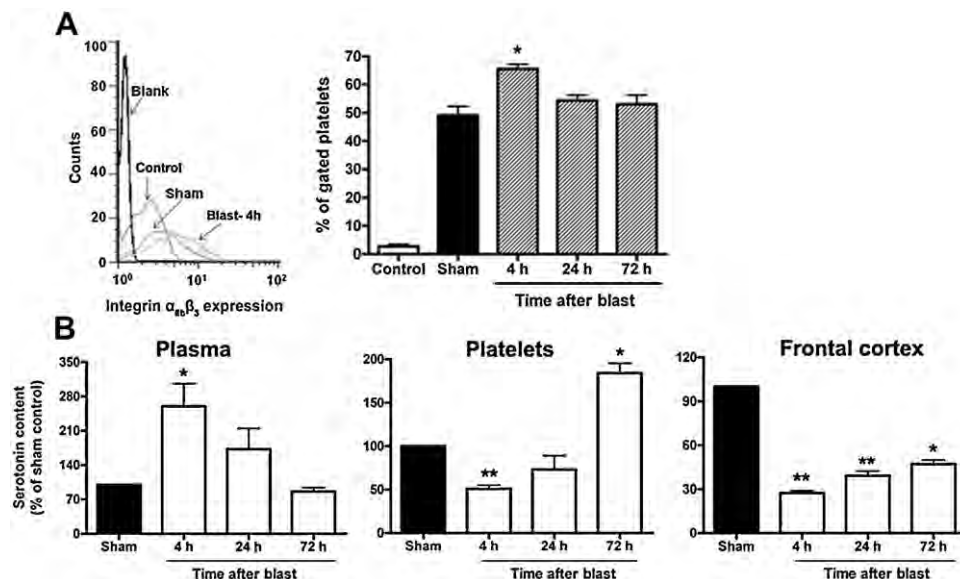


Fig. 1. Flow cytometric analysis of platelet activation and serotonin content after blast exposure. C57BL/6J mice were anesthetized with isoflurane and exposed to repeated blast overpressure (20.6 psi, 3 times with duration of 1–30 min). At indicated time points, the blood and brain was collected from blast exposed and sham control mice. (A) Aliquots of platelets were activated with 0.1 U/ml of thrombin and the surface expression of integrin $\alpha_{\text{IIb}}\beta_3$ were analyzed in a Guava flow cytometer using PE-conjugated anti- $\alpha_{\text{IIb}}\beta_3$ antibody. Representative diagram of flow cytometric analysis of platelets from sham and blast exposed (4 h post-blast) animals and quantification of the data (mean $\pm$ SEM). (B) The concentration of serotonin in the plasma, platelets and frontal cortex were estimated by ELISA as described in Section 2 and the results were expressed as % of sham control. Sham, $n=6$; blast 4–72 h, $n=4$; *0.05 < p < 0.01; and **0.01 < p < 0.001.

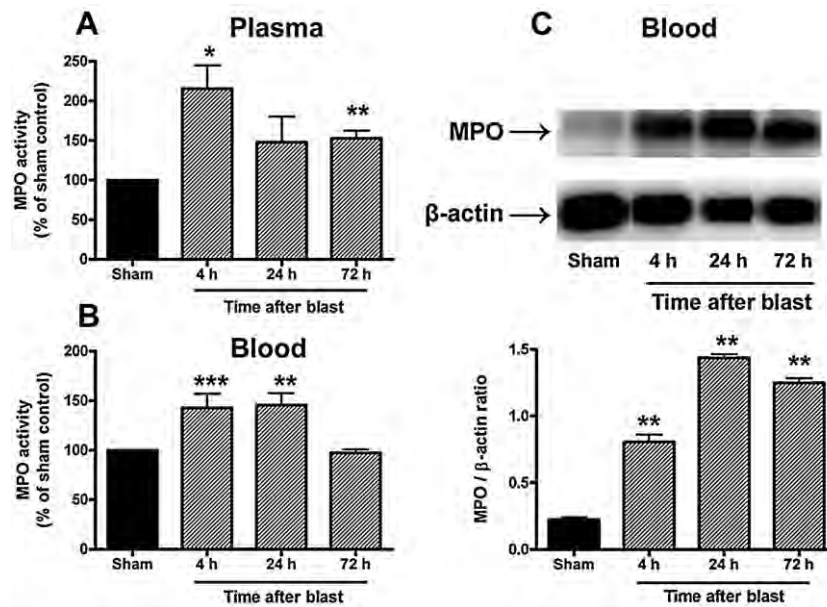


Fig. 2. Myeloperoxidase activity/expression change after repeated blast exposures. The activity of MPO was analyzed in the plasma (A) and whole blood (B) of sham and repeated blast exposed mice as described in Section 2. (C) Western blotting analysis of MPO expression in the whole blood after blast exposures; quantification of the expression data. Sham, $n=6$; blast 4–72 h, $n=4$ –8; * $0.05 < p < 0.01$; ** $0.01 < p < 0.001$; and *** $p < 0.001$.

MPO in the frontal cortex of blast exposed mice also showed a significant acute increase until 24 h post-blast and gradually returned to normal levels by 72 h (Fig. 3A). The increased expression of MPO in the whole blood and frontal cortex after repeated blast exposures indicate acute inflammation after repeated blast exposures.

3.3. Histopathology of brain sections after blast exposure

Sham and repeated blast exposed mice were perfused with formalin as described in the methodology and the sections of cerebral cortex were stained with hematoxylin and eosin. As shown in Fig. 3B, at 4 h post-blast, cerebral cortex of the blast exposed mice

showed significant constriction of the blood vessels compared to respective sham controls (indicated by arrows). These results may indicate potential cerebral vasoconstriction after blast exposure, most probably as an ensuing injury from increased systemic factor contribution.

4. Discussion

Platelet activation and aggregation has been considered as a critical step in hemostasis and inflammatory and immunopathogenic responses [6]. Activation of circulating platelets and leukocytes play a significant role in microcirculation occlusion and development of ischemic brain lesions [11]. Recently, Zhang et al. reviewed the significance of TBI-associated coagulopathy and suggested that TBI can augment the platelet hyperactivity [31]. Here we evaluated the activation of platelets after repeated blast exposures and report an acute increase in the thrombin mediated activation of platelets, suggesting the presence of hyperactive platelets which can play a pathological role in blast neurotrauma. Similarly, increased activation of PMNs after repeated blast exposure indicated by enhanced activity and expression of MPO suggests the early onset of inflammation as one of the major contributing factors for hyperactivation of platelets. Minimum activation of platelets or PMNs was observed after single blast exposure (data not shown).

Previous studies on the systemic effects of blast injury reported activation of PMNs resulting in increased expression of MPO in the circulation or periphery [14]. Circulating and locally released MPO in the brain can catalyze the formation of highly reactive oxygen species causing cellular injury [3,15]. The increased activity and expression of MPO in the blood and plasma as observed in our studies can augment blast-induced neurotrauma, resulting in both acute and chronic neuropathological and neurobehavioral changes. Platelets are also reported to be activated by MPO, mainly through MPO mediated oxidized lipoproteins and reactive oxygen species formation [22]. The increased expression and activity of MPO in the blood and plasma along with increased platelet activity after repeated blast exposure confirms this phenomenon indicating that platelet and PMN activation can be considered as major systemic

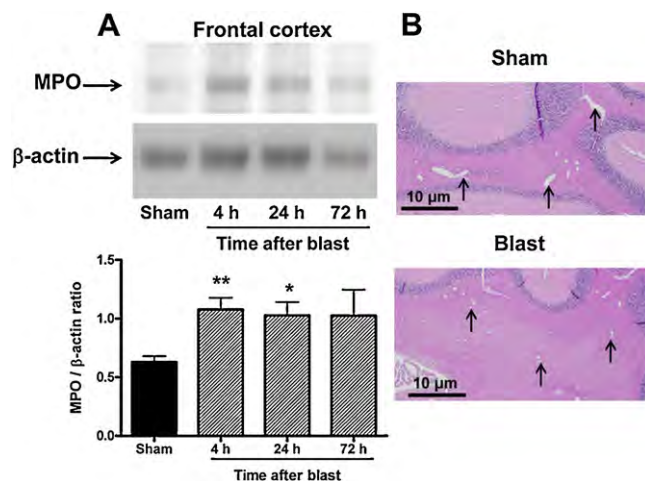


Fig. 3. Expression of MPO in the frontal cortex and hematoxylin/eosin staining of cerebral cortex after repeated blast exposures. (A) Western blot analysis of MPO expression in the frontal cortex of sham and blast exposed mice and quantification of the data. (B) Perfused brain samples of sham and blast exposed mice were sectioned and stained with hematoxylin and eosin as described in Section 2. Shown are the representative photographs of stained cerebral cortex of sham and blast exposed mice at 4 h post-blast. The blood vessels are indicated by arrows and the constriction of blood vessels is evident in the blast exposed animals. Sham, $n=4$ –6; blast 4–72 h, $n=4$; * $0.05 < p < 0.01$; and ** $0.01 < p < 0.001$.

factors potentially contributing to the severity of brain injury in blast neurotrauma.

Another molecule of interest is serotonin, which was increased in the plasma after blast exposure while both platelet and frontal cortex serotonin levels decreased. Serotonin is a monoamine neurotransmitter derived from the amino acid tryptophan and is primarily found in the gastrointestinal tract, platelets and central nervous system [2,30]. In platelets, serotonin is stored in granules and serves as a vasoconstrictor regulating hemostasis and blood clotting [28,30]. Serotonin level in the platelet and brain are reported to be involved in aggression and suicidal behavior, common features in various types of TBI associated psychiatric deficits [17,21,24]. Serotonergic abnormalities have been established in the frontal cortex and platelets of victims who are predisposed to suicidal behavior [17,21]. Our results on the level of serotonin in the frontal cortex and platelets after repeated blast exposures can be addressed from these perspectives.

Histopathological analysis of cerebral cortex of blast exposed animals showed acute constriction of blood vessels, an indication of potential vasoconstriction after repeated blast exposures [23]. Although, the observed effects in platelet and leukocyte activation resulting in changes in serotonin and MPO after repeated blast exposures can be major contributing factors involved in the cerebral vasoconstriction process, various other factors contributing to this complex phenomenon need to be studied further. The acute increase in the concentration of serotonin in the plasma can be a major contributor for this process because of its vasoconstriction property [28].

Platelets are a rich source of the bioactive lipid, lysophosphatidic acid (LPA), which is reported to be released from activated platelets and functions as a potent inflammatory agent in multiple injuries [7,9]. The increased expression of LPA and its receptors in the brain after TBI and spinal cord injury has been reported, which in turn can initiate a network of pathological process [10,13]. Recently, targeting LPA using anti-LPA antibodies has been proposed as a successful treatment strategy in TBI and spinal cord injury [12]. Increase in the activation of platelets/leukocytes after blast exposure shows the significance of LPA and the potential use of anti-LPA antibody in blast-induced neurotrauma which need to be explored further. The acute changes observed after repeated blast exposures underscore the evaluation of early treatment strategies in blast-induced neurotrauma to alleviate the exacerbation and development of secondary injuries and long-term neuropathological and neurobehavioral changes.

5. Conclusions

In summary, repeated blast exposures of mice showed significant acute activation of platelets and PMNs. Hyperactivation of platelets was evident from the increased expression of $\alpha_{IIb}\beta_3$ in blast exposed animals. Increased expression and activity of MPO in the blood, plasma and brain of repeated blast exposed animals indicate activation of PMNs. Histopathologic analysis of cerebral cortex of blast-exposed animals showed constriction of blood vessels suggesting possible role of systemic factors in vasoconstriction after repeated blast exposures. Thus, the acute changes in the activation of platelets and leukocytes after repeated blast exposures warrants the need for therapeutic evaluation of platelet/leukocyte targeted drugs to mitigate the development and exacerbation of brain injury after blast exposure.

Conflicts of interest statement

There is no potential conflict of interest related to this manuscript.

Disclosure

The opinions and assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Army, the Navy, or the Department of Defense, USA.

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Research Article

Neurobehavioral, cellular, and molecular consequences of single and multiple mild blast exposure

Mild traumatic brain injury, caused by the exposure to single or repeated blast overpressure, is a principal concern due to its pathological complexity and neurobehavioral similarities with posttraumatic stress disorder. In this study, we exposed rats to a single or multiple (five total; administered on consecutive days) mild blasts, assessed their behavior at 1 and 16 days postinjury and performed histological and protein analyses of brains and plasma at an early (2 h) and a late (22 days) termination time point. One day postinjury, multiple-injured (MI) rats showed the least general locomotion and the most depression- and anxiety-related behaviors among the experimental groups; there were no such differences at 16 days. However, at the later time point, both injured groups displayed elevated levels of select protein biomarkers. Histology showed significantly increased numbers of TUNEL+ (terminal-deoxy-transferase-mediated dUTP nick-end labeling)-positive cells in the dorsal and ventral hippocampus (DHC and VHC) of both injured groups as early as 2 h after injury. At 22 days, the increase was limited to the VHC of MI animals. Our findings suggest that the exposure to mild blast overpressure triggers early hippocampal cell death as well as neuronal, glial, and vascular damage that likely contribute to significant, albeit transient increases in depression- and anxiety-related behaviors. However, the severity of the observed pathological changes in MI rats failed to support the hypothesized cumulative effect of repeated injury. We infer that at this blast frequency, a potential conditioning phenomenon counteracts with and reduces the extent of subsequent damage in MI rats.

Keywords:

Blast / Cumulative effect / Neurobehavior / Protein biomarkers / Traumatic brain injury
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Abbreviations: AD, amygdala; BM, Barnes maze; BOP, blast overpressure; bTBI, blast-induced traumatic brain injury; DCX, doublecortin; DHC, dorsal hippocampus; FLK-1, fetal liver kinase-1 or VEGF receptor-2; GCL, granular cell layer; GFAP, glial fibrillary acidic protein; mbTBI, mild blast-induced traumatic brain injury; MI, multiple-injured; MS, multiple sham; mTBI, mild traumatic brain injury; NCad, N-cadherin; NF-H, neurofilament-heavy chain; NSE, neuron-specific enolase; OF, open field; PFC, prefrontal cortex; PTSD, posttraumatic stress disorder; SI, single-injured; SS, single sham; Tau, tau protein; TUNEL, terminal-deoxy-transferase-

1 Introduction

Mild traumatic brain injury (mTBI) is the most frequent form of neurotrauma among civilian and military populations [1–4]. While sports injuries, motor vehicle accidents, and assaults represent the bulk of mTBI incidence among civilians, improvised explosive devices account for the majority (~80%) of the casualties sustained during recent military conflicts. Most of these casualties (~60%) involved injuries to the head, resulting in various severities of blast-induced traumatic brain injury (bTBI). Of the bTBIs, mild blast-induced traumatic brain injury (mbTBI), caused by the exposure to low levels of explosive blast, is the most frequent and its true occurrence is probably underreported [2, 3, 5, 6]. mbTBI

mediated dUTP nick-end labeling; USU, Uniformed Services University; VEGF, vascular endothelial growth factor; VHC, ventral hippocampus; vWF, von Willebrand factor; Y-axis intercept

represents a major challenge for the military healthcare system due to its high incidence, partly overlapping symptoms with posttraumatic stress disorder (PTSD), and a lack of objective diagnostics and specific treatments [4, 7–10].

Soldiers exposed to low levels of blast typically do not lose consciousness or experience very brief periods of unconsciousness [10–13]. Consequently, most soldiers pass the Automated Neuropsychological Assessment Metrics or the Military Acute Concussion Evaluation as they exhibit transient and mild symptoms, and are sent back into the field where many of them may be re-exposed to one or more additional blasts. Some individuals develop various neurobehavioral problems following a single blast exposure while others do so after several exposures [2, 5, 12]. Importantly, practically no mbTBI occurs without psychological and physiological stress in a battlefield scenario.

The exposure to psychological stress, particularly chronic and repeated stress, can cause substantial neurobehavioral abnormalities and occasionally result in severe affective disorders such as anxiety and memory dysfunctions, even without physical injury. Therefore, stress as a cofactor in mbTBI can be significant, particularly in the case of repeated exposure [2, 3, 12, 14, 15]. Among the leading neurobehavioral abnormalities observed in mbTBI are increased anxiety and memory impairments, both of which have been observed as a part of PTSD's symptomatology [3, 7, 15–17]. While reports suggest that increased anxiety may be transient in nature and can disappear over time, memory deficits typically have a delayed onset and may last for an extended period of time (months and even years).

On a cellular and molecular level, these neurobehavioral changes imply damage to the hippocampus. The ventral hippocampus (VHC) and its afferent and efferent connections is predominantly involved in mediating depression- and anxiety-related behavior, while the dorsal hippocampus (DHC) and its afferent and efferent connections is predominantly involved in mediating spatial learning and memory [18, 19]. Previous works using various animal models of bTBI have shown that the pathobiology of bTBI includes inflammation, neuronal and glial cell loss, gliosis, as well as axonal and vascular damage [2, 14, 20–22]. It has been found that even a single exposure to mild blast can cause lasting increases in serum levels of neuron- and glia-specific markers, implicating neuronal and glial cell damage and/or loss in mbTBI [22, 23]. Interestingly, some of these pathological changes have shown distinct anatomical localization and corresponded with the observed functional deficits.

Studies have shown that exposing an organism to brief periods of various physiological stressors such as ischemia and hypoxia can lead to an increased tolerance to subsequent insults; a phenomenon known as conditioning [24–27]. Conversely, it has been well demonstrated that some patients who have suffered an mTBI, usually in sports accidents, have an elevated risk for sustaining severe damage if exposed to subsequent mTBIs [28–30]. This condition, known as secondary impact syndrome, suggests that the repeated exposure to mild blast can have a similar cumulative effect that increases the

severity of the functional outcome. While epidemiological data indicate that repeated exposure to mild levels of blast can increase the severity of the neurobehavioral outcome in some cases [2, 3, 5, 6, 11, 13, 31, 32], the key factors responsible for the observed cumulative effect (e.g., predisposing genetics, preexisting and comorbid conditions, frequency of impacts) are currently unknown.

In order to assess the validity of a presumed damage accumulation hypothesis as it relates to functional outcome severity, we determined the effects of single and multiple mild blast overpressure (BOP) exposure in a rodent model of bTBI on basic neurobehavior, plasma and brain tissue levels of select protein biomarkers, and cellular changes at two differing postinjury time points.

2 Materials and methods

2.1 Animals and housing conditions

A total of 66 male Sprague Dawley rats [weight at arrival: 245–265 g] (Charles River Laboratories, Wilmington, MA, USA) were used in our study. For the duration of the experiments, all animals were housed in pairs in standard rat cages in a reverse 12–12 h light–dark cycle with food and water *ad libitum*. Animals were handled according to protocol, approved by the Institutional Animal Care and Use Committee at the Uniformed Services University (USU; Bethesda, MD, USA).

2.2 Experimental groups and manipulations

This study is composed of two separate experiments that vary with respect to the time animals were terminated after the completion of the blast exposure(s); all other experimental manipulations were otherwise identical (Fig. 1). Experiment One was terminated ~2 h after blast (or sham) injury, while Experiment Two was terminated following the completion of two sets of behavioral analyses at 22 days postinjury. Experiment One was carried out to completion before the commencement of Experiment Two.

All animals underwent an acclimation and handling period of 5 days and were then assigned to the following experimental groups: Naïve, single sham (SS), single-injured (SI), multiple sham (MS), and multiple-injured (MI). Animal group numbers in Experiments One and Two are: ($N = 30$; Naïve = 3, SS = 6, SI = 7, MS = 6, MI = 8) and ($N = 36$; Naïve = 6, SS = 6, SI = 10, MS = 6, MI = 8), respectively.

For the duration of the experiments, Naïve animals were kept in the animal facility at USU without any manipulation except on behavioral testing days. SS animals were transported once from USU to Walter Reed Army Institute of Research (Silver Spring, MD, USA) and anesthetized for 6 min in an induction chamber with 4% isoflurane (Forane; Baxter Healthcare Corporation, Deerfield, IL, USA) without being exposed to BOP. MS animals were similarly transported and anesthetized daily for five consecutive days. Sham rats were

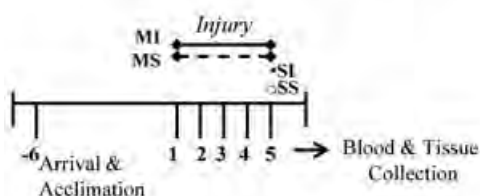
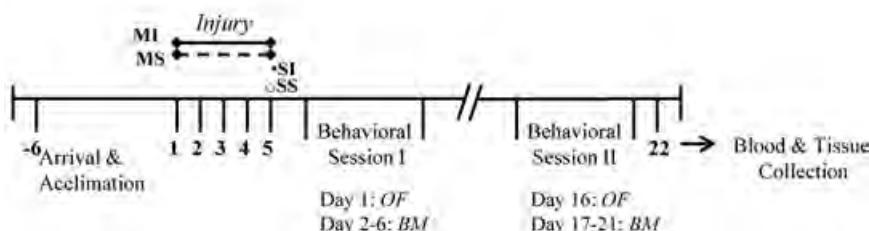
Experiment One: Early Termination Time Point**Experiment Two: Late Termination Time Point**

Figure 1. Outline of the experimental schedules. Blood and brain tissue were collected from animals in Experiment One approximately 2 h after the last blast on injury day 5; Experiment Two was terminated 22 days after. The timelines are not drawn to scale.

kept in the procedure room adjacent to the shock tube for the length of the injured animals' exposures.

2.3 Injury

SI and MI animals underwent the same procedures as their respective sham groups in addition to being exposed to a single or multiple (five total) mild blasts, respectively. Blast injury was administered to rats (weight at injury: 300–330 g) while wearing chest protection using a compressed air-driven shock tube as described earlier in detail [15, 22, 23, 33]. Briefly, rats were placed in the shock tube holder in a transverse prone position and exposed to mild BOP (average peak total pressure: ~138 KPa at the animal level); the right side of the animal faced the direction of the membrane and the incidence of the blast waves. All animals were transported back to the USU animal facility at the conclusion of the daily exposures.

2.4 Behavioral tests

All animals in Experiment Two were tested for general locomotion as well as depression- and anxiety-related behaviors using the open field (OF) system, and for spatial learning and memory using the Barnes maze (BM). Behavioral assessments were performed on separate days starting at day 1 (Behavioral Session I) and day 16 (Behavioral Session II) after the last exposure (Fig. 1). Behavioral tests were conducted during animals' dark cycle.

2.4.1 Open field

Horizontal and vertical activity as well as time spent in the center of the OF were measured during the 60 min testing

sessions as described earlier [15, 22, 23]. The Omnitech Electronics Digiscan infrared photocell system (Test box model RXYZCM; Omnitech Electronics, Columbus, OH, USA), consisting of a 40 × 40 × 30 cm clear Plexiglas arena equipped with infrared photocells to track the movement of the testing subject, was used. Each animal was placed on the floor of the testing arena, the perforated lid was secured, and the subject was left undisturbed for the duration of testing. Data were automatically recorded and transmitted to a computer via an Omnitech Model DCM-BBU analyzer. Cage mates were tested concurrently to avoid additional separation anxiety for any of the subjects, and the testing arenas were thoroughly cleaned with a 30% ethanol solution between animals.

2.4.2 Barnes maze

The latency to locate and enter the escape box was measured in the BM as described earlier [15, 22, 23]. Briefly, each rat was tested twice per day for five consecutive days except on the first day of BM (three trials). The first of the three trials was an untimed training trial for animals to locate and enter the escape chamber. During training, each animal was placed in the escape box and covered for 30 s, after which the rat was removed while in the escape box and placed in the center of the maze. The animal was then allowed to explore the maze for a few seconds before it was returned to its cage. During the testing trials, the same rat was placed under a start box in the center of the maze for 30 s. The start box was then removed and the animal was allowed to explore the maze freely to locate the escape chamber. The trial ended when the animal entered the escape box or when a predetermined time (240 s) elapsed without finding the escape box. In the case that the animal was unable to find the escape box within the allotted time, it

was placed in the escape box for 1 min and then returned to its cage. The second timed trial for the animal commenced after the remaining subjects in the same experimental group completed their first timed trial. The latency to locate and enter the escape box was measured with ANY-maze 4.2 Software (Stoelting Company, Wood Dale, IL, USA). Throughout the behavioral sessions, animals were tested in the same order each day, the position of the escape box (relative to the maze and the testing room) was kept constant. The escape chamber and the maze were thoroughly cleaned with a 30% ethanol solution between animals to eliminate any olfactory cues.

2.5 Blood and tissue collection

For protein measures, rats from Experiment One ($N = 17$; Naïve = 3, SS = 3, SI = 4, MS = 3, MI = 4) and Experiment Two ($N = 18$; Naïve = 3, SS = 4, SI = 4, MS = 4, MI = 3) were deeply anesthetized with Isoflurane inhalant; blood was collected and samples were promptly centrifuged at 10 000 revolutions per minute for 15 min at 4°C. The supernatants were aliquoted, flash-frozen, and stored at –80°C until processing. Animals were then decapitated using a guillotine (Harvard Apparatus Co.; Dover, MA, USA) and the brains were immediately removed. The prefrontal cortex (PFC), amygdala (AD), VHC, and DHC were dissected over wet ice and the dissected brain regions were then flash-frozen and stored at –80°C until processing.

For histology, rats from Experiment One ($N = 12$; SS = 3, SI = 3, MS = 3, MI = 3) and Experiment Two ($N = 12$; Naïve = 2, SS = 2, SI = 2, MS = 2, MI = 4) were similarly anesthetized and transcardially perfused with cold 1× PBS followed by a 4% paraformaldehyde in 1× PBS solution. Fixed brains were then cryoprotected, frozen, and sectioned coronally using a cryostat (Cryocut 1800; Leica Microsystems; Bannockburn, IL, USA) as described in detail [15, 22, 23].

2.6 Protein measures

Relative protein concentrations of the selected biomarkers were determined using reverse phase protein microarray. Sample preparation, printing, scanning, and data analysis were performed as described in detail [15, 22, 23]. Briefly, dissected brain regions were pulverized in liquid nitrogen and then sonicated in the presence of protease and phosphatase inhibitors. Protein concentrations were measured using a bicinchoninic acid assay (Thermo Scientific, PI-23250). Samples were diluted in print buffer to a final protein concentration of 1 mg/mL and printed on ONCYTE Avid (tissue samples) or ONCYTE Nova (serum samples) single-pad NC-coated glass slides using an Aushon 2470 Arrayer (Aushon Biosystems, Billerica, MA, USA). Primary antibodies were diluted to 10× the optimal Western analysis concentration in antibody incubation buffer as described [21–23, 34]. Primary antibodies were used in the following dilutions: vascular endothelial growth factor (VEGF; 1:50) (Abcam, ab53465), neurofilament-heavy chain (NF-H; 1:20) (Sigma-

Aldrich, N4142), neuron-specific enolase (NSE; 1:50) (Abcam, ab53025), glial fibrillary acidic protein (GFAP; 1:500) (Abcam, ab7260), tau protein (Tau; 1:20) (Santa Cruz Biotechnology, sc-1995), N-Cadherin (NCad; 1:20) (Santa Cruz Biotechnology, sc-31031), von Willebrand factor (vWF; 1:20) (Santa Cruz Biotechnology, sc-8068), and VEGF receptor-2 or fetal liver kinase-1 (FLK-1) (1:50) (Santa Cruz Biotechnology, sc-315). Slides were incubated with the primary antibody solutions overnight at 4°C, then washed and incubated with the secondary antibodies Alexa Fluor® 635 goat antimouse (A-31574), goat antirabbit (A-31576), or Alexa Fluor® 633 rabbit antigoat Ig G (heavy + light chains) (A-21086) (Molecular Probes®, Invitrogen) at 1:6000 dilution in antibody incubation buffer for 1 h at room temperature. Fluorescent signals were measured in a Scan Array Express HT microarray scanner (Perkin Elmer, Waltham, MA, USA), and data were imported into a Microsoft Excel-based bioinformatics program developed in house for analysis [21–23, 34]. The linear regression of the log–log data was calculated after the removal of flagged data, which include S/N ratios of less than 2, spot intensities in the saturation or noise range, or high variability between duplicate spots (>10–15%). The total amount of antigen is determined by the Y-axis intercept or Y-cept [21–23, 34].

2.7 Histology

2.7.1 Immunohistochemistry

Every first and tenth coronal section containing the DHC or the VHC were mounted on positively charged glass slides two sections per slide. Three slides per animal, containing sections with identical Z-axes, were selected per brain region for each immunostaining. Immunohistochemical staining was performed as described in detail [22, 23]. Briefly, sections were incubated with the primary antibodies mouse anti-GFAP (Millipore, MAB360) at 1:400 dilution and rabbit antidoublecortin (DCX; Cell Signaling Technology, 4604) at 1:1000 overnight at 4°C. After washing with 1× PBS, the slides were incubated with the secondary antibodies Alexa Fluor® 555 goat antimouse (A-21422) or 488 goat antirabbit Ig G (heavy + light chains) (A-11008) at 1:100 for 1 h at room temperature; Hoechst 33342 was then applied for 2 min at 1 µg/mL (Molecular Probes®, Invitrogen). After a second wash, sections were coverslipped using antifading media (Vectashield; Vector Laboratories, Burlingame, CA, USA).

2.7.2 Histological data acquisition

Histological sections were visualized in an Olympus IX-71 microscope using the appropriate filters, and images were collected using a SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI, USA).

2.7.3 TUNEL assay

DNA fragmentation as a result of apoptotic signaling cascades was determined using a terminal-deoxy-transferase-mediated

dUTP nick-end labeling (TUNEL) in situ cell death detection kit, POD (Roche, Indianapolis, IN, USA), according to the manufacturer's instructions and as described earlier [22]. TUNEL+ cells were then visualized by 3,3'-diaminobenzidine substrate and counted.

2.8 Statistical analyses and data comparison

2.8.1 Behavior

ANOVA, repeated measures ANOVA, and Dunnett's *t* tests were conducted for each of the behavioral variables using IBM SPSS Statistics 19. OF activity scores were separated into three subscales: horizontal activity, vertical activity, and center time. Latency to find the escape box was analyzed for BM. All animals in Experiment Two (*N* = 30; Naïve = 5, SS = 6, SI = 6, MS = 6, MI = 7) were used for the behavioral analyses. Data are presented as the mean $\pm$ S.E.M. All tests were two tailed using α = 0.05.

2.8.2 Protein measures

Differences in the mean protein biomarker levels measured in plasma and in brain tissue were analyzed with ANOVA followed by the Tukey–Kramer Multiple Comparisons Test for all pairwise comparisons across the five experimental groups. The statistical analyses for Experiments One and Two were performed separately using Graph Pad InStat software. Statistical significance within each experiment was reported for blast injury (MS versus MI* and SS versus SI⁵) and frequency (SI versus MI[#]). A total of 17 animals from Experiment One (Naïve = 3, SS = 3, SI = 4, MS = 3, MI = 4) and 18 from Experiment Two (Naïve = 3, SS = 4, SI = 4, MS = 4, MI = 3) were used for the analyses. Data are presented as the mean $\pm$ S.E.M. A two-sided *p* value of <0.05 is depicted by one special character, *p* <0.01 by two, and *p* <0.001 by three.

2.8.3 Histology

TUNEL+ cells were counted from four brain sections per animal in the hilus and in the granular cell layer (GCL) of the DHC and the VHC. Data were analyzed with ANOVA followed by the Tukey–Kramer Multiple Comparisons Test as described above. A total of 12 animals from Experiment One (SS = 3, SI = 3, MS = 3, MI = 3) and 12 from Experiment Two (Naïve = 2, SS = 2, SI = 2, MS = 2, MI = 4) were used for the analyses. Data are presented as the mean $\pm$ S.E.M. A two-sided *p* value of <0.05 is depicted by one special character, *p* <0.01 by two, and *p* <0.001 by three. GFAP- and DCX-immunoreactive cells were not quantified for group comparison; the images are for illustrative purposes only.

3 Results

3.1 Behavioral changes

3.1.1 Open field

Figure 2A represents the horizontal activity, an index of general health and locomotion, of all animals at day 1 and 16 after the injury. Overall, animals had significantly lower horizontal activity at day 1 ($18\,222.93 \pm 764.94$) than at day 16 ($23\,054.80 \pm 836.49$), ($F(1, 25) = 27.03$, $p < 0.001$, $\eta^2 = 0.520$). There was also a significant group by time interaction on day 1, ($F(4, 25) = 4.35$, $p = 0.008$, $\eta^2 = 0.410$). Pairwise comparisons revealed that the MI group had significantly less horizontal activity than the Naïve, SS, and SI groups ($p < 0.05$). A Dunnett's *t* test was conducted to decrease type I error (four comparisons compared to ten with pairwise), revealing that MI animals were significantly different from Naïve ($p < 0.05$). No such differences were observed between the groups at day 16.

Figure 2B represents the vertical activity, an index of depression-related behaviors, of all animals at day 1 and 16 postinjury; decreased vertical activity indicates more depression-related behaviors. Overall, animals had lower vertical activity at day 1 (2279.22 ± 143.19) than at day 16 (3290.05 ± 188.02), ($F(1, 25) = 30.91$, $p < 0.001$, $\eta^2 = 0.553$). There was also a significant Group by Time interaction, ($F(4, 25) = 6.47$, $p = 0.001$, $\eta^2 = 0.509$). At day 1, there was a main effect for Group $F(4, 25) = 6.32$, $p = 0.001$, $\eta^2 = 0.50$, such that the Naïve group had significantly greater vertical activity than the SS, MS, and MI groups ($p < 0.05$). Similarly, SI animals had greater vertical activity than MI animals ($p < 0.05$). Dunnett's *t* test supported the finding that Naïve animals had significantly greater vertical activity than SS, MS, and MI animals ($p < 0.05$). At day 16, there were no differences in vertical activity between the groups.

Figure 2C represents the amount of time animals spent in the center of the OF chamber. Center time is an index of anxiety-related behaviors: a greater time spent in the center (as opposed to the margins and corners of the testing chamber) indicates less anxiety-related behaviors. Overall, animals spent less time in the center of the chamber at day 1 postinjury (718.03 ± 60.87) than at day 16 (1364.24 ± 63.53), ($F(1, 25) = 75.25$, $p < 0.001$, $\eta^2 = 0.751$). At day 1, pairwise comparisons revealed that the Naïve group spent significantly more time in the center than the SS, MS, and MI groups ($p < 0.05$). At this early time point, Dunnett's *t* test revealed that MS and MI animals were significantly different from Naïve ($p < 0.05$). No significant differences were observed in anxiety-related behaviors on day 16.

3.1.2 Barnes maze

Figure 3A and B represent the latency to find the escape box on days 2–6 (Test Session I) and 17–21 (Test Session II) after the injury. Data for Test Session I showed a significant effect

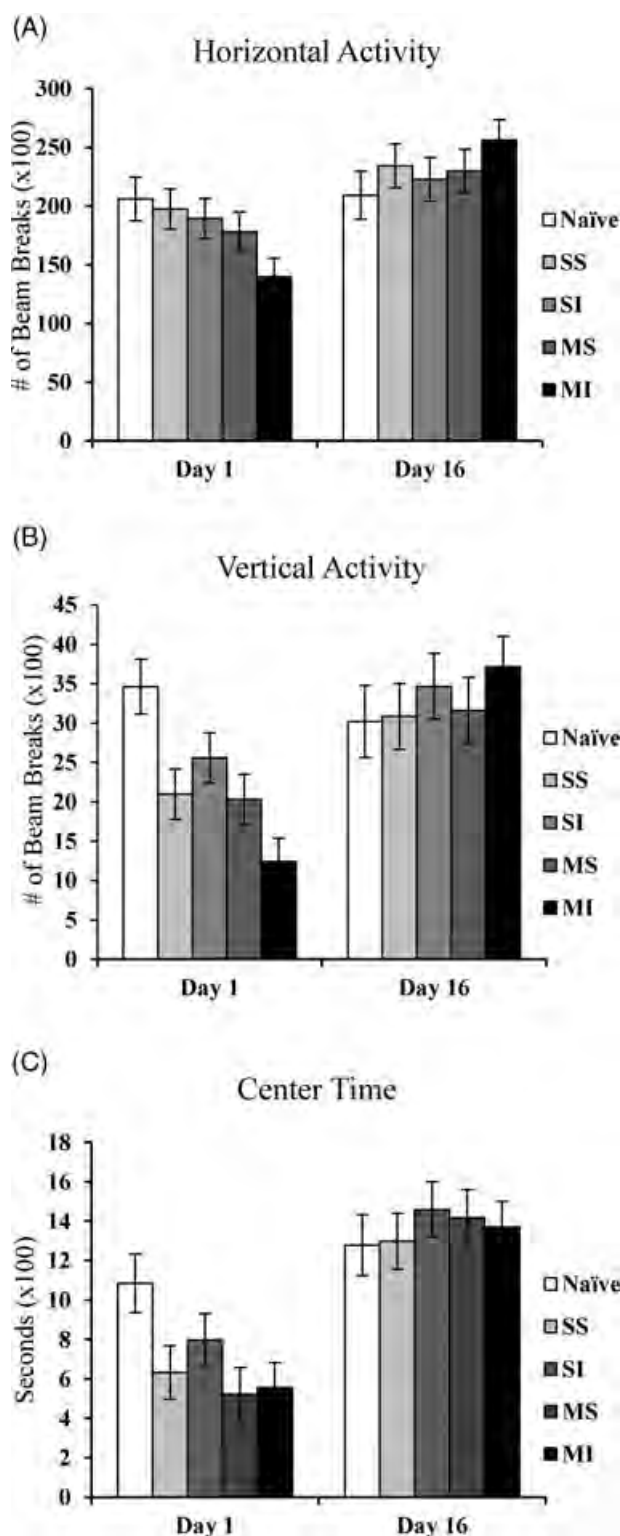


Figure 2. The general health and depression/anxiety-related behavior of animals in Experiment Two; an open field system was used to measure horizontal activity (number of beam breaks) (A), vertical activity (number of beam breaks) (B), and center time (seconds) (C) at day 1 and 16 postinjury. Data are presented as the mean $\pm$ S.E.M.

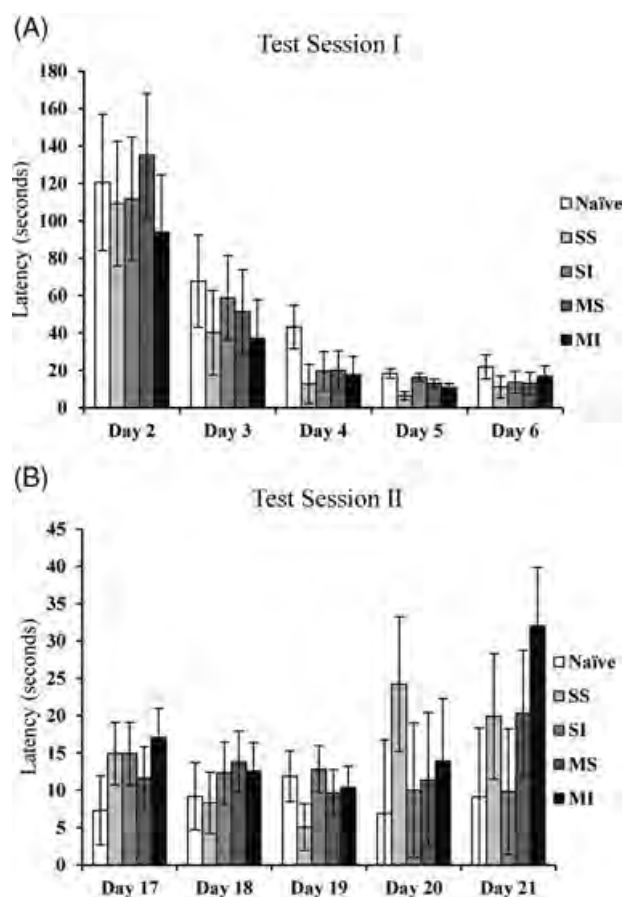


Figure 3. The spatial learning and memory of animals in Experiment Two; a Barnes maze was used to determine the latency (seconds) to find the escape box for five consecutive days starting at day 2 (A) and day 17 (B) postinjury. The depicted values represent the averages of two timed trials per animal in each experimental group. Data are presented as the mean $\pm$ S.E.M.

for time, such that the time to locate the escape box decreased over time ($F(4, 100) = 25.89$, $p < 0.001$, $\eta^2 = 0.509$). There was also a significant difference at day 5 of Test Session I ($F(1, 25) = 4.00$, $p = 0.012$, $\eta^2 = 0.390$), where Naïve animals spent a longer time to find the escape box than SS and MI animals but these differences were minimal.

The data for Test Session II showed no significant differences between the experimental groups on any of the testing days. It is noteworthy that on the final testing day, the MI group took substantially longer (32.07 ± 7.79) to locate the escape box than the other groups, and that the difference between the MI and Naïve group (9.10 ± 9.22) approached significance (pairwise comparison, $p = 0.06$).

3.2 Molecular changes

3.2.1 Protein markers in plasma

We analyzed the effects of single and multiple mild blast exposure on the plasma levels of VEGF, NF-H, NSE, and

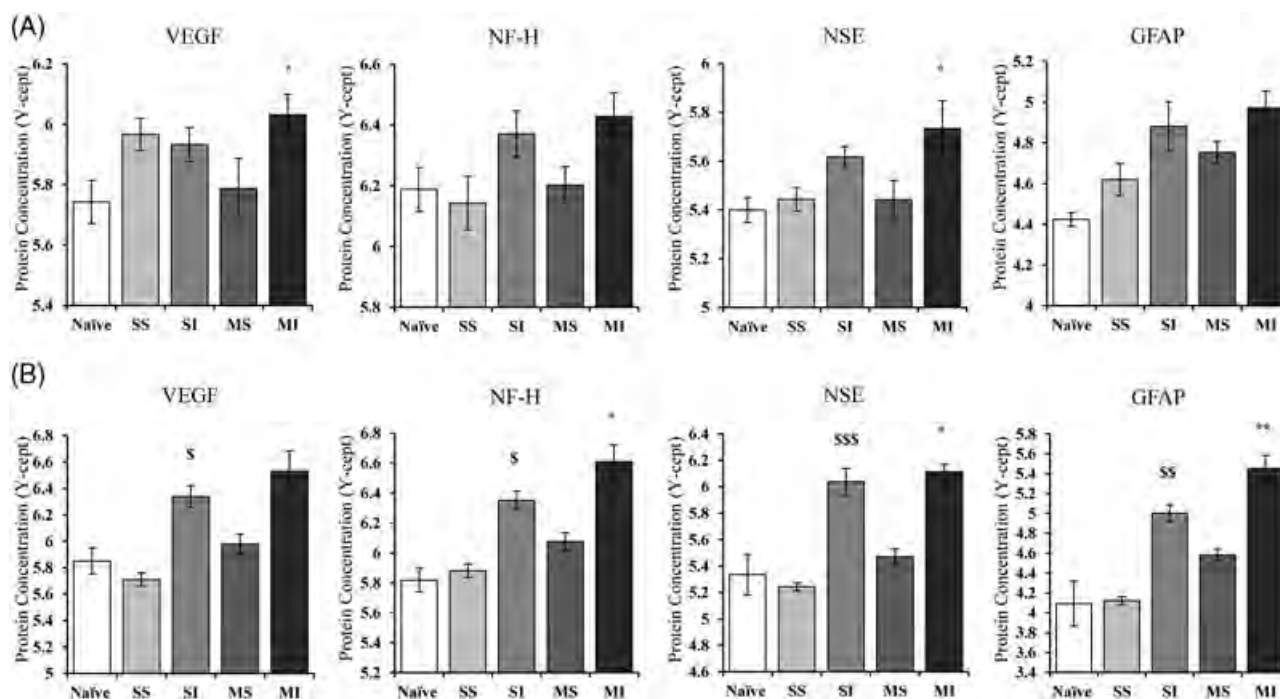


Figure 4. Plasma levels of select protein biomarkers at the early (A) and late (B) termination time points. Protein levels were assayed using reverse phase protein microarray; the reported Y-cept values (log10) indicate relative protein concentrations. Data are presented as the mean $\pm$ S.E.M. (SS versus SI $^{**}p < 0.01$ and $^{***}p < 0.001$; MS versus MI $^{*}p < 0.05$ and $^{**}p < 0.01$).

GFAP at the two described termination time points following injury (or sham). At the early time point (Experiment One; ~ 2 h after the last exposure), VEGF and NSE plasma levels were significantly increased in MI animals compared to their sham controls (MS) (Fig. 4A). The plasma levels of the other two markers, NF-H and GFAP, were not statistically different in either one of the injured groups (SI and MI) compared to their respective sham groups (SS and MS). At this time point, there were no statistically significant differences between the protein values measured in SI and SS animals for any of the four markers.

At the late termination time point (Experiment Two; 22 days after the last exposure), the plasma levels of all four markers were significantly elevated in both injured animal groups compared to their sham controls, except for VEGF protein levels in MI animals (Fig. 4B). Importantly, there were no significant differences between the plasma levels of any of the markers between SI and MI animals.

3.2.2 Protein markers in select brain regions

To identify some of the molecular consequences of single and multiple mild BOP exposure, we analyzed Tau, NCad, vWF, and FLK-1 concentrations in the AD, PFC, DHC, and VHC. At the early time point, Tau levels were only significantly elevated in the VHC of SI animals compared to their sham group. At the late time point, Tau levels were significant in

the DHC as well as in the AD of SI animals. Interestingly, Tau levels were significantly decreased in the AD of MI rats compared to SI rats. Contrary to Tau, tissue levels of NCad were significantly increased in the PFC of MI animals at the early time point. Similarly significant increases were measured in the DHC of MI rats as well as SI rats at the late time point; MI animals also had significant NCad increases in the VHC compared to MS animals (Supporting Information Table 1).

No significant changes were observed in vWF or FLK-1 at the early time point; tissue levels of both markers were relatively similar in each brain region for injured animals and their corresponding sham groups. At the late time point, significantly increased vWF tissue levels were measured in the PFC and in the VHC of SI animals. Similarly, MI rats had significant vWF increases in the PFC in addition to the DHC. FLK-1 was only significantly elevated in the AD of SI rats as well as in the VHC of both, SI and MI rats. There were no significant differences in tissue levels for any of the measured markers between SI and MI animals (Supporting Information Table 1).

3.3 Cellular changes

3.3.1 GFAP and DCX immunoreactivities

To determine the effects of single and multiple mild blast exposure on astroglial response and hippocampal de novo

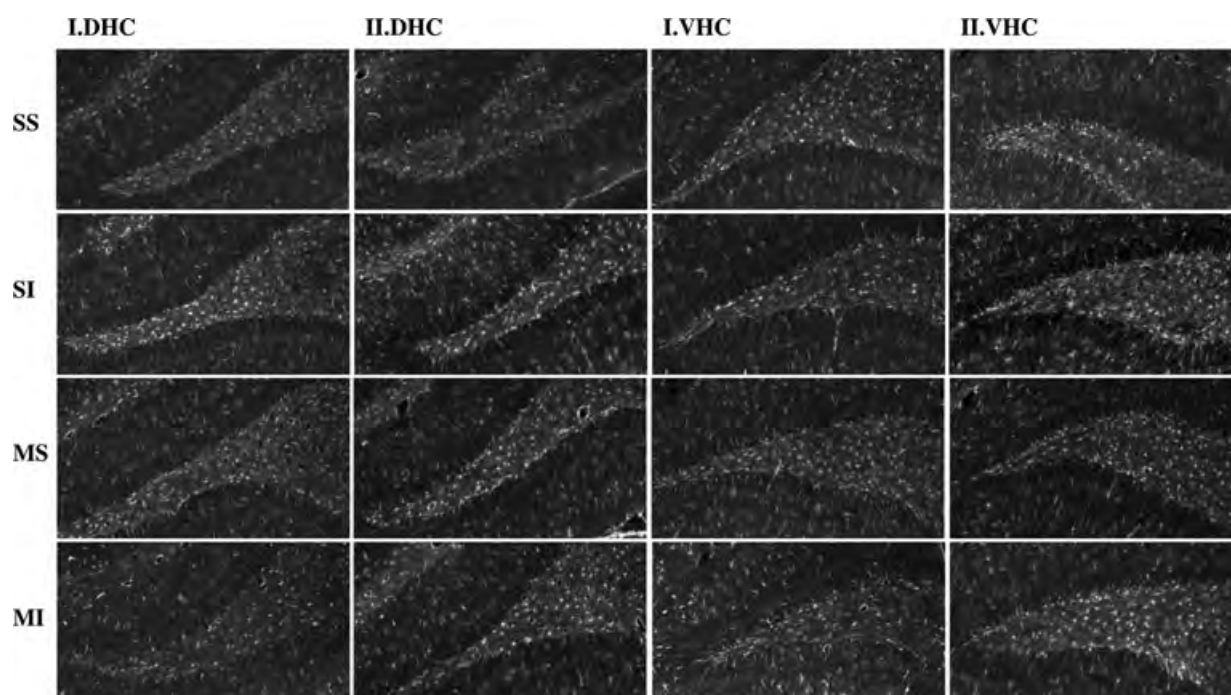


Figure 5. GFAP immunoreactivity in the dorsal and ventral hippocampus of SS, SI, MS, and MI animals at the early (I.DHC and I.VHC) and the late (II.DHC and II.VHC) termination time point, respectively.

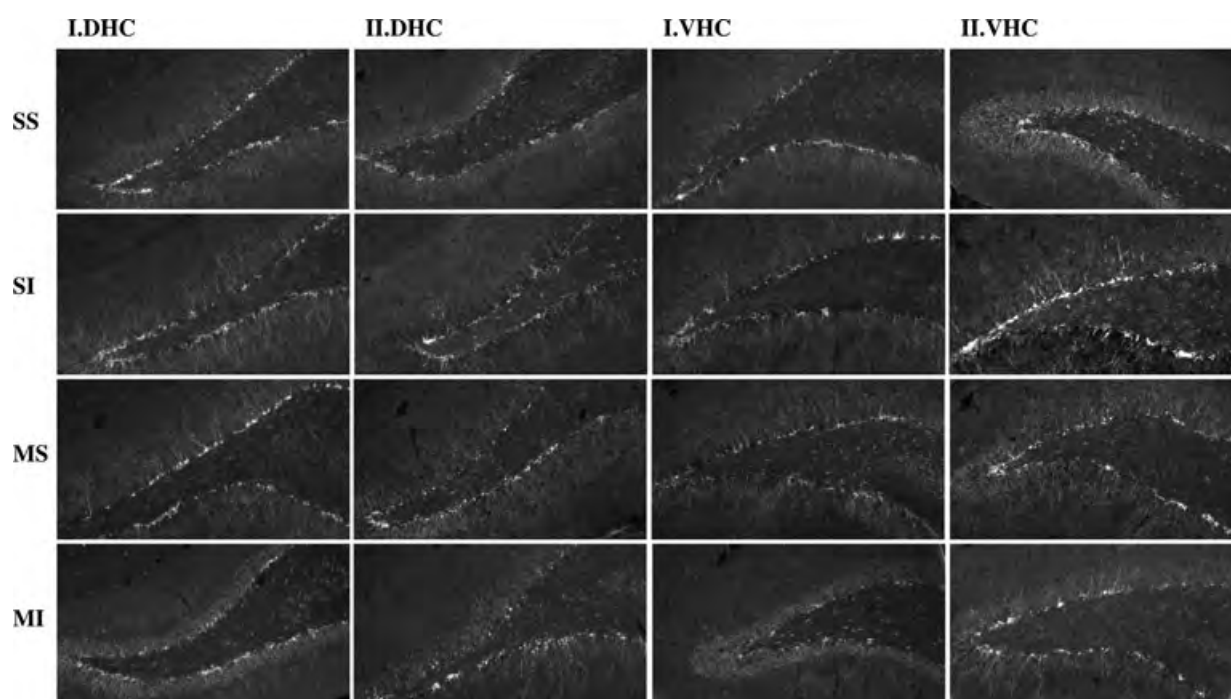


Figure 6. DCX immunoreactivity in the dorsal and ventral hippocampus of SS, SI, MS, and MI animals at the early (I.DHC and I.VHC) and the late (II.DHC and II.VHC) termination time point, respectively.

neurogenesis, we analyzed the DHC and the VHC by GFAP (Fig. 5) and DCX (Fig. 6) immunohistochemistry, respectively. At the early time point, we observed an apparent increase in GFAP immunoreactivity in the DHC (Fig. 5 I.DHC) as well as the VHC of SI animals (Fig. 5 I.VHC). Inter-

estingly, no such increase was seen in MI animals at the early termination time point (Fig. 5 I.DHC and I.VHC). However, there was an apparent increase in GFAP immunoreactive cells at the late time point in both, the DHC and the VHC of MI rats (Fig. 5 II.DHC and II.VHC).

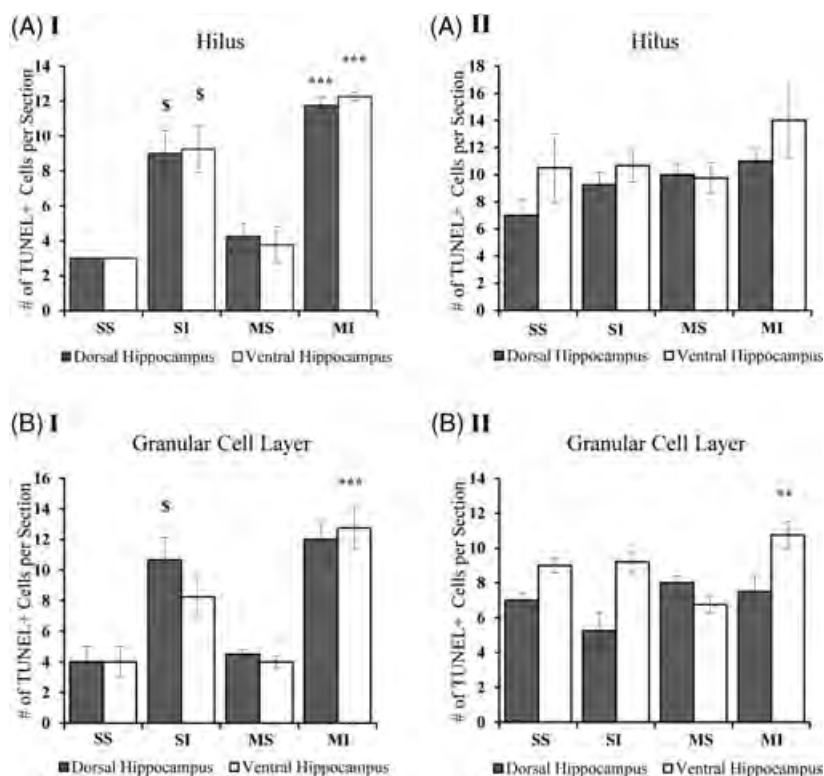


Figure 7. Presumed apoptotic cells marked by DNA fragmentation in the dorsal and ventral hippocampus. TUNEL+ cells per brain section in the hilus (A) and in the granular cell layer (B) of SS, SI, MS, and MI animals at the early (A.I and B.I) and the late (A.II and B.II) termination time point. Data are presented as the mean $\pm$ S.E.M. (\$ $p < 0.05$ SS versus SI; ** $p < 0.01$ and *** $p < 0.001$ MS versus MI; # $p < 0.05$ SI versus MI).

We detected an apparent increase in DCX immunoreactivity in the DHC of SI animals at the late but not at the early time point compared to SS animals (Fig. 6 I. and II.DHC). No such effect was observed in the DHC or the VHC of MI animals compared to their sham group at either termination time point (Fig. 6 I. and II. DHC; I. and II.VHC).

3.3.2 TUNEL histology

We used TUNEL histology to assess the extent of DNA fragmentation, and in turn the number of presumed apoptotic cells in the DHC and the VHC, following single and multiple mild blasts. At the early time point, the number of TUNEL+ cells was significantly increased in the hilus of the DHC and the VHC of SI and MI animals compared to their sham groups (Fig. 7 A.I). At the same time point, differences were only significant in the GCL of the DHC in SI animals and in the VHC of MI animals (Fig. 7 B.I). At the late time point, we detected no significant differences in the number of TUNEL+ cells in the hilus of injured and sham animals (Fig. 7 A.II). However, significant numbers of TUNEL+ cells were present in the GCL of the VHC in MI rats (Fig. 7 B.II).

4 Discussion

The goal of our study was threefold: (i) to compare the effects of single and multiple exposures to mild BOP on select neurobehavioral, cellular, and molecular (protein) outcomes; (ii) to assess the extent of the cellular and molecular dam-

age immediately following injury and at a later time point; (iii) to determine whether a presumed cumulative effect of repeated blast exposure increases the severity of the observed neurobehavioral abnormalities. We found that the exposure to mild BOP results in specific time-dependent functional, cellular, and molecular changes. However, we failed to detect the anticipated cumulative effect of repeated mild blast exposure. We hypothesize that at this particular frequency of blast exposure (i.e., once per day for five consecutive days), a conditioning phenomenon among other factors reduces the extent of the cumulative damage.

4.1 Behavioral changes

At the functional level, clinical observations have indicated mood disorders including depression, increased aggression, anxiety, and memory impairments as hallmarks of mbTBI [11, 13, 32]. Some of these neurobehavioral abnormalities, particularly memory impairment, develop over time while other affective symptoms, such as anxiety, can be transient in nature. In our current study, we found that anxiety- and depression-related behaviors were greatest in MI rats 1 day following the injury. The only other study comparing the effects of single and repeated (3 $\times$) exposure to moderate levels of BOP used a basic locomotor test (Rotarod) to evaluate the functional outcome in a mouse model [35]. Interestingly, the Rotarod test failed to show any cumulative effect on basic motor function following repeated blast exposure [35].

Consistent with our previous reports, the observed locomotor differences (including anxiety- and depression-related activities) were transient [22, 23]. In a long-term study (69 day survival following a single mild blast injury), we also found that while anxiety was significantly increased in injured animals 1 day after the exposure, at 1 and 2 months after the injury, animals displayed no signs of increased anxiety [22, 23]. Importantly, stress alone without any injury triggered a similar temporal increase in anxiety levels at the early testing point. Stress alone and as a cofactor in mbTBI is extremely important because at the clinical level, mbTBI and PTSD exhibit partly overlapping neurobehavioral symptoms [12, 17, 36, 37].

Consistent with our previous observations [22, 23], spatial learning and memory were not affected immediately following the injury; all experimental groups learned and performed the task of locating the escape box in the BM at about the same rate (Test Session I). On the other hand, on day 21 after the injury MI rats displayed signs of memory impairment. This finding is consistent with the chronic nature of mbTBI and the delayed functional deficits that develop in humans [12, 17, 38].

4.2 Molecular changes

Clinical as well as experimental findings indicate that the exposure to BOP causes a specific form of TBI (i.e., bTBI). The highly complex environment caused by explosive blast consists of (supersonic) pressure waves, kinetic energy, heat, and toxic gases among others. All of these components likely contribute to the complex pathology of bTBI. In fact, evidence suggests that the various severities of blast induce distinct biological responses that are reflected in different temporal profiles of protein biomarkers, which may be used to develop blood-based diagnostics. At the biological interface of each of these components, specific molecular and cellular responses can be initiated as a part of the secondary injury mechanism. In the case of mbTBI, these biological responses include neuronal and glial loss, neuroinflammation, and gliosis [2, 14, 20–22].

In our assessment of the temporal profile of four commonly used blood-based biomarkers, we found that plasma VEGF and NSE levels were significantly increased within 2 h of the injury. The rapid increase in plasma VEGF levels is consistent with the observation of a rapid and transient increase in brain water content following repeated injury in the mouse model [39]. VEGF is involved in the regulation of various endothelial functions including vascular permeability. The increase in plasma VEGF concentrations after repeated (but not a single) BOP exposure suggests that multiple exposures may cause early and sufficient damage to the vasculature that can contribute to the elevated brain water content [40–43]. Neuronal damage and loss, reflected in the elevated plasma levels of neuron-specific proteins, has been documented after various types of brain insults [44].

NSE is a soluble neuron-specific enzyme that is frequently used as a blood-based marker in various TBI studies. The detected rapid and early rise in NSE's plasma concentrations in response to multiple but not to a single blast injury indicates significant neuronal cell damage incurred by repeated mild blast, which causes the release of NSE from injured neuronal cell bodies. This protein should be further investigated as a potential early blood-based marker of repeated mbTBI.

At the later time point, that is, 22 days after injury, the plasma concentrations of all four markers were significantly increased indicating axonal and glial damage (NF-H and GFAP, respectively), in addition to the vascular and neuronal damage that was detected previously at the early termination time point. The increase in NF-H plasma concentrations in both SI and MI rats is indicative not only of axonal damage, but also of a compromised blood brain barrier and/or increased permeability [34, 45–47]. The significant increases in GFAP levels at this time point may reflect a gliotic response to injury that can be associated with repair processes in the central nervous system. Importantly, at both time points, we found no correlation between biomarker plasma levels and the number of blast exposures. Although protein levels for each marker were slightly higher in the MI group compared to the SI group, the expected cumulative effect of repeated injury as indicated by substantially increased vascular, neuronal, axonal, and glial damage was not observed in MI rats compared to SI rats.

In addition to the biomarkers measured in plasma, we analyzed changes in protein markers in select brain regions with direct neurobehavioral implications; these include the AD, PFC, DHC, and VHC. Our measurements revealed brain region- and time-dependent changes in some of the markers. While there were practically no changes in the tissue concentrations of markers 2 h after the injury, we found significant increases in the tissue levels of Tau in the VHC of SI rats and NCad in the PFC of MI rats at this early time point. Tau is a marker of axonal function while NCad is involved in cell adhesion [23, 48–50]. The other two markers, vWF and FLK-1, are involved in mediating inflammation and the neuron-specific effects of VEGF, respectively [51, 52].

At the later time point, changes in tissue protein levels were induced by both types of injury, single and repeated, with the VHC and the DHC being more affected than the other two brain regions. The hippocampus is involved in mediating anxiety as well as spatial learning and memory [18, 19]. Increased tissue levels of the selected markers in the DHC and the VHC may be a part of the injury-related response and/or the compensatory mechanism. Consistent with our measurements in sera, we found no indication of the hypothesized cumulative effect on the tissue levels of the measured markers. The preliminary characterization of the mouse model of repeated blast also found no cumulative effect of repeated blast on cortical levels of reactive oxygen species [39].

4.3 Cellular changes

The hippocampus has been long implicated in various neuropsychiatric conditions due to its central role in mediating learning and memory, as well as anxiety [19,53]. In bTBI, damage to hippocampal structures can occur due to the primary injury (i.e., the physical forces of the BOP), as well as various secondary injury processes such as metabolic changes or inflammation. Considering the nature of the neurobehavioral abnormalities associated with mbTBI, our histological analyses focused on different subregions within the hippocampus. While the VHC is implicated in anxiety-related behaviors, the DHC is involved in cognitive functions such as learning and memory [18,53,54]. The pathological changes we observed hereinafter are consistent with our previous findings showing distinct anatomical localizations in mbTBI [22,23]. However, it is important to note that due to the exploratory nature of this study and the absence of stereological analyses, our immunohistochemistry data are nonquantitative and are for illustrative purposes only.

Our immunohistochemical analysis showed an apparent increase in GFAP immunoreactivity, especially in the hilus of both parts of the hippocampus (the DHC and the VHC), within 2 h after a single exposure. However, multiple blast injuries failed to produce the anticipated (i.e., greater) increase in GFAP immunoreactivity in MI rats compared to their MS controls in both of the tested brain regions. Interestingly, at the later time point, GFAP immunoreactivity is comparable in both injured groups as well as the MS group, particularly in the DHC. The apparent increase in GFAP immunoreactive astrocytes in MS animals is consistent with our previous finding that the repeated exposure to environmental and psychological stressors alone (without injury) is capable of inducing pathological changes and altering functional outcome [15]. Increased GFAP expression by astrocytes has been observed after various neuronal insults and is generally considered to be a part of the gliotic response to injury [55–58]. This gliotic response has been viewed as both, beneficial and detrimental. Astroglia have been shown to be involved in eliminating toxic molecules and in providing trophic factors in support of recovery after injury. De novo hippocampal neurogenesis, partly supported by astroglia, has been implicated as a part of the recovery/regenerative process following neuronal insults including TBI.

Unlike the apparent early onset of the GFAP response, the increase in DCX immunoreactivity was more apparent at the later termination time point. The apparent increase in DCX immunoreactivity would be consistent with the known temporal pattern of hippocampal de novo neurogenesis following TBI. New neurons marked by DCX expression typically become detectable 2–3 wk after injury [59–62]. Importantly, the largest increase in DCX immunoreactivity was observed in the subgranular zone of the dentate gyrus in the VHC of SI animals. It should be noted that the VHC is predominantly involved in mediating anxiety, and that at this late time point injured animals' anxiety levels were similar to those of their respective shams. While it is intriguing to think

about de novo neurogenesis playing a role in the normalization of anxiety levels, testing this hypothesis would require highly complex experiments. The lack of increase in DCX immunoreactivity in MI rats (in either hippocampal region) compared to their sham controls can be potentially explained by the presence of negative growth signals generated by the repeated exposure to blast, which interfere with the injury-induced neurogenesis apparent after a single exposure; this, too, warrants further investigation.

Our TUNEL histology provided evidence for a very early onset of DNA fragmentation indicating an increased rate of apoptotic cell death [41,62–64]. It was at the early time point (2 h after injury) that we detected the most significant increase in the number of TUNEL+ cells in the hilus as well as in the GCL of the DHC and the VHC of injured animals. The detected increases may reflect the damage caused by the primary injury process, which results in an acute cellular injury that can lead to programmed cell death. Interestingly, the number of TUNEL+ cells was significant only in the GCL of the VHC of MI animals at the late time point. Again, there were no significant differences in the number of TUNEL positive cells following single or multiple exposures.

4.4 Conclusions

This exploratory study is the first to compare single and multiple BOP exposure on select functional, cellular, and molecular outcomes in an effort to assess the extent of the damage in repeated mbTBI. Although the apparent damage was not substantially higher following five blast exposures as opposed to one, a slight cumulative effect was observed in MI rats. We believe that several factors can account for this potentially important finding. These include obvious species differences, the frequency of BOP exposures, and a conditioning phenomenon, which may all be interconnected.

Species differences have been a significant issue in experimental biomedical research, especially in TBI. In addition to the anatomical, biomechanical, and physiological differences that exist across species, there are significant differences in metabolism, life expectancy, and the dynamics of disease progression. A “human year” is calculated to be between 10 and 12 “rat days” [65]. Consequently, the daily blast exposures administered to MI rats in this preliminary study can be roughly equivalent to monthly exposures in humans. Although there are currently no publicly available epidemiological data that correlate between the frequency of blast exposures and the observed cumulative effect, it is safe to assume that this blast frequency (coupled with rodent physiology) provides too long of a recovery period between exposures.

Another possible and intriguing explanation is that this rate of exposure triggers a conditioning effect in rats. It has been known that brief periods of physiological stress provide temporary protection or an increased tolerance to subsequent stressors. It should be noted that the conditioning effect we postulate in this study is related to the injury process itself, and not to the neuroprotective effect of repeated anesthesia.

In our experimental design, we deliberately compare injured animals to their respective sham groups (as opposed to Naïve animals) to account for all variables (i.e., handling, transportation, and anesthesia) not related to the BOP. Therefore, significant differences between injured animals and their respective shams can be attributed to the blast injury alone.

Conditioning has been particularly well studied in various models of ischemia affecting the heart as well as the brain. Ischemic conditioning can significantly improve the outcome of an otherwise fatal ischemic attack. In a rodent model of conditioning, repeated mild focal TBI delivered once a day for 3 days resulted in a significantly improved functional outcome after a severe focal TBI was delivered 3–5 days after the last mild (conditioning) injury targeting the same site [24–26]. In this model, the (focal) injury activated the local astroglia population, which upregulated their Hsp27 expression. Even though there are differences between the pathologies of focal and global types of TBI (e.g., bTBI), our group as well as others have observed astroglial activation in various models of bTBI. Future testing of the effects of repeated BOP exposures on astroglial expression of Hsp27 can serve as a useful marker, which can potentially identify the extent of vulnerability and/or conditioning after injury.

A limitation of this study (and more generally the rodent model for studying bTBI) is that under controlled experimental conditions, animals are positioned to receive the blast unilaterally, at the same anatomical location, and at consistent BOP peak ranges each time. Although it is standard procedure to use identical experimental conditions, this is rarely, if ever, the case in real life. Considering that BOP exposure in our blast model is unilateral, it is noteworthy to mention that there is evidence of brain hemisphere specialization in rodents [66,67]. The right hemisphere controls emotional expression and behavioral responses to novel events and threats as supported by the significant increases in anxiety- and depression-related behaviors in MI rats following the injury. Learning, a left hemisphere specialization, is not affected as seen in Test Session I of the BM. Animals, irrespective of their experimental manipulations, learned the task of locating the escape box at a similar rate. Therefore, the functional outcome of injury as it relates to the incidence of blast can be a substantial confounding factor in behavioral analyses. Given the nonfocal, global nature of bTBI, a more randomly targeted delivery of blast waves (with varying peak pressures) can more closely mimic operationally relevant scenarios of repeated mbTBI; an issue that will be investigated at length in future studies.

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ulations related to animals and experiments involving animals, and adhered to principles stated in the Guide to the Care and Use of Laboratory Animals, National Research Council. The facilities are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

The authors have declared no conflict of interest.

5 References

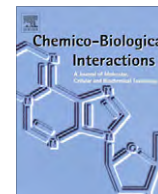
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Modulation of cholinergic pathways and inflammatory mediators in blast-induced traumatic brain injury

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ABSTRACT

Cholinergic activity has been recognized as a major regulatory component of stress responses after traumatic brain injury (TBI). Centrally acting acetylcholinesterase (AChE) inhibitors are also being considered as potential therapeutic candidates against TBI mediated cognitive impairments. We have evaluated the expression of molecules involved in cholinergic and inflammatory pathways in various regions of brain after repeated blast exposures in mice. Isoflurane anesthetized C57BL/6 J mice were restrained and placed in a prone position transverse to the direction of the shockwaves and exposed to three 20.6 psi blast overpressures with 1–30 min intervals. Brains were collected at the 6 h time point after the last blast exposure and subjected to cDNA microarray and microRNA analysis. cDNA microarray analysis showed significant changes in the expression of cholinergic (muscarinic and nicotinic) and gammaaminobutyric acid and glutamate receptors in the midbrain region along with significant changes in multiple genes involved in inflammatory pathways in various regions of the brain. MicroRNA analysis of cerebellum revealed differential expression of miR-132 and 183, which are linked to cholinergic anti-inflammatory signaling, after blast exposure. Changes in the expression of myeloperoxidase in the cerebellum were confirmed by Western blotting. These results indicate that early pathologic progression of blast TBI involves dysregulation of cholinergic and inflammatory pathways related genes. Acute changes in molecules involved in the modulation of cholinergic and inflammatory pathways after blast TBI can cause long-term central and peripheral pathophysiological changes.

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1. Introduction

Blast exposure has been described as one of the major factors involved in mild to moderate brain injury in service members returning from Iraq and Afghanistan which can lead to chronic neurological disabilities [1–4]. Acute changes in the central and peripheral nervous systems after blast TBI can exacerbate the pathological outcomes resulting in long-term chronic effects [5,6]. Neuroinflammation including cross-talk between central and peripheral immune systems is considered to be a primary event after blast exposure exacerbating the brain injury [6,7]. Inflammation and innate immune responses are primarily regulated by neural mechanisms [8,9]. In particular, cholinergic systems involving the neurotransmitter acetylcholine and the enzyme acetylcholinesterase (AChE) have been proposed as components of an anti-inflammatory pathway regulating neuroimmunomodulation [8–11].

Recently we reported regional specific alterations in the brain AChE activity after repeated blast exposures [12]. AChE inhibitors are possible therapeutic candidates against Alzheimer's disease and TBI [13–15]. In this study, we analyzed the expression of cholinergic and inflammatory related genes in different regions of the brain of repeated blast exposed mice using cDNA microarray. We also analyzed the microRNA expression profile in the cerebellum of blast exposed mice. MicroRNAs are endogenous tissue specific non-coding ribonucleic acids of approximately 18–26 nucleotides which modulate gene expression by binding to complementary mRNA, either targeting degradation or inhibiting translation, potentially play major roles in neuropathophysiology.

2. Materials and methods

2.1. Materials and animals

Trizol reagent, Tris–Bis gradient gels (4–12%), protein molecular weight markers, and SDS–PAGE running and transfer buffers were

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purchased from Invitrogen Life Technology (Carlsbad, CA); tissue protein extraction reagent and bicinchoninic acid (BCA) protein assay kit were purchased from Pierce Chemical Co. (Rockford, IL); acetylthiocholine, tetra monoisopropyl pyrophosphotetramide (iso-OMPA), and 4,4'-dipyridyl disulfide, 4,4'-dithiodipyridine (DTP) were purchased from Sigma-Aldrich (St. Louis, MO); polyvinylidene difluoride (PVDF) membrane and anti-myeloperoxidase (MPO) antibody were purchased from Millipore (Billerica, MA). C57BL/6J mice (male, 8–10 weeks old, 21–26 g) were purchased from Jackson Laboratory, Bar Harbor, ME. Animal experiments were performed at Walter Reed Army Institute of Research (WRAIR) in compliance 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 (National Research Council Publication, 1996 edition) with an approved Institutional Animal Care and Use Committee protocol. Isoflurane anesthetized mice were exposed to 20.6 psi blast overpressure for three times with 1–30 min intervals as described earlier [5,12,16].

2.2. Brain acetylcholinesterase activity assay

Brain samples collected at various time points (3, 6, 24 h and 3, 7, 14 days) were dissected into different parts (frontal cortex, hind cortex, hippocampus, cerebellum, mid brain and medulla) and homogenized with tissue protein extraction reagent at 4 °C using a tissue homogenizer and centrifuged. AChE activity in the supernatant of brain extracts were measured by using modified Ellman assay with 1 mM of acetylthiocholine substrate and 0.2 mM DTP as chromogen in the presence of 4 µM of iso-OMPA, a butyrylcholinesterase inhibitor, as described earlier [12,17–19]. Brain AChE activity was expressed as milliunits/mg protein.

2.3. Microarray analysis

Various regions of the brain (frontal cortex, cerebellum, mid brain, and hippocampus) of sham and repeated blast exposed mice at 6 h time point after the last blast exposure were collected and total RNA was isolated using Trizol reagent according to manufacturer's protocol. The concentration and purity of RNA were determined by using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Total RNA (5 µg) was labeled using the Agilent Low Input Quick Amp labeling Kit in conjunction with the Agilent two-Color Spike-Mix according to the RNA Spike-In Kit protocol and amplified in a thermal cycler (Mycycler, Bio-Rad Laboratories, Hercules, CA). Labeled RNA samples were subjected to fragmentation followed by 17 h hybridization against universal mouse reference RNA (Stratagene, La Jolla, CA) using the Agilent Gene Expression Hybridization Kit. Agilent 60-mer whole mouse genome 44 K oligo microarrays (Agilent Technologies, Santa Clara, CA) printed with Agilent SurePrint technology were used for microarray analysis as outlined in the Two-Color Microarray-Based Gene Expression Analysis (version 6.5) protocol. Microarray slides were scanned using an Agilent G2565CA fluorescence dual laser scanner for Cy3 and Cy5 excitation and the generated data were feature extracted using default parameters in Agilent Feature Extraction Software (version 10.7.1). GeneSpring 10.1 Software was used to carry out the data filter and normalization.

2.4. Western blot analysis of MPO expression

Total protein was extracted from the cerebellum of sham and repeated blast exposed mice at 6 h time point using tissue protein extraction reagent and the total protein content was estimated by using BCA assay kit. Equal aliquots (30 µg) of protein extracts were

separated on 4–12% SDS-PAGE, transferred to PVDF membranes and probed with anti-MPO antibody. Blots were developed by using chemiluminescent substrate, photographed by Alphamager (CellBioSciences, Santa Clara, CA) and quantified by using ImageJ software.

2.5. MicroRNA analysis

For the microRNA analysis, total RNA was extracted from cerebellar region of sham and repeated blast exposed mice (6 h time point after the last blast exposure) as described above followed by analysis by µParaflo™ MicroRNA microarray at LC Sciences (Houston, TX). Briefly, 4–8 µg total RNA was size fractionated using a YM-100 Microcon centrifugal filter (Millipore, Billerica, MA) and the small RNAs (<300 nt) isolated were 3'-extended with a poly(A) tail using poly(A) polymerase. An oligonucleotide tag was then ligated to the poly (A) tail for later fluorescent dye staining; two different tags were used for the two RNA samples in dual-sample experiments. Hybridization was performed overnight on a µParaflo microfluidic chip using a micro-circulation pump. On the microfluidic chip, each detection probe consisted of a chemically modified nucleotide coding segment complementary to target microRNA (from miRBase, version 16) or other RNA (control or customer defined sequences) and a spacer segment of polyethylene glycol to extend the coding segment away from the substrate. The detection probes were made by *in situ* synthesis using photogenerated reagent chemistry. After RNA hybridization, tag conjugating Cy3 and Cy5 dyes were circulated through the microfluidic chip for dye staining. Fluorescence images were collected using a laser scanner (GenePix 4000B, Molecular Device, Sunnyvale, CA) and digitized using Array-Pro image analysis software. Data were analyzed by first subtracting the background and then normalizing the signals using a LOWESS filter (Locally-weighted Regression).

2.6. Data and statistical analysis

Statistical analysis of brain AChE enzyme activity was performed by using GraphPad Prism software with Mann-Whitney test. The statistical analysis of the microarray data was performed by using GeneSpring 10.1 Software. Differentially regulated genes (between sham control and blast exposed samples) were selected using Welsh's *t*-test analysis ($p < 0.05$), followed by the Benjamini-Hochberg multiple correction test to find genes that varied between control and blast exposed samples with a false discovery rate of 5%. To account for the small sample size, we used the reference design and filtered for genes with signal intensities that are twice the standard deviation of the background intensity levels. We determined that by performing gene-by-gene *t*-tests, for a samples size of 3% and 5% false discovery rate and a standard deviation of 0.5, the power is 75%. We also applied pathway and gene ontology analyses that offer extra power because it is statistically unlikely that a larger fraction of false positive genes end up in one specific pathway.

3. Results

3.1. Summary of AChE activity changes in different regions of brain after blast exposure

Changes in AChE activity in different regions of brains following repeated blast exposure have been reported earlier and the summary is shown in Fig. 1 [12]. Except for frontal cortex, all other brain regions of blast exposed mice showed an acute decrease in the activity of AChE. In the cerebellum and midbrain regions, a significant increase in AChE activity was observed at 3 days, while the

medulla region showed a significant increase in AChE activity at 14 days post-blast. These data indicate heterogeneous changes in brain AChE activity after blast overpressure.

3.2. Changes in the expression of cholinergic pathway related genes in the midbrain

Gene expression analysis of the midbrain of blast exposed mice using cDNA microarray identified the modulation of multiple genes involved in cholinergic transmission (Table 1). The expression profiles of cholinergic receptors muscarinic 2 and nicotinic alpha 7 were down-regulated after the blast exposure. Similarly, expression of gammaaminobutyric acid receptors beta 3 and gamma 3 was also down-regulated in blast exposed mice. The expression of glutamate receptor metabotropic 8 showed significant reduction after blast exposure.

3.3. Changes in the expression of inflammatory pathway related genes in various brain regions of blast exposed mice

The cDNA microarray analysis showed significant changes in the expression of tumor necrosis factor (TNF), interleukins (IL) and their multiple receptors after blast exposure (Table 1). Several IL receptors showed significant up-regulation in the midbrain. In the frontal cortex, cerebellum and hippocampus the expression of IL receptors was significantly low, while TNF and its receptors showed higher expression (Table 1). There was a significant increase in the expression of carboxylesterase in the midbrain region and myeloperoxidase expression in the cerebellum of blast exposed mice indicating increased inflammation. These results indicate that repeated blast exposure leads to modulation of multiple inflammatory pathway related genes.

3.4. MPO expression in the cerebellum after blast exposure

Western blot analysis of MPO in the cerebellum showed significant increase at 6 h after blast exposure (~1.6-fold increase) (Fig. 2), confirming the results from cDNA microarray data (Table 1).

3.5. Changes in microRNA expression in the cerebellum of blast exposed mice

It has been reported earlier that the cerebellum is injured more than other brain regions after blast exposure [5,16]. MicroRNA analysis of blast exposed mice showed significant reduction in the cerebellar expression of miR132, which is reported to be involved in cholinergic anti-inflammatory signaling pathway

(Fig. 3) [20]. The expression of miR183 in the cerebellum of blast exposed mice also showed an increase which was not statistically significant. MicroRNA 134 expression showed no significant change after blast exposure. Both miR183 and 134 are involved in stress related cholinergic transmission (Fig. 3) [21]. These results indicate that blast exposure modulates microRNAs involved in both cholinergic and inflammatory pathways after blast exposure.

4. Discussion

The cholinergic anti-inflammatory pathway has been proposed as a link in neuroimmunomodulation, especially during stress conditions [8–11]. Neuroinflammation is one of the major causes for increased neuropathology and neurobehavioral changes after single or repeated exposures to blast overpressure [6,7,22–25]. Our recent studies on the brain regional specific alterations of the activity of AChE in repeated blast exposed mice prompted us to evaluate gene expression related to cholinergic and inflammatory pathways [12]. The results presented in this paper describe significant alterations in the expression of multiple receptors involved in cholinergic and inflammatory pathways after blast exposure.

The significance of cholinergic pathways (nicotinic and muscarinic) in controlling both central and peripheral inflammation has been thoroughly reviewed by Pavlov et al. [9–11]. In our experiments, we found significant down-regulation of cholinergic receptors muscarinic 2 and nicotinic alpha 7 in the midbrain of mice exposed to repeated blasts (Table 1). Previous studies indicate that acetylcholine receptors muscarinic and nicotinic alpha 7 are essential elements of the anti-inflammatory pathways [9–11]. Down-regulation of these receptors in the midbrain region after blast can induce the pro-inflammatory pathways leading to neuropathology and neurobehavioral deficits. Concurrently, GABA and glutamate receptors in the midbrain are down-regulated after repeated blast exposure. Additional studies using RT-PCR and Western blotting with specific antibodies need to be performed to assess the levels of target mRNAs and proteins after blast exposure. These results indicate the modulation of cholinergic and other neurotransmitter signaling pathways after blast exposure which can potentially play multiple roles in the neuroinflammatory process.

Pro-inflammatory cytokines and chemokines are reported to be released both centrally and peripherally after blast exposure [7,22,26]. The cDNA microarray analysis of various brain regions of repeated blast exposed mice showed significant up-regulation of multiple ILs, TNF and their receptors confirming these observations (Table 1). The pro-inflammatory marker, myeloperoxidase expression was significantly higher in the cerebellum of blast exposed mice, which was further confirmed by Western blotting

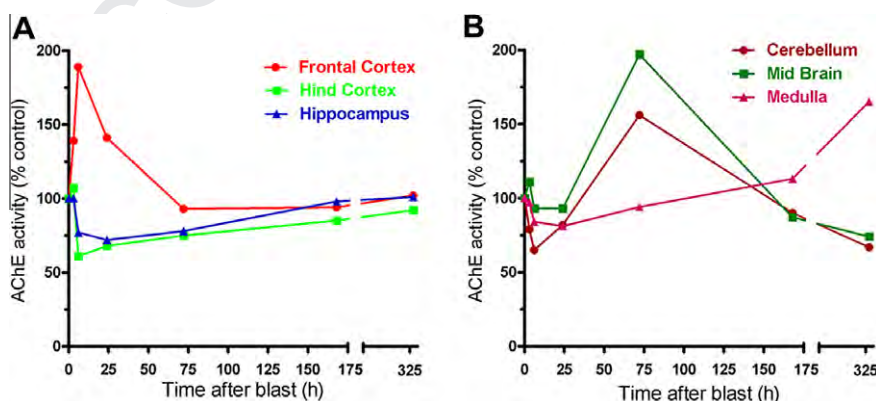
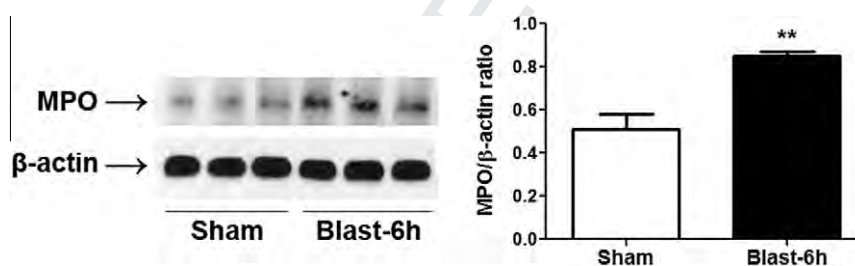


Fig. 1. Summary of AChE activity changes in various regions of the brain after blast exposure. Brain AChE activity of sham and blast exposed mice were analyzed as described in "Section 2". The values were expressed as % of sham control and plotted over time (h). Shown are the mean values from 6 different blast exposed mice.

Table 1
Changes in the expression of cholinergic and inflammatory pathways related genes in various regions of brain after repeated blast exposures.

GenBank accession#	Gene symbol	Gene product	Fold change*	Significance (p value)**
<i>Mid brain</i>				
NM_203491	Chrm2	Cholinergic receptor muscarinic 2	−2.01	0.032
NM_007390	Chrna7	Cholinergic receptor nicotinic alpha 7	−1.53	0.015
NM_008071	Gabrb3	Gammaaminobutyricacid receptor beta 3	−1.38	0.035
NM_008074	Gabrg3	Gammaaminobutyricacid receptor gamma 3	−1.43	0.034
NM_008174	Grm8	Glutamate receptor metabotropic 8	−1.54	0.026
NM_008365	Il18r1	Interleukin 8 receptor 1	−1.42	0.049
NM_009425	Tnfsf10	TNF superfamily 10	−1.65	0.035
NM_021456	Ces1	Carboxylesterase 1	+1.94	0.006
NM_031168	Il6	Interleukin 6	+1.58	0.020
NM_008374	Il9r	Interleukin 9 receptor	+1.48	0.007
NM_139299	Il31ra	Interleukin 31 receptor A	+1.65	0.035
<i>Frontal cortex</i>				
NM_008356	Il13ra2	Interleukin 13 receptor alpha 2	−2.76	0.033
NM_008354	Il12rb2	Interleukin 12 receptor beta 2	−2.47	0.043
NM_021349	Tnfrsf13b	TNF receptor superfamily 13b	−1.36	0.019
NM_134437	Il17rd	Interleukin 17 receptor D	+1.20	0.012
NM_008371	Il7	Interleukin 7	+1.48	0.047
NM_009423	Traf4	TNF receptor associated factor 4	+1.11	0.045
<i>Cerebellum</i>				
NM_177396	Il28	Interleukin 28	−1.54	0.004
NM_010824	Mpo	Myeloperoxidase	+1.56	0.019
NM_011614	Tnfsf12	TNF superfamily 12	+1.95	0.038
NM_178931	Tnfrsf14	TNF receptor superfamily 14	+1.82	0.016
NM_175649	Tnfrsf26	TNF receptor superfamily 26	+1.53	0.036
<i>Hippocampus</i>				
NM_008366	Il2	Interleukin 2	−1.29	0.030
NM_028075	Tnfrsf13c	TNF receptor superfamily 13c	+1.64	0.021

* Average values from 3 different animals ($n = 3$ for sham and blast).** Significance was calculated using Welch's *t*-test analysis followed by the Benjamini–Hochberg multiple correction test.**Fig. 2.** Western blot analysis of MPO expression in the cerebellum after blast exposure. Cerebellar regions were collected from sham and repeated blast exposed animals at 6 h time point and the total protein was extracted, separated on 4–12% Tris–Bis gels and immunoblotted for MPO expression as described in “Section 2”. Blots were re-probed with β -actin antibody and quantified using ImageJ software. Shown are the mean $\pm$ SEM values of densitometric measurements of Western blot data. $n = 3$ for sham and blast; and (**) $p < 0.01$.

(Table 1 and Fig. 2) [6]. More studies are required to understand the differential effects of blast exposure on gene expression in various regions of the brain. Initial studies indicate that frontal cortex and cerebellum are the two brain regions that may be affected more by blast exposure [3,16]. Also, it is possible that acute effects of blast may be different than chronic effects in various regions of the brain. In addition to blast shock wave (primary blast injury) there can be also a component of acceleration mediated injury (tertiary blast injury) [27]. Thus, blast exposure using shock tube may cause differential effects and the brain damage may not be uniform.

MicroRNAs have been studied as regulators of cholinergic anti-inflammatory signaling pathways [20,21]. MicroRNA 132 is reported to attenuate the inflammatory process by targeting AChE activity [20]. Significantly decreased expression of miR132 in the cerebellum of blast exposed mice indicates augmentation of pro-inflammatory signaling cascades probably through cholinergic pathways (Fig. 3). There are multiple splice variants of AChE (AChE-S, -R, and -E), among which AChE-R (R stands for ‘read-

through’) is usually generated during stress conditions [28]. Similarly, stress is also reported to up-regulate the expression of splicing factor SC35 which is involved in the formation of AChE-R from AChE-S (S stands for ‘synaptic’) [21,29]. In our studies, no significant changes in the expression of miR134 and 183 were observed after blast exposure. Both the microRNAs 134 and 183 are known to share a common predicted mRNA target encoding the splicing factor SC35 [21,29]. If there was significant modulation of these microRNAs after blast exposure, it would support the previously reported up-regulation of miR183 and 134 during acute stress targeting two different transcription factors (ZPFM2 and CBFA2T1), which are involved in oxidative stress, inflammation and neuropathology [21,30]. More studies are required to conclude whether neuroimmunomodulatory miRNAs, ‘neurimmirs’ can be eventually used for potential therapy of blast-induced traumatic brain injury [31].

In summary, our results indicate that acute changes in the expression of genes related to cholinergic and inflammatory pathways in various regions of the brain can play significant roles in the

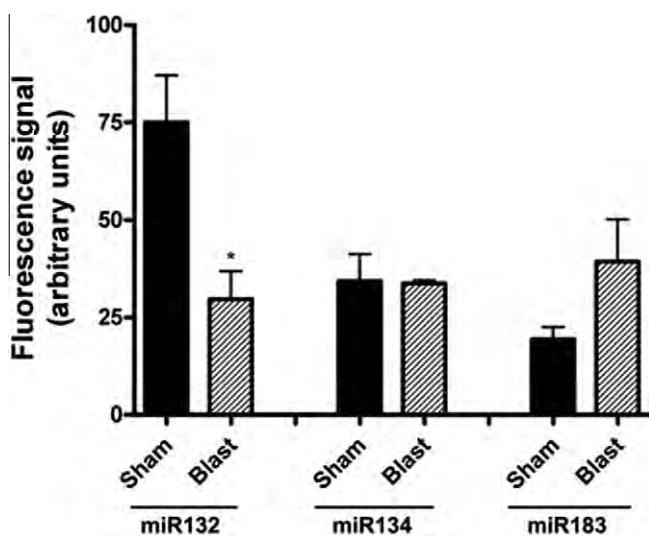


Fig. 3. MicroRNA expression profile in the cerebellum of blast exposed mice. Animals were exposed to 20.6 psi blast overpressure, at 6 h time point the cerebellum was collected by necropsy and the tissue samples were for processed for microRNA analysis as described in "Section 2". Shown are the mean $\pm$ SEM values of fluorescence intensity expressed in arbitrary units. $n = 3$ for sham and blast; and (*) $0.05 < p < 0.01$.

development of chronic neuropathology and neurobehavioral effects after blast exposure. Additionally, modulation of stress and inflammation related microRNAs also play role in blast induced TBI. Targeted therapies focused on cholinergic pathways and microRNA regulation can be feasible approaches to combat against chronic pathophysiological changes after blast exposure.

Conflicts of interest

There is no potential conflict of interest related to this manuscript.

Disclosure

The opinions and assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Army, the Navy, or the Department of Defense, USA.

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Modulation of hearing related proteins in the brain and inner ear following repeated blast exposures

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Abstract: Emerging studies show that blast exposure causes traumatic brain injury (TBI) and auditory dysfunction without rupture of tympanic membrane suggesting central auditory processing impairment after blast exposure. There is limited information on the mechanisms of blast-induced TBI and associated peripheral and central auditory processing impairments. We utilized a repetitive blast exposure mouse model to unravel the mechanisms of blast TBI and auditory impairment. C57BL/6J mice were exposed to three repeated blasts (20.6 psi) using a shock tube, and the cerebellum was subjected to proteomic analysis. The data showed that calretinin and parvalbumin, two major calcium buffering proteins, were significantly up-regulated after repeated blast exposures, and this was confirmed by Western blotting. Since these proteins are reportedly involved in auditory dysfunction, we examined the inner ear and found both calretinin and parvalbumin were up-regulated, suggesting that modulation of these proteins plays a role in blast-induced peripheral and central auditory processing impairments. Expression of cleaved caspase-3 was also up-regulated in both regions indicating ongoing cellular apoptosis, possibly due to altered calcium homeostasis. These results provide a molecular basis for changes in central and peripheral auditory processing involving abnormal calcium homeostasis resulting in hearing impairment after blast exposure.

Keywords: blast exposure, calcium signaling, calretinin, cerebellum, inner ear, NMDA receptor, parvalbumin, traumatic brain injury

Introduction

Hearing impairment and tinnitus are the most widespread dysfunctions associated with traumatic brain injury (TBI) in the current wars [1]. Blast injury produces up to 60% hearing loss compared to non-blast related TBI [2]. It is also one of the most frequent occupational disorders in the United States which is linked to occupational and recreational high intensity noises [3]. In a minor scale, blast exposure includes natural gas explosions, industrial accidents, fireworks, mining and building constructions, and demolitions and homemade bombs [4]. The symptoms of auditory impairment present particular challenges for blast research community due to possible overlapping with post-traumatic stress disorder, mental illness and cognitive deficits, where apparent hearing loss may arise from different underlying psycho-traumatic mechanisms [1, 5]. There are limited approaches to properly assess the severity of central auditory processing impairment after blast exposure, when the tympanic membrane is intact, especially in animal models. Accurate differentiation of auditory impair-

ments from TBI related psychiatric symptoms using specific biomarkers and development of effective treatment strategies require an understanding of the molecular basis of abnormalities in auditory signal processing and perception of sound by the brain after blast exposure.

The incidence of central auditory processing damage in service members exposed to blast in the current war is unknown. Anecdotal evidence from United States Department of Veterans Affairs suggests that significant number of blast victims maintain hearing sensitivity but have difficulties in hearing noise owing to central auditory processing damage [1]. Blast exposure damages the central auditory processing involved in auditory patterns essential for speech perception and sound localization (<http://asha.org/policy/>) [1]. Blast shockwave transmission through the skull and reflection in the brain can lead to shearing and stretching resulting in axonal and microcapillary injuries, and subsequent disruption of signal inputs to auditory processing brain stem nuclei [1]. It has been shown that blast exposure damages auditory processing regions in the brain such as inferior colliculus and medial geniculate body, suggesting that

blast exposure causes significant effects on the auditory system through the auditory pathway in addition to the possible direct impact on the brain parenchyma through the skull [6]. Detailed investigations on central auditory processing impairments after blast exposure are required to locate the damages in the brain and develop novel tools for clinical diagnosis, prevention, and effective treatments for rehabilitation.

There are limited studies on the pathophysiology of auditory dysfunction following repeated blast exposures. It has been reported that repeated blast exposure causes more severe auditory impairment than a single high level exposure [1]. Repeated blast exposure also caused more severe brain injury especially in the cerebellum [7]. Repeated blast exposure studies using sub-lethal blast levels showed decreased threshold for auditory dysfunction [8].

We had postulated potential involvement of shock-wave/impulse noise transmission through the auditory/vestibular system in the etiology of blast-induced TBI and associated central auditory/vestibular injuries. In this paper, we utilized the mouse model of repetitive blast exposure with shock tube and studied differentially expressed proteins after blast exposure in the cerebellum and inner ear. We demonstrate that blast exposure leads to significant changes in the brain and inner ear levels of proteins involved in auditory function and apoptosis and proposed a potential mechanism of blast-induced central and peripheral auditory processing defect.

Materials and Methods

Animals and blast injury model

Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals. It adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, National Research Council, published by the National Academy Press, 1996, and the Animal Welfare Act of 1966, as amended. Male C57BL/6J mice (8–10 weeks old, 21–26 g) obtained from Jackson Laboratory (Bar Harbor, ME) were used in this study, since the blast TBI model was developed using the same species and the possibility of utilizing genetic knockout/transgenic technologies in the future. Moreover, the same species has been widely used for studying auditory impairment. The animals were housed on a 12-h/12-h light-dark cycle and were provided standard mice chow and water *ad libitum*. All the mice were used in accordance with an experimental protocol that was approved by the Institute Animal Care and Use Committee, Walter Reed Army Institute of Research, and all the experiments were conducted in Association for Assessment and Accreditation of Laboratory Animal Care approved laboratories.

Blast exposure

A well-characterized blast overpressure exposure using a compressed air-driven shock tube described earlier was used for the study [7, 9]. Animals were subjected to repeated blast exposures as described earlier [7]. Briefly, after anesthetizing with 4% isoflurane gas (O₂ flow rate 2 L/min) for 8 min, mice were placed 2.5 feet inside the shock tube in a prone position perpendicular to the direction of shockwaves. The animals were exposed to blast overpressure (20.6 psi) twice with 1 min interval between each blast followed by a third blast exposure at 30 min after the second blast [7]. The blast overpressure of 20.6 psi was selected from earlier studies that showed significant injury with low mortality [7]. Repeated blasts were used to mimic multiple blast exposures in the battlefield. Sham controls received anesthesia but were not exposed to blast. The animals were sacrificed at 6 and 24 h after the third blast exposure. Brain tissues were removed and cerebellum was initially separated for analysis. The inner ear/cochlea was dissected using a dissection microscope and frozen immediately.

Extraction of proteins

Proteins were isolated from the cerebellum of sham control and repeated blast exposed mice (three animals/group) using the ToPI-DIGE™ total protein isolation kit (ITSI-Biosciences, Johnstown, PA). Briefly, the tissue was rapidly homogenized in 50 µL of ToPI Buffer-2 (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% NP-40, 5 mM magnesium acetate, 30 mM Tris-HCl, pH 8.5) using clean disposable plastic pestles supplied with the kit. After homogenization, samples were incubated on ice for 30 min, with four intermittent vortex mixings, and centrifuged at 16,000 *g* for 10 min. Supernatant was collected and the total protein concentration was determined using the ToPA™ protein assay kit (ITSI-Biosciences, PA) according to the manufacturer's instructions.

Two-dimensional differential in-gel electrophoresis (2D-DIGE)

For 2D-DIGE, 50 µg each of total protein was labeled with 200 pmol of Cy3 or Cy5, and Cy2 labeling was used as internal standard using the 'minimal labeling' protocol [10]. The Cy2, and Cy3/Cy5 labeled samples were mixed and co-separated by isoelectric focusing (IEF) with pH 3–10 linear Immobiline Drystrips (GE Healthcare) in the first dimension. IEF was for a total of 65,500 V h in an IPGphor electrophoresis unit (GE Healthcare). The focused strips were equilibrated for 15 min in sodium dodecyl sulfate (SDS) equilibration buffer containing 1% dithiothreitol followed by a second 15-min equilibration

in SDS equilibration buffer containing 2.5% iodoacetamide. The strips were then placed on 24×20 cm, 12.5% SDS-polyacrylamide gels and electrophoresed in an Ettan DALT6 (GE Healthcare) at 15 W per gel for about 4.5 h.

Image analysis

After the second dimension electrophoresis, all the gels were scanned on a DIGE-enabled Typhoon Trio Variable Mode Digital Imager (GE Healthcare, Piscataway, NJ) using the following excitation/emission wavelengths: Cy2, 488/520 nm; Cy3, 532/580 nm; and Cy5, 633/670 nm. All the images generated (three per gel) were imported into the Biological Variation Analysis module of DeCyder™ software (Version 6.5, GE Healthcare, Piscataway, NJ) for matching, normalization, and identification of differentially abundant spots, with the False Discovery algorithm enabled. The images obtained from sham control samples were compared to the images of the corresponding repeated blast exposed samples to identify the protein spots that showed ≥two-fold difference in abundance.

Identification of differentially expressed proteins by liquid chromatography/tandem mass spectrometry (LC/MS/MS)

The candidate spots were picked with the Ettan Spot Picker (GE Healthcare, Piscataway, NJ) and in-gel digested overnight with trypsin using the Ettan Spot Digester (GE Healthcare, Piscataway, NJ). The in-gel digested samples were extracted in 50 µL of 50% acetonitrile/0.1% formic acid for 20 min, dried down completely at 45 °C, and sequenced by LC/MS/MS using a nanobore electrospray column constructed from 360 mm outside diameter and 75 mm inside diameter fused silica capillary with the column tip tapered to a 15-µm opening. The column was packed with 200-Å, 5-µm C₁₈ beads (Michrom BioResources, Auburn, CA) to a length of 10 cm. The mobile phase used for gradient elution consisted of (a) 0.3% acetic acid, 99.7% water, and (b) 0.3% acetic acid, 99.7% acetonitrile at a flow rate of 350 nL/min. All tandem mass spectra were acquired in a Thermo LTQ ion trap mass spectrometer (Thermo Corp., San Jose, CA) with the needle voltage set at 3 kV. The MS/MS spectra were searched against the NCBI non-redundant protein sequence database using the SEQUEST computer algorithm to establish the protein identity.

Western blot analysis

Polyclonal rabbit antibodies against calretinin and parvalbumin were obtained from Sigma-Aldrich (St. Louis, MO), and rabbit polyclonal antibodies against cleaved caspase-3 was obtained from Chemicon International (Billerica, MA). Secondary antibody labeled with horseradish peroxidase (HRP) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibody to β-actin conjugated with HRP (Sigma-Aldrich) was used as gel loading control. Western blot analysis was performed using tissue homogenates of inner ear and cerebellum using tissue protein extraction reagent (Pierce Chemical Co, Rockford, IL). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 30 µg total protein with precast 10% Tris-glycine gels (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After electrophoresis, the proteins were transferred to polyvinylidene difluoride membrane (GE Healthcare, Piscataway, NJ) using Novex transfer apparatus (Invitrogen, Carlsbad, CA). The membrane was blocked with blocking buffer [4% powdered milk made in phosphate-buffered saline containing 0.001% Tween-20 (PBST)] for 1 h, washed once with PBST buffer, and kept overnight at 4 °C in primary antibody made in blocking buffer. Calretinin, cleaved caspase-3, and parvalbumin antibodies were used at a dilution of 1:1000, and β-actin at a dilution of 1:50,000. The membranes were washed five times with PBST and incubated with secondary antibody made in blocking buffer for 1 h. Secondary antibody was not used in the case of β-actin. The membranes were washed again with TBST, the protein bands were detected using ECL-Plus Western blot detecting reagent (GE Healthcare, Piscataway, NJ), and the chemiluminescence was measured in an AlphaImage reader (Cell Biosciences, Santa Clara, CA).

Results

Effect of blast exposure on the expression of calretinin and parvalbumin

Figure 1 shows representative 2D-DIGE images depicting differential expression of calretinin and parvalbumin at 6 h in the cerebellum after repeated blast exposures. Protein identification by LC/MS/MS showed six matching peptides of calretinin from the protein spot labeled as calretinin. In the case of protein spot labeled as parvalbumin, only one peptide sequence correspond-

ing to parvalbumin was obtained possibly due to the low abundance of the protein in the cerebellum. Both proteins were consistently up-regulated after the repeated blast exposures with calretinin showing the highest increase compared to parvalbumin (Fig. 1).

Confirmation of differential expression of calretinin and parvalbumin by Western blotting

Western blotting of cerebellar proteins of sham control and repeated blast exposed mice using calretinin and parvalbumin specific antibodies is shown in Fig. 2. Western blotting confirmed the proteomic analysis data showing the up-regulation of both calretinin and parvalbumin after repeated blast exposures. Both proteins showed increased expression at 24 h compared to 6 h post-blast exposures.

Expression of calretinin and parvalbumin in the inner ear

Figure 3 shows the expression of calretinin and parvalbumin in the inner ear of mice at different intervals after repeated blast exposures. The level of both calcium buffering proteins increased in the inner ear after blast exposure. The up-regulation of calcium buffering proteins after blast exposure was significantly higher in the inner ear compared to cerebellum (Figs 2 and 3). Calretinin showed higher expression at 24 h whereas parvalbumin showed higher expression at 6 h post-blast exposures.

Expression of cleaved caspase-3

Expression of caspase-3 in the cerebellum and inner ear showed significant increase at 6 and 24 h post blast (Figs 2 and 3). The increased expression was similar at both the time points after blast exposure.

Discussion

Our studies by proteomic and Western blot analyses indicate that repeated blast exposures in mice results in alteration in multiple proteins in the brain which are reported to be associated with hearing impairment. Calretinin and parvalbumin, the calcium binding proteins which are found to be up-regulated in the mouse brain cerebellum after repeated blast exposures, are the major calcium buffering proteins present in the auditory system including auditory neurons [11–13]. Evidences indicate that calcium binding proteins play major roles in central auditory processing [14, 15], and our data suggest that blast exposure results in central auditory processing

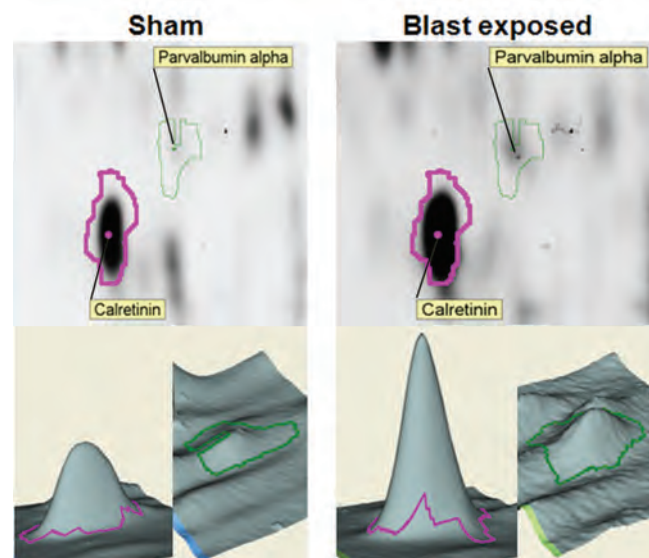


Fig. 1. Proteomic analysis of sham and repeated blast exposed mouse cerebellum samples using 2D-DIGE followed by densitometry and mass spectroscopy. Representative picture (from three different animals) showing the differential expression of calretinin and parvalbumin in the mouse brain cerebellum after repeated blast exposures

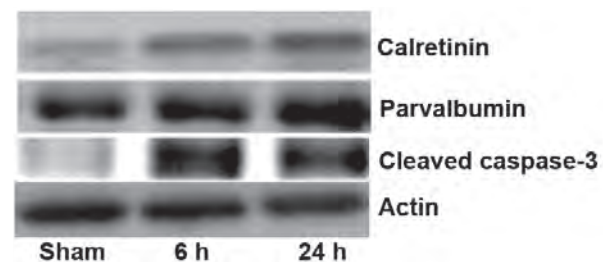


Fig. 2. Western blotting analysis of sham and repeated blast exposed mouse cerebellum samples. Representative picture from three animals in each group. Cerebellum extracts were subjected to Western blotting using specific antibodies to confirm the modulation and identity of calretinin and parvalbumin after repeated blast exposures. Modulation of cleaved caspase-3 was used as a marker of ongoing apoptosis. Western blotting with β -actin is used as loading control

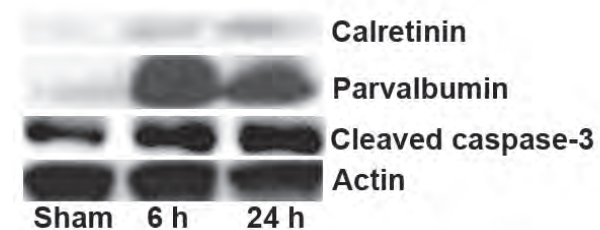


Fig. 3. Western blotting analysis of sham and repeated blast exposed mouse inner ear samples. Representative picture from three animals in each group. Inner ear extracts were subjected to Western blotting using specific antibodies to determine the expression of calretinin and parvalbumin and cleaved caspase-3 after repeated blast exposures. Western blotting with β -actin is used as loading control

signaling abnormalities. In the cochlear nucleus, they function more specifically in hearing function rather than mere buffering of intracellular calcium fluctuations during signaling. Calretinin and parvalbumin modulation in the brain including cochlear nucleus has been reported to be associated with hearing impairment [16, 17]. Based on the biological functions of the proteins, we further studied the expression of the proteins at the peripheral auditory region and observed their differential expression after blast exposure.

The cerebellum is considered as one of the key regions in the brain involved in auditory signal processing and sound perception [18]. The role of cerebellum in auditory signal processing was first observed when studies showed that the cerebellum of cats was found to receive auditory signal senses and transmit them to the cortical auditory pathways [19]. Later, multiple studies confirmed the role of cerebellum in auditory signal processing and determined different cerebellar auditory processing areas and their connections to the central and peripheral auditory system [20–23]. Thus, our results involving the cerebellar modulation of proteins involved in auditory function indicate the possible involvement of auditory neurons of cerebellum in the pathogenesis/protection of hearing impairment and tinnitus after blast exposure. Cerebellar re-

gion was investigated first mainly due to the pronounced effects of blast exposures in the cerebellum compared to other brain regions [7, 24–28]. It is quite likely that similar changes can be observed in other regions of the brain involved in auditory signal processing after repeated blast exposures which needs to be investigated further.

Noise exposure in mice resulted in the up-regulation of calretinin and parvalbumin in the cochlear nucleus in a noise intensity dependent manner, suggesting a possible protective role of the calcium binding proteins in the brain stem after noise exposure [16]. In addition to cochlear nucleus, noise stimulation also leads to up-regulation of these proteins in other regions of the brain including dorsal cortex, inferior colliculus, and commissural nucleus [29]. No studies so far reported the increased expression of calretinin and parvalbumin in the inner ear and/or brain after blast exposure. Our results for the first time indicate a possible role of these proteins in the development/prevention of hearing impairment and tinnitus commonly seen in service members returning from the battlefield.

It has been demonstrated that 24 h after cochlear ablation, a significant increase in calretinin immunoreactivity was observed in the superior and inferior colliculus of adult ferrets, indicating that cochlear-driven activity

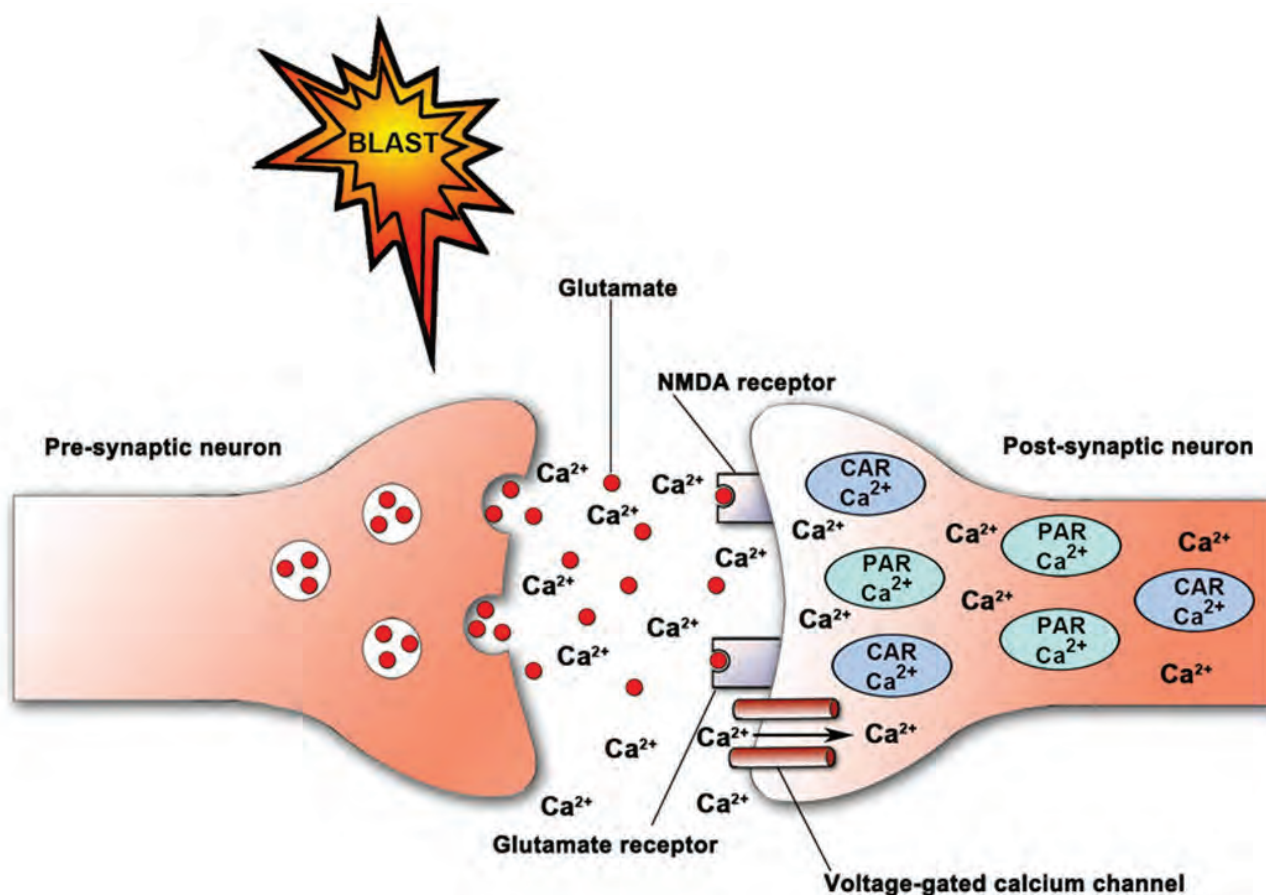


Fig. 4. Schematic representation of the potential mechanism of auditory injury and the role of up-regulated calretinin (CAR) and parvalbumin (PAR) in the brain after blast exposure

appears to affect calcium binding protein levels not only in auditory nuclei but also in other neural structures whose response properties may be influenced by auditory-related activities [17, 30]. The expression of calretinin and parvalbumin was found to be up-regulated in the cochlear nucleus with aging and/or associated hearing impairment, suggesting the role of these proteins in age-related auditory dysfunction [31]. A recent study showed that the expression of calcium buffering proteins including calretinin and parvalbumin decreased significantly in the hippocampus of circling mouse, a murine model of deafness, and suggested that the decreased expression could be related to the loss of auditory information modulating processes in various hippocampal areas [32].

As shown in *Fig. 4*, blast and impulse noise exposure through the auditory system can cause long-lasting depolarization of auditory neurons and release of glutamate leading to glutamate excitotoxicity resulting in calcium influx via voltage-gated calcium channels. Such a potential mechanism for auditory dysfunction has been reported in the case of chemical and noise-induced hearing impairment [33–35]. Thus, therapeutics which can counteract glutamate excitotoxicity such as N-methyl-D-aspartic acid (NMDA) receptor antagonists could be used as potential treatments against blast-induced hearing impairment. NMDA receptor antagonists have been found to be effective for protection against noise-induced hearing impairment [36–38]. Voltage-gated calcium channels are reported to be involved in the pathogenesis of acoustic injury in the cochlea [36, 39]. Thus, voltage-gated calcium channel blockers can reduce calcium influx and subsequent damage to the auditory neurons after blast exposure. The up-regulation of calretinin and parvalbumin in the inner ear and cerebellum after repeated blast exposures could be due to the very high demand of calcium buffering in the auditory system. It is quite likely that, in the auditory neurons, the up-regulated calretinin and parvalbumin will bind to the free calcium ions entering through the calcium channels after blast exposure and protect against calcium-induced cell death (*Fig. 4*). Such a protective role for these calcium binding proteins in auditory neurons has been proposed earlier [29, 40].

In addition to different brain regions involved in central auditory processing system, the inner ear/cochlea involved in peripheral auditory system also significantly expresses the calcium binding proteins suggesting their roles in peripheral auditory processing [13, 41]. Our present data on the up-regulation of calretinin and parvalbumin in the inner ear after repeated blast exposures suggest similar roles for these calcium buffering proteins in the peripheral auditory processing. The up-regulation of calretinin and parvalbumin was highest in the inner ear compared to cerebellum suggesting significant alteration in calcium homeostasis in the peripheral auditory system compared to the central auditory processing regions after blast exposure.

Since altered calcium homeostasis is associated with cellular apoptosis, we determined the expression of cleaved caspase-3 in the cerebellum and inner ear, and our results indicate ongoing cellular apoptosis in both regions. Previous studies have shown increased cellular apoptosis in the brain at different intervals after blast exposure [7, 42]. Cellular apoptosis in the cochlea has been reported as a mechanism involved in noise-induced hearing loss [43]. Thus, increased cellular apoptosis in the central and peripheral auditory processing regions could be a molecular mechanism of hearing impairment associated with blast exposure.

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Disclaimer

The contents, opinions, and assertions contained herein are private views of the authors and are not to be construed as official or reflecting the views of the Department of the Army or the Department of Defense. The authors report no conflict of interest.

Abbreviations

DIGE: differential in-gel electrophoresis; NMDA: N-methyl-D-aspartic acid; SDS: sodium dodecyl sulfate; TBI: traumatic brain injury

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Preliminary studies on differential expression of auditory functional genes in the brain after repeated blast exposures

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Abstract—The mechanisms of central auditory processing involved in auditory/vestibular injuries and subsequent tinnitus and hearing loss in Active Duty servicemembers exposed to blast are not currently known. We analyzed the expression of hearing-related genes in different regions of the brain 6 h after repeated blast exposures in mice. Preliminary data showed that the expression of the deafness-related genes *otoferlin* and *otoancorin* was significantly changed in the hippocampus after blast exposures. Differential expression of *cadherin* and *protocadherin* genes, which are involved in hearing impairment, was observed in the hippocampus, cerebellum, frontal cortex, and midbrain after repeated blasts. A series of calcium-signaling genes that are known to be involved in auditory signal processing were also found to be significantly altered after repeated blast exposures. The hippocampus and midbrain showed significant increase in the gene expression of hearing loss-related antioxidant enzymes. Histopathology of the auditory cortex showed more significant injury in the inner layer compared to the outer layer. In summary, mice exposed to repeated blasts showed injury to the auditory cortex and significant alterations in multiple genes in the brain known to be involved in age- or noise-induced hearing impairment.

Key words: auditory functional genes, auditory process, blast injury, *cadherin*, hearing loss, neurotrauma, *otoancorin*, *otoferlin*, *protocadherin*, tinnitus.

INTRODUCTION

Battlefield blast exposure is reported to cause auditory impairment in a large population of military personnel deployed to Iraq and Afghanistan [1–2]. Auditory/vestibular injuries from blast traumatic brain injury (TBI) can cause increased incidence of tinnitus and hearing loss, which worsens over time if not treated [1–4]. Shock waves generated from explosive blasts are reported to be destructive to both gas- and fluid-filled structures of the body, including the lungs, intestines, brain, eyes, nose, and middle ear [5–9]. Blast-induced damage to the auditory system can be the consequence of either direct exposure of the auditory canal to blast shock waves or TBI and impairment in the central auditory processing involving different brain regions after blast exposure. The literature on the neurobiological mechanisms of hearing impairment and development of tinnitus from blast TBI is limited.

Abbreviations: cDNA = complementary DNA, TBI = traumatic brain injury.

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A number of genes and their protein products have been reported to be involved in both age- and noise-related hearing loss [10–15]. Cadherin and protocadherin mutations were linked to digenic inheritance of deafness and have specific functional roles in noise-induced hearing loss [13–14,16–17]. Other groups of proteins involved in deafness are otoferlin and otoancorin, which are also reported to have major roles in auditory functions, including central auditory processing [18–21]. Another large class of molecules involved in auditory signaling is centered on the calcium regulating proteins, which are known to have broad functions in age- and noise-related hearing loss or protection [18,22–26]. The significance of reactive oxygen species and heat shock proteins in age- and noise-related auditory impairments are also reviewed in detail [10,27–34].

Recent research on age- or noise-related hearing loss preferred mice as a suitable animal model because of the vulnerability of mice to sound compared to other rodents [15,35]. We have developed a preclinical mouse model of repeated blast exposures using an air-blast shock tube that closely mimics the repeated exposure to improvised explosive devices, grenades, or firing weapons used in the battlefield or breacher's studies [36–37]. The newly developed repetitive blast animal TBI model showed significant levels of neuropathology and neurobehavioral deficits after repeated blast exposures at 20.6 psi [36]. Using this mouse model of repeated blast exposures, we sought to determine differential expression of auditory-related genes in various regions of the brain by complementary DNA (cDNA) microarray analysis.

METHODS

Animal Blast Exposure Model

Experiments were performed in male mice (C57BL/6J, age 8–10 weeks, Jackson Laboratory; Bar Harbor, Maine). Groups of isoflurane (4%) anesthetized animals ($n = 6$ for sham and $n = 6$ for blast) were exposed to repeated blast exposures (20.6 psi), as reported previously, using a shock tube [9,36,38–39]. At a 6 h time point after the last blast exposure, three animals each from sham and blast groups were euthanized, and the brain tissue was collected after necropsy and separated into various regions as described earlier [39]. Different regions of the brain samples were immediately snap frozen and stored at -80°C until use. Remaining animals ($n = 3$) in each group

were sacrificed at 24 h after the last blast exposure and used for histopathology.

Preparation of RNA

Total RNA was isolated using Trizol reagent (Invitrogen Life Technology; Carlsbad, California) following the manufacturer's protocol. RNA quality and quantity were determined by using an Agilent 2100 Bioanalyzer (Agilent Technologies; Santa Clara, California).

cDNA Microarray Analysis

Microarray analysis was performed using Agilent 60-mer mouse genome 44K oligo microarrays (Agilent Technologies). We labeled 5 μg of purified RNA with a commercially available kit (Agilent Low Input Quick Amp) by polymerase chain reaction amplification (Bio-Rad Laboratories; Hercules, California). Samples were fragmented and hybridized against universal mouse reference RNA (Stratagene; La Jolla, California) with a kit from Agilent. A 2-Color Microarray-Based Gene Expression Analysis (version 6.5) protocol was used for labeling and microarray processing. An Agilent G2565CA fluorescence scanner was used to quantitate the slides, and the resultant data were extracted using software (Agilent Feature Extraction, version 10.7.1). For filtering and normalization of the data, GeneSpring 10.1 software (Agilent) was used.

Statistical Analysis

The statistical analysis of the microarray data was performed with GeneSpring 10.1 software. Changes in the level of expression of various genes after blast exposure in comparison to sham controls were identified by Welch's *t*-test statistical method (p -values < 0.05) in conjunction with multiple correction test (Benjamini-Hochberg) with 5 percent false discovery rate. To account for the small sample size, we used the reference design and filtered for genes with signal intensities that are twice the standard deviation of the background intensity levels. We determined that by performing gene-by-gene *t*-tests, for a samples size of 3 and 5 percent false discovery rate and a standard deviation of 0.5, the power is 75 percent. We also applied pathway and gene ontology analyses that offer extra power because it is statistically unlikely that a larger fraction of false positive genes end up in one specific pathway.

Histopathology of Auditory Cortex

Histopathology was performed in blast-exposed and sham control mice ($n = 3$ in each group), as described previously [36]. Brain sections were silver stained and microscopically examined for neurodegeneration exclusively in the auditory cortex region, and the severity of injury was scored as mild (+), moderate (++) and severe (+++).

RESULTS

Expression of Auditory-Related Genes in Hippocampus After Repeated Blast Exposures

The hippocampus of mice exposed to repeated blasts showed significant changes in the expression of multiple genes that are reported to be involved in age- or noise-induced hearing loss (Table 1). Otoancorin, a gene defective in autosomal recessive deafness, showed a significant increase (3.4-fold), while otoferlin, which is essential for glutamate exocytosis at the auditory ribbon synapse, showed a 1.8-fold decrease in the expression after repeated blast exposures. The expression of calcium binding protein 2 showed a 1.6-fold increase, whereas calcitonin-related polypeptide expression showed a 1.9-fold decrease after

blast exposures. The expression of antioxidant enzyme superoxide dismutase 3 showed a 2.0-fold increase in the hippocampus of mice exposed to repeated blasts. The expression of heat shock protein 8 and heat shock transcription factor 5 showed significant increase in the hippocampus after repeated blast exposures. Protocadherin alpha 4 expression showed a 1.3-fold decrease after blast exposures.

Expression of Auditory-Related Genes in Cerebellum After Repeated Blast Exposures

The cerebellum of mice exposed to repeated blasts showed a 1.2-fold increase in protocadherins alpha 4 and beta 20 expression (Table 2). The expression of S100 calcium binding protein A7A showed a 1.4-fold increase, while multiple calcium channel proteins and calcium binding protein 2 expression showed a 1.1 to 1.2-fold decrease in the cerebellum after repeated blast exposures. Heat shock protein 8 expression also showed a 1.1-fold decrease after blast exposures.

Expression Profile of Auditory-Related Genes in Frontal Cortex After Repeated Blast Exposures

The expression of calcium signaling-related molecules showed significant increase in the frontal cortex of mice

Table 1.

List of auditory-related genes significantly altered in hippocampus after repeated blast exposures in mice.

Gene Symbol	GenBank Accession No.	Gene Product	Fold Change	p-Value
Otoa	NM_139310	Otoancorin	+3.4	0.04
Cabp2	NM_013878	Calcium binding protein 2	+1.6	0.003
Sod3	NM_011435	Superoxide dismutase 3	+2.0	0.03
Hspb8	NM_030704	Heat shock protein 8	+1.3	0.02
Hsf5	NM_001045527	Heat shock transcription factor member 5	+1.8	0.02
Otof	NM_031875	Otoferlin	-1.8	0.04
Calca	NM_007587	Calcitonin/calcitonin-related polypeptide alpha	-1.9	0.04
Pcdha4	NM_007766	Protocadherin alpha 4	-1.3	0.001

Table 2.

List of auditory-related genes significantly altered in cerebellum after repeated blast exposures in mice.

Gene Symbol	GenBank Accession No.	Gene Product	Fold Change	p-Value
Pcdhb20	NM_053145	Protocadherin beta 20	+1.2	0.03
Pcdha4	NM_007766	Protocadherin alpha 4	+1.2	0.03
S100a7a	NM_199422	S100 calcium binding protein A7A	+1.4	0.03
Cacng1	NM_007582	Calcium channel, voltage-dependent, gamma subunit 1	-1.2	0.003
Cacna2d1	NM_009784	Calcium channel, alpha 2, delta subunit 1	-1.1	0.049
Efcab2	NM_026626	EF-hand calcium binding domain 2	-1.1	0.01
LOC641192	XM_918536	Similar to heat shock protein 8	-1.1	0.046

exposed to repeated blasts, including calpain 3 (1.5-fold), S100 calcium binding protein A3 (1.4-fold), calcium/calmodulin-dependent protein kinase kinase 1 (1.2-fold), and calcium binding domain 4A alpha polypeptide 7 (1.4-fold) (**Table 3**). Protocadherin beta 11 and calreticulin expression showed significant decrease (2.2- and 1.2-fold, respectively) in the frontal cortex of mice exposed to repeated blasts.

Expression of Auditory-Related Genes in Midbrain After Repeated Blast Exposures

The changes in the expression of auditory-related genes in the midbrain of repeated blast-exposed mice are shown in **Table 4**. Expression of cadherin-like 24 showed a 1.8-fold increase, while expression of cadherin 12 and protocadherin 8 showed significant decrease (1.7- and 1.4-fold, respectively) after the blast exposures. Multiple calcium signaling molecules, including calpain 9 (2.1-fold), S100

calcium binding protein A3 (1.2-fold), and calcium activated potassium channel beta 3 (2.1-fold), showed significantly increased expression in the midbrain after repeated blast exposures. At the same time, the expression of calcium binding protein 7 (2.2-fold), calcium channel voltage dependent L type alpha 1D subunit (1.6-fold), and calcium/calmodulin-dependent protein kinase 2 gamma (1.1-fold) showed significant decrease after repeated blast exposures. The midbrain of repeated blast-exposed mice also showed significant decrease in the expression of heat shock protein 2 (1.3-fold), nicotinic alpha polypeptide 7 cholinergic receptor (1.5-fold), and stanniocalcin 2 (1.3-fold).

Histopathology of Auditory Cortex After Repeated Blast Exposures

To investigate whether blast exposure induces pathology of the auditory cortex, neuropathology analysis of the

Table 3.

List of auditory-related genes significantly altered in frontal cortex after repeated blast exposures in mice.

Gene Symbol	GenBank Accession No.	Gene Product	Fold Change	p-Value
Capn3	NM_007601	Calpain 3	+1.5	0.03
S100a3	NM_011310	S100 calcium binding protein A3	+1.4	0.03
Camkk1	NM_018883	Calcium/calmodulin-dependent protein kinase kinase 1	+1.2	0.01
Efcab4a	NM_001025103	EF-hand calcium binding domain 4A alpha polypeptide 7	+1.4	0.04
Pcdhb11	NM_053136	Protocadherin beta 11	-2.2	0.004
Calr	NM_00759	Calreticulin	-1.2	0.04

Table 4.

List of auditory-related genes significantly altered in midbrain after repeated blast exposures in mice.

Gene Symbol	GenBank Accession No.	Gene Product	Fold Change	p-Value
Cdh24	NM_199470	Cadherin-like 24	+1.8	0.02
Capn9	NM_023709	Calpain 9	+2.1	0.03
S100a3	NM_011310	S100 calcium binding protein A3	+1.2	0.048
Gpx4	NM_008162	Glutathione peroxidase 4	+1.1	0.02
Ccs	NM_016892	Copper chaperone for superoxide dismutase	+1.1	0.03
Kcnmb3	NM_171828	Calcium activated potassium channel beta 3	+2.1	0.03
Pcdh8	NM_021543	Protocadherin 8	-1.4	0.02
Cadh12	NM_001008420	Cadherin 12	-1.7	0.01
Cabp7	NM_138948	Calcium binding protein 7	-2.2	0.04
Cacna1d	NM_028981	Calcium channel, voltage-dependent, L type, alpha 1D subunit	-1.6	0.03
Camk2g	NM_178597	Calcium/calmodulin-dependent protein kinase 2 gamma	-1.1	0.03
Hspb2	NM_178597	Heat shock protein 2	-1.3	0.04
Chrna7	NM_007390	Cholinergic receptor, nicotinic alpha polypeptide 7	-1.5	0.02
Stc2	NM_011491	Stanniocalcin 2	-1.3	0.04

brain of repeated blast-exposed mice was performed by silver staining. As shown in the **Figure**, a significant level of neurodegeneration occurred in the auditory cortex at 24 h after repeated blast exposures. The pathology index in the inner layer of auditory cortex (**Figure(b2)**) was scored as + to ++, while the pathology index of the outer layer (**Figure(a2)**) was – to + compared to the respective sham controls.

DISCUSSION

Previous studies showed a significant level of neuropathology and neurobehavioral changes, with ~20 percent mortality rate after repeated blast exposures in mice at 20.6 psi [36]. The pathology was more evident in the pre-frontal cortex and cerebellum of repeated blast-exposed mice. More recent results showed regional-specific changes in acetylcholinesterase activity in various regions of the brain after repeated blast exposures, indicating that the effects of blast exposure is heterogeneous in the brain [39]. The majority of the neurobiological changes in the brain were significant at 6 h after the last blast exposure [36]. Based on these observations, we analyzed the changes in the gene expression profile in different regions of the brain at 6 h after blast exposures in the present study.

The expression of otoferlin, which is known to be present in the brain and is essential for glutamate exocytosis at the auditory ribbon synapse and reported to be defective in a recessive form of human deafness, showed significant decrease in the hippocampus of mice exposed to repeated blasts [19–20,40–42]. In contrast, otoanchorin, another hearing-related gene defective in autosomal recessive deafness and known to mediate the contact between the apical surface of sensory epithelial cells and acellular gels of the inner ear and the tectorial and otoconial membranes for proper auditory processing, showed significant increase in the hippocampus after repeated blast exposures [21,43]. Significant increase in the expression of otoanchorin in the hippocampus after repeated blast exposures seems to be a compensatory mechanism to increase the sensitivity of hearing following injury to the auditory system and needs to be investigated in detail as a potential mechanism involved in the development of tinnitus.

Cadherins and protocadherins are another set of genes that showed differential expression in various regions of the brain after repeated blast exposures. Cadherin and protocadherin mutations are reported to be involved in noise-induced

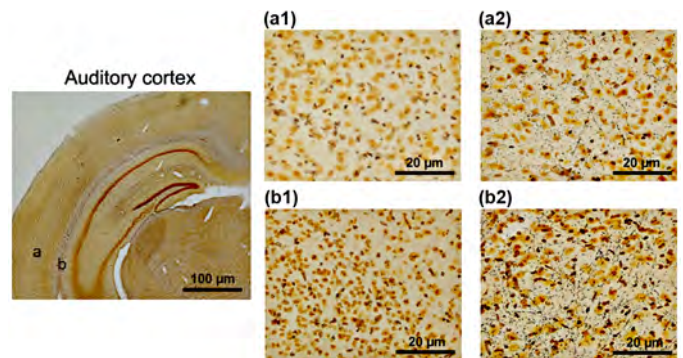


Figure.

Neuropathology of auditory cortex exposed to repeated blasts. Paraformaldehyde-fixed brain samples were sectioned into 50 µm sections and stained with Neurosilver Kit II. Two different close proximities of auditory cortex indicated as **(a)** and **(b)** in left-most panel (labeled as “Auditory cortex”) were analyzed for severity of injury in sham control (**(a1)** and **(b1)**) and repeated blast-exposed (**(a2)** and **(b2)**) mice at 24 h after last blast exposure. Positive silver staining is evident in auditory cortex of repeated blast-exposed mice confirming neurodegeneration ($n = 3$ for sham and blast).

hearing loss [13–14,16–17,44]. The altered expression profile of cadherins and protocadherins in different regions of the brain after repeated blast exposures may impair central auditory processing. The functional significance of these gene modifications in the blast-induced impairment of central auditory processing has to be studied in detail to exploit them for therapeutic applications.

Molecules involved in calcium influx and calcium-dependent proteins/enzymes are predominant signal transducers in auditory neurons [22–23,45–48]. The frontal cortex and midbrain of blast-exposed mice showed significant increase in the expression of calcium-dependent cysteine proteases and calpain 3 and 9, respectively (**Tables 3** and **4**). Calpains are essential for initiation and promotion of cell death, and treatment with calpain inhibitors are known to prevent the hearing loss induced by aminoglycoside ototoxicity [45]. The hippocampus of blast-exposed mice showed a significant decrease in the expression of calcitonin-related peptide, a suggested peptide therapeutic treatment for hearing loss (**Table 1**) [22,48]. The cerebellum and midbrain regions showed significant decrease in the expression of voltage-dependent calcium channel genes after repeated blast exposures, while multiple calcium binding proteins showed differential expression in the hippocampus, cerebellum, frontal cortex, and midbrain after repeated blast

exposures (**Tables 1–4**). It is known that L-type voltage-gated calcium channels are involved in the pathogenesis of acoustic injury in the cochlea, and treatment with calcium channel blockers can reduce the damage to the auditory neurons [26].

Other types of molecules involved in calcium regulation, such as calreticulin and calmodulin-dependent protein kinase expression, showed significant decrease in the frontal cortex and midbrain of blast-exposed mice, respectively (**Tables 3 and 4**) [23,47]. Interestingly, two calcium binding proteins, calretinin and parvalbumin, that were upregulated in the cerebellum at 24 and 48 h after blast exposures by proteomic analysis were not found to be altered in cDNA microarray analysis at 6 h after blast exposures.* One possible reason for this difference is that calretinin and parvalbumin expression might be regulated at the translational level after blast exposures, which needs to be investigated further. Second, cDNA microarray analysis at 24 and 48 h after blast exposures needs to be done to find any significant changes in calretinin and parvalbumin gene expression. Western-blotting of the hippocampal region at 6 h after blast exposures showed significant increase in calretinin, further supporting the idea that blast exposure possibly modulates the protein expression at the translational level. The differential expression of calcium-dependent proteins/receptors in the brain after repeated blast exposures could be the consequence of increased/decreased calcium buffering in the auditory neurons. Thus, these results suggest that repeated blast exposures lead to an imbalance in the regulation of calcium homeostasis in different regions of the brain that can directly influence the central auditory processing and lead to auditory impairment.

Heat shock proteins or factors are one of the best-characterized families of protective proteins that are usually upregulated after stress, offering cellular protection and survival [10,27–28]. Repeated blast exposures in mice showed significant increase in the expression of heat shock protein 8 and factor 5 in the hippocampus, while cerebellum and midbrain showed significant decrease in heat shock protein 8 and heat shock protein 2, respectively (**Tables 1, 2, and 4**). The functional significance of heat shock proteins in hyper-

thermia and noise overstimulation is well documented [10,28]. The differential expression of heat shock proteins in the brains of repeated blast-exposed mice needs to be investigated further. Additionally, repeated blast exposure in mice showed significant reduction in the expression of the cholinergic receptor nicotinic alpha polypeptide 7 in the midbrain, suggesting a possible role of these receptors in aberrant central auditory processing (**Table 4**). The nicotinic receptor of cochlear hair cells has been proposed by others as a potential therapeutic target in acoustic trauma [11,49].

The functional role of reactive oxygen species and the protective efficacy of antioxidants in noise-induced hearing loss are well documented [32,50–51]. Repeated blast exposure in mice showed significant increase in the expression of antioxidant enzymes, superoxide dismutase 3, and glutathione peroxidase 4 in the hippocampus and midbrain, suggesting a protective mechanism in central auditory processing (**Tables 1 and 4**). The influence of glutathione peroxidase and superoxide dismutase in noise-induced hearing loss has also been reported [12,31,33–34]. Reactive oxygen species showed an increase in the brain following repeated blast exposures [36].

Neuropathology analysis of the auditory cortex of repeated blast-exposed mice showed significant injury (**Figure**). The injury level was more on the medial contralateral side of the brain than the ipsilateral side. The neuropathology of the auditory cortex is in line with the significant level of auditory-related gene expression changes in the brain of blast-exposed mice. It is not clear whether the neuropathology is responsible for the changes in gene expression or vice versa. It has been reported that blast-induced mild to moderate TBI leads to neurobiological and behavioral changes with multifocal axonal injury [52–53]. In these reports, neuropathological changes were observed at 7 and 14 d after blast exposure, although gene expression changes were observed at day 1, indicating that molecular changes contributes to the neuropathology. In our studies, neuropathology was prominent at 24 h after repeated blast exposures, but changes in gene expression were observed much earlier, suggesting that molecular changes can occur earlier as a direct effect of blast exposures. Preliminary data on brain DNA damage after blast exposure using comet assay showed breakage of DNA after repeated blast exposures. Studies with rats exposed to low-levels of explosive blast showed terminal dUTP nick end labeling-positive cells in the white matter in day 1 without any changes in day 7 [54].

*Arun P, Valiyaveetil M, Biggemann L, Alamneh Y, Wei Y, Oguntayo S, Wang Y, Long JB, Nambiar MP. Modulation of hearing related proteins in the brain and inner ear following repeated blast exposures. *Intervent Med Appl Sci*. Forthcoming.

Changes in many hearing-related genes after blast exposure in the brain indicate that these genes play specific roles in central auditory processing. The gene expression changes may be the consequence of initial protection against blast-induced central auditory processing and later as injury mechanism of central auditory processing. Gene expression changes can vary with respect to blast overpressure or number of blasts and may also depend on the severity of injury. The contribution of the shock waves transmitting through the auditory canal or directly through the skull in central auditory processing impairment is currently being investigated in the laboratory by using ear protection. Unraveling the functional role of these genes in central auditory processing and how they cross-talk with each of the brain regions to perform sound perception, hearing, speech recognition, and long-term memory will help us to understand how exactly their modulation plays a role in central auditory processing impairments. The linkage of these gene modulations to concurrent neuropsychiatric changes after blast exposure is also important to understand the complex neurobiological mechanisms of blast affecting central auditory processing and aid in rehabilitation.

CONCLUSIONS

In summary, preliminary results indicate that repeated blast exposures in mice showed significant alterations in multiple genes that are reported to be involved in age- or noise-related hearing loss at 6 h after blast exposure. The repeated blast exposure also showed significant neuropathology at 24 h in the auditory cortex, suggesting that blast exposure damages central auditory processing systems. Gene expression changes occur at early time points after blast exposure and may not be the consequence of apoptotic or necrotic changes in the brain. The gene expression profile showed differential pattern in various regions of the brain of mice exposed to repeated blasts. Otoferlin and otoancorin, which are involved in deafness, showed significant alteration in the hippocampus after repeated blast exposure. Similarly, cadherins and protocadherins, which are involved in noise-induced hearing loss, showed significant changes in all the brain regions tested. The expression profile of calcium-regulating proteins/receptors in various brain regions also showed differential expression, indicating an imbalance in calcium homeostasis after repeated blast exposures. The heat shock proteins and antioxidant enzyme expressions

also showed significant changes in various regions of the brain after repeated blast exposure, indicating possible protective effects. The differential expression of multiple auditory-related genes in various regions of the brain after repeated blast exposures in mice needs to be investigated further to draw specific biochemical pathways involved in the functional significance of central auditory processing in blast-induced auditory dysfunction and tinnitus.

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Author contributions:

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Rapid Release of Tissue Enzymes into Blood after Blast Exposure: Potential Use as Biological Dosimeters

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Abstract

Explosive blast results in multiple organ injury and polytrauma, the intensity of which varies with the nature of the exposure, orientation, environment and individual resilience. Blast overpressure alone may not precisely indicate the level of body or brain injury after blast exposure. Assessment of the extent of body injury after blast exposure is important, since polytrauma and systemic factors significantly contribute to blast-induced traumatic brain injury. We evaluated the activity of plasma enzymes including aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH) and creatine kinase (CK) at different time points after blast exposure using a mouse model of single and repeated blast exposures to assess the severity of injury. Our data show that activities of all the enzymes in the plasma were significantly increased as early as 1 h after blast exposure. The elevated enzyme activity remained up to 6 h in an overpressure dose-dependent manner and returned close to normal levels at 24 h. Head-only blast exposure with body protection showed no increase in the enzyme activities suggesting that brain injury alone does not contribute to the systemic increase. In contrast to plasma increase, AST, ALT and LDH activity in the liver and CK in the skeletal muscle showed drastic decrease at 6 h after blast exposures. Histopathology showed mild necrosis at 6 h and severe necrosis at 24 h after blast exposures in liver and no changes in the skeletal muscle suggesting that the enzyme release from the tissue to plasma is probably triggered by transient cell membrane disruption from shockwave and not due to necrosis. Overpressure dependent transient release of tissue enzymes and elevation in the plasma after blast exposure suggest that elevated enzyme activities in the blood can be potentially used as a biological dosimeter to assess the severity of blast injury.

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Introduction

The frequency of blast-induced traumatic brain injury (blast TBI) has been increased tremendously in the recent conflicts due to the high use of improvised explosive devices [1,2]. Although the precise cause and mechanisms of blast TBI remains unclear, blast TBI shares clinical features of both penetrating TBI and closed-head TBI [3]. The uniqueness of blast TBI compared to other types of TBI is concurrent organ injury and polytrauma due to the whole body exposure to blast. Hemorrhage, inflammation and oxidative stress after blast exposure are not only confined to brain but also occur in other gas filled body organs such as lungs, gastrointestinal tracts and auditory systems [4–8].

The neuropathology and subsequent cognitive deficits after blast exposure are proposed to be a cumulative effect of direct blast overpressure effect on the brain along with damage to other body organs [9–11]. Protective Kevlar body vests decreased the mortality, neuropathology and behavioral deficits in rats exposed to blast overpressure supporting the notion that polytrauma and systemic effects significantly contribute to blast TBI [12]. Recently it has been reported that blast exposure to torso after head protection produce more severe neurotrauma compared to head-only exposure supporting that body injury, polytrauma and

systemic factors play a significant role in blast TBI [13,14]. Furthermore, torso protection but not head protection significantly attenuated blast neurotrauma indicating that blast mediated organ injury, polytrauma and systemic response plays a vital role in the development of primary blast neurotrauma [13,14]. Systemic response along with transient torso to brain hydraulic overpressure transmission leads to blood-brain barrier breakdown, neuroinflammation, cerebral vasospasm and ultimately blast neurotrauma [3,13,15–21].

One of the drawbacks in the identification as well as timely treatment of blast TBI is the difficulty in early diagnosis of the extent of brain and body injury to blast in the absence of any physical injury in most cases. Also, the threshold of blast overpressure for TBI is not well understood and not uniform among the population. Moreover, the nature of exposure, orientation of the subject and surrounding environment plays decisive roles in blast TBI or body injury [22]. Recently, a colorimetric blast injury dosimeter which can detect high blast overpressure exposure by changing the color and ultrastructure of the photonic crystalline material in an overpressure dependant manner has been reported [23]. Blast overpressure measurement alone may not exactly reflect the extent of body injury or blast

TBI. Early determination of the extent of body exposure to blast not only enables assessing the severity of injury but also helps providing appropriate medical care and prevent the victims from immediately returning to the duty which can further exacerbate the injury with more exposures or other co-morbidity factors [23].

Organ specific proteins/enzymes as well as microRNAs have been reported to be secreted in to the blood after an insult and are being studied as specific biomarkers of injury including TBI [24–28]. Significant amount of proteins which are abundant in the brain including glial fibrillary acidic protein, neuron-specific enolase and S100B have been found to be present in the blood after injury to the brain [27,28]. The release of these proteins/enzymes to blood requires blood-brain barrier breakdown after injury. Proteins/enzymes abundant in liver, kidney and heart are also found to be elevated in the blood circulation after injury to the respective organs [29–31]. Aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) are highly expressed in the liver and their elevated levels in blood circulation have been used for the diagnosis and prognosis of liver damage [32,33]. Similarly, the levels of creatine kinase (CK), which is abundant in the skeletal muscle, increase significantly in the plasma after damage to muscle [34,35]. Using an *in vitro* blast TBI model system with shock tube, we have shown that blast exposure leads to the release of LDH from the cells to the culture medium without cell mortality and suggested that blast-induced plasma membrane damage as a potential mechanism of this enzyme release [36]. We considered that activities of enzymes can increase in the blood if there is any type of injury to the organs, including cell membrane rupture due to shockwave after blast exposures and may reflect the severity of injury and the intensity of exposure.

In the present study, by using a mice model of single and repeated blast exposures, we measured the activities of AST, ALT, LDH and CK in the plasma at different time intervals after blast exposures for potential application as biological dosimeters of blast injury.

Results

Effect of blast overpressure dose on plasma enzyme activity

Increase in plasma enzyme activities after different levels of blast overpressure exposure is shown in Fig. 1. Plasma enzyme activity increase was directly proportional to the blast overpressure levels. At 10 psi overpressure, except ALT, none of the enzyme activities measured were increased significantly at 1 h after single blast exposure suggesting that ALT is the most sensitive enzyme for blast exposure. All the enzymes studied showed significant increase in the plasma at 1 h after exposure to 15 and 21 psi blast overpressures.

Enzyme activities in the plasma after single, repeated and head-only blast exposures

Table 1 shows the activities of AST, ALT, LDH and CK respectively at 1, 6 and 24 h in the plasma of mice exposed to single and repeated blasts at 21 psi with and without protective vest. All the enzymes studied in the plasma showed a transient increase as early as 1 h and remained elevated at least up to 6 h post-blast. Compared to single blast exposure, repeated exposures did not further increase the enzyme activities at 6 h (Table 1). The activities of LDH and CK in the plasma of blast exposed mice returned to normal levels by 24 h post-blast exposures, whereas the activities of AST and ALT were still significantly elevated compared to respective sham controls. The highest increase was

observed in the activity of ALT and was approximately 131-fold at 6 h after single blast exposure. The activities of LDH and AST showed approximately 34 and 20-fold increase respectively at 6 h after repeated blast exposures. Activity of CK showed a maximum of 6-fold increase in the plasma at 1 h after repeated blast exposures. Repeated blast exposure of animals with protective vest covering the whole body except the head completely prevented the increase of enzyme activities in the plasma (Table 1).

Effect of blast exposure on activities of enzymes in the tissue

The enzymes AST, ALT and LDH are abundant in the liver. The activities of these enzymes in the liver were determined at 6 h post-blast (21 psi), the time period at which their activity was maximum in the plasma. The activity of CK was determined at 6 h after blast exposure in the skeletal muscle, a tissue which is rich with this enzyme. The results in Table 2 show that the activities of these enzyme decreased significantly in the tissues after blast exposure. At 24 h, the activities of the enzymes were higher in the tissues compared to 6 h after repeated blast exposures.

Histopathology of the liver and skeletal muscle after blast exposure

Histopathology of liver and skeletal muscle at 6 and 24 h in the mice exposed to repeated blasts is shown in Fig. 2. No histopathological changes were observed in the skeletal muscle at both the time points studied. On the other hand, in the case of liver, there were foci of moderate coagulative necrosis with pyknosis, karyolysis, or nuclear absence randomly and multifocally at 6 h time point after exposure. At 24 h post-blast, the histopathological changes of liver were more severe with significant neutrophil infiltration.

Discussion

In this study, we demonstrated for the first time, a transient increase in the activities of tissue enzymes in the plasma after single and repeated blast exposures. The enzymes activities of AST, ALT and LDH which are highly abundant in the liver were found to be increased several folds in the blood circulation as early as 1 h after blast exposure (Table 1). The activity of all the enzymes remained elevated in the plasma at least up to 6 h post-blast exposure. The activities of ALT and AST also showed statistically significant elevation in the plasma at 24 h. Plasma LDH activity reached to almost normal levels by 24 h post-blast. The decrease in the activities of enzymes in the plasma at 24 h compared to 6 h suggests possible rapid elimination by the renal clearance system probably due to their lower molecular weights.

The significant increase in the activities of all the three liver enzymes in the plasma at 6 h post-blast exposure was associated with a simultaneous decrease in the liver tissue (Table 2) suggesting that they are released rapidly from the liver after blast exposure. The mechanism of enzyme release seems to be a consequence of cell membrane disruption. Instantaneous cellular pressure rise and drop after blast exposure may be a factor responsible for the disruption of cell membrane. Histopathological examination of liver showed severe necrosis at 24 h post-blast whereas only mild necrosis at 6 h (Fig. 2) indicating that the release of liver enzymes is independent on blast-induced necrosis of liver and further support the hypothesis of cell membrane disruption after blast exposure as a mechanism of transient enzyme release. Moreover, the activities of the enzymes in the liver tissue at 24 h was significantly higher compared to 6 h post-blast exposures suggesting that there is no positive correlation between the severity of liver

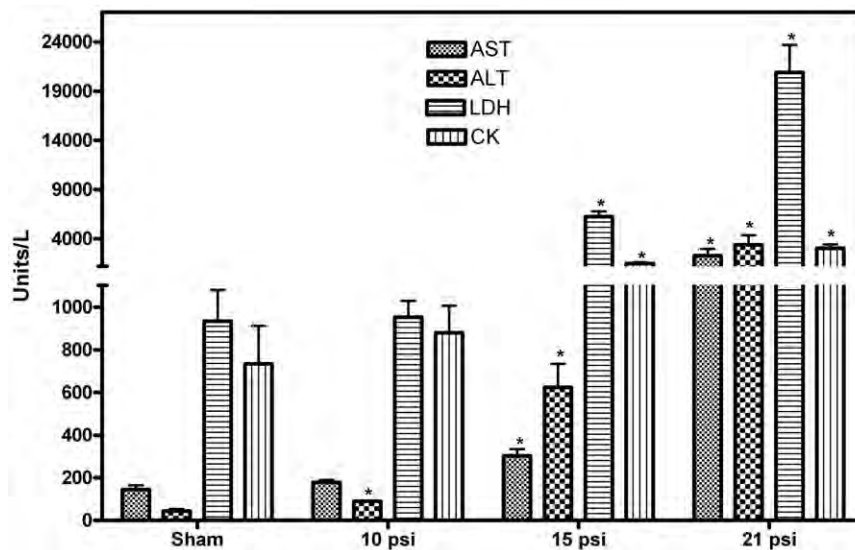


Figure 1. Blast overpressure dose response on the activities of enzymes in the plasma after 1 h. The activities of AST, ALT, LDH and CK increases in the plasma in an overpressure dose dependant manner. Mean $\pm$ SEM values of enzyme activities after exposure to different levels of blast overpressures are compared to sham. Statistical analysis was carried out by ANOVA test ($n=6$). * $p<0.05$. doi:10.1371/journal.pone.0033798.g001

damage/necrosis and plasma or liver enzyme activities at 6 or 24 h. However, the initial increase in the blood enzyme activity may predict the severity of eventual liver damage after blast.

Similar results were observed with CK activity in the plasma and skeletal muscle (Tables 1 and 2). Histopathological analysis of skeletal muscle showed no any significant changes at 6 or 24 h after repeated blast exposures (Fig. 2) which support the possible release of CK from skeletal muscle through transient membrane rupture after blast exposure and rules out the possibility of muscle fiber necrosis leading to release of CK.

In the mice model of tightly coupled repetitive blast-induced TBI, the neurobiological effects of blast exposure increased significantly with the number of exposures [37]. However, the increase in plasma enzyme activities was similar between single or repeated blast exposures suggesting that maximum enzyme release occurs after single blast and multiple blasts do not lead to further increase in the plasma enzyme activities. The reason for the lack of cumulative effects is not clear, but these results implicate that systemic factors contributing to TBI may be similar between single or repeated blasts and increase in TBI after repeated blasts probably involves more direct effects of blast exposure to the brain.

Also, different organs may have differences in vulnerability to repeated blast exposures.

Brain water content or edema was significantly higher as early as 4 h after repetitive blast exposures where as no significant difference was observed at 24 h post-blast exposures compared to sham controls [37] suggesting possible transient neuronal membrane rupture leading to rapid movement of water molecules into the cells. In rats exposed to underwater shockwaves using a micro explosion devise, leakage of administered Evans blue dye in and around the area of lesion has been reported indicating increase in cell membrane permeability as an impending mechanism of brain edema [21]. The view of abrupt membrane rupture is also supported by our earlier studies showing potential plasma membrane disruption and release of LDH from cell lines to extracellular medium after blast exposure without cell mortality [36]. Shockwaves have been utilized as a method for increasing the cell permeability to introduce macromolecules and small polar molecules into the cells for gene therapy and anticancer drug delivery [38–42]. Shockwave exposure was found to increase the permeability of leukemia cells and it has been reported that the

Table 1. Enzyme activities in the plasma at different time intervals after single and repeated blast exposures at 21 psi with and without protective vest.

	AST (Units/L)	ALT (Units/L)	LDH (Units/L)	CK (Units/L)
Sham	144.57 $\pm$ 52.86	44.14 $\pm$ 22.52	935.14 $\pm$ 384.36	734.00 $\pm$ 469.38
1 blast, 6 h, no vest	2739.33 $\pm$ 1281.63*	5786.00 $\pm$ 3845.01*	31776.33 $\pm$ 24597.79*	2318.33 $\pm$ 894.89*
3 blasts, 1 h, no vest	2708.33 $\pm$ 1490.17*	3924.33 $\pm$ 2049.52*	26399.67 $\pm$ 8146.89*	4765.66 $\pm$ 27.37*
3 blasts, 6 h, no vest	2940.66 $\pm$ 1075.15*	5216.50 $\pm$ 2165.76*	32135.17 $\pm$ 4153.18*	1855.83 $\pm$ 968.52*
3 blasts, 6 h, with vest	147.20 $\pm$ 63.40	91.00 $\pm$ 44.23	978.80 $\pm$ 333.81	676.80 $\pm$ 267.65
3 blasts, 24 h, no vest	392.80 $\pm$ 156.25*	1133.40 $\pm$ 1010.11*	2627.60 $\pm$ 2307.97	548.20 $\pm$ 270.07

Mean $\pm$ SEM values of different blast exposure groups were compared to those of sham. AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; CK, creatine kinase; Statistical analysis was carried out by ANOVA test ($n=7$).

* $p<0.05$.

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Table 2. Enzyme activities in the liver and muscle tissues at 6 and 24 h after repeated blast exposures.

Tissue	Enzyme	Sham	6 h post-blast	24 h post-blast
Liver	AST (Units/mg protein)	4.70±1.29	1.28±0.70*	2.68±0.97*
Liver	ALT (Units/mg protein)	7.41±3.27	0.89±0.65*	3.47±1.02*
Liver	LDH (Units/mg protein)	38.01±18.62	0.56±0.43*	12.59±3.87*
Muscle	CK (Units/μg protein)	101.03±16.62	50.55±18.99*	71.48±12.57*

Mean ± SEM values at each time points are compared to those of sham. AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; CK, creatine kinase. Statistical analysis was carried out by ANOVA test (n=7).

*p<0.05.

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shockwaves rather than the overpressure increases membrane permeability without any significant cell mortality [38].

Significant increase in the incidence of blast TBI in the current wars urged the need to determine blast thresholds that can induce TBI. Recently a material-based colorimetric blast injury dosimeter has been reported [23]. The blast overpressures used in that study (59 to 158 psi) were significantly higher than that was used in our experiments (10 to 21 psi) suggesting that measurement of plasma enzyme activity is a more sensitive method. Significant elevation of all the four enzymes studied in the plasma by 1 h after 15 psi blast overpressure exposure (Fig. 1), suggest that the increases in the activities of these enzymes in the plasma can be used as a biological dosimeter of the severity of blast exposure. Increase in the enzyme activity is a direct measure of the extent of blast-induced body injury. It might also represent the severity of TBI, since more severe body injury release higher levels of enzymes and systemic factors after single blast exposure and exacerbate brain

injury [13,14]. Since the basal levels of these enzymes are established in human populations, significant increase after an incidence can indicate the extent of blast injury in the absence of protective vests and aid in medical countermeasures. On the other hand, measurement of these enzymes in the plasma before deployment will address basal level variation of these enzymes in military population and will help to more precisely determine the severity of blast injury. Increase in the activities of plasma enzymes can also distinguish blast versus non-blast injuries or other types of trauma which are more localized.

Materials and Methods

Chemicals and reagents

Diagnostic kits to measure the activities of AST, ALT, LDH and CK in tissues were purchased from Randox Laboratories (Kearneysville, WV, USA). Phosphate buffered saline (PBS) and 4% paraformaldehyde (PFA) were purchased from Fisher Scientific (Pittsburgh, PA, USA).

Animals and blast exposure

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 (NRC Publication 1996 edition). The protocol was approved by Institutional Animal Care and Use Committee, Walter Reed Army Institute of Research. C57BL/6J male mice (8–10 weeks old) that weighed between 21–26 g (Jackson Laboratory, Bar harbor, ME, USA) were used in this study. Mice were housed at 20–22°C and 12 h light/dark cycle with free access to food and water *ad libitum*. A compressed air-driven shock tube was used for blast exposure of mice [12,37]. Mice were exposed to single and repeated blasts as described earlier [37]. Briefly, mice were anesthetized with 4% isoflurane gas (O₂ flow rate 1.5 L/min) for 8 min and quickly placed on a holder. The animals were restrained with a net to minimize the movements and any related injury during blast exposure. The holder was placed 2.5 feet inside from the open end of the 15 feet long shock tube and animals were placed in prone position, perpendicular to the direction of shock waves. Animals were exposed to different levels of single blast overpressure (10, 15 and 21 psi) by rupture of Mylar membranes of various thicknesses. For repeated blast exposures, mice were subjected to three consecutive blasts (21 psi) with 1–30 min intervals between blasts. The first two blasts were with 1 min intervals and the third blast exposure was at 30 min after the second blast. These exposures were similar to breachers exposure (8–10 kg trinitrotoluene, 10–12 times in a day

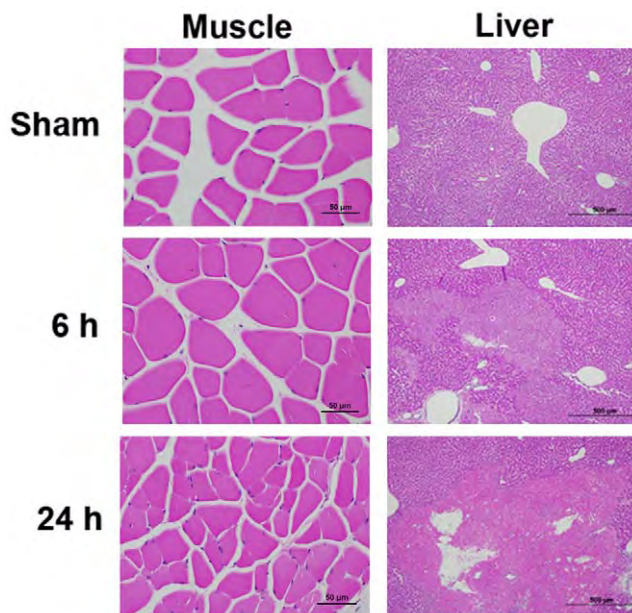


Figure 2. Hematoxylin and Eosin staining of liver and muscle at different time intervals after blast exposure. In the liver of blast exposed mice, the area with maximum lesion was showed. 6 h post-blast exposure showed the initiation of necrosis with mild discoloration of a large area of the tissue, whereas after 24 h, there is more widespread necrosis with increased neutrophilic infiltration resulting in significant tissue damage and stromal collapse. Liver –10× (500 μm) and muscle –40× (50 μm) magnifications.
doi:10.1371/journal.pone.0033798.g002

or more in the night) (USAARL Report No. 2010-16, www.dtic.mil) or multiple blast exposures in the battlefield.

At the end of each time period, the animals were anesthetized and blood was collected by cardiac puncture into heparinized tubes. Plasma was separated and used for enzyme analyses. After blood collection, the animals were perfused transcardially using PBS to remove blood from tissues and liver and skeletal muscle were dissected for enzyme analyses. For histopathological analysis, separate groups of mice were exposed to blast and perfused transcardially with 4% PFA and post-fixed in 4% PFA after blood collection.

Determination of the contribution of direct head exposure to blast on the plasma enzyme activity

To determine the contribution of direct head/brain blast exposure on the activity of enzymes in the plasma, the mice were protected with a snugly fitting vest covering the whole body except the head under anesthesia. Sham controls were treated in the same way except that the mice were not exposed to blast. The blood samples were collected after 6 h and subjected to enzyme analysis.

Analysis of enzymes in the plasma

The activities of AST, ALT, LDH and CK in the plasma were determined using automated Vitros® 350 Chemistry System, Ortho Clinical Diagnostics (Rochester, NY, USA).

Enzyme analyses in tissue homogenates

Activities of AST, ALT and LDH were determined in the liver, which is abundant source of these enzymes. CK is mostly present in the muscle and the activity was determined from the skeletal muscle. For enzyme analyses in the liver and skeletal muscle, PBS perfused tissues were homogenized with 1:7 (wt/vol) tissue protein extraction reagent (Pierce Chemical Co, Rockford, IL, USA) containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis,

MO, USA). The homogenate was centrifuged at 5000×g for 10 min and collected the supernatant. The activities of AST, ALT, LDH and CK were determined in the supernatant by using the diagnostic kits according to manufacturer's instructions.

Histopathology

Histopathology analysis of the liver and skeletal muscle of mice was performed at 6 and 24 h post-blast exposure. Tissues were collected after transcardially perfusing the animals with 4% PFA and processed by routine histology procedures. Tissue sections (5 µm) were stained with Hematoxylin and Eosin and evaluated using an Olympus Model AX80 (Olympus, Center Valley, PA, USA) inverted microscope and photos were taken using Olympus Model DP70 camera attached to the microscope.

Statistical analysis

Results were expressed as mean ± SEM. Statistical analysis was carried out by Analysis of Variance (ANOVA). A *p* value < 0.05 was considered significant.

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Disclaimer

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Author Contributions

Conceived and designed the experiments: PA MPN. Performed the experiments: PA SO YA CH. Analyzed the data: PA YA YW MV MPN. Contributed reagents/materials/analysis tools: MPN JBL. Wrote the paper: PA MPN MV.

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Transient changes in neuronal cell membrane permeability after blast exposure

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The biochemical mechanisms of explosive blast-induced traumatic brain injury and the subsequent long-term neurobehavioral abnormalities are still not completely understood. We studied the biochemical mechanism of blast traumatic brain injury using our recently reported in-vitro model system with a shock tube. Primary blast exposure of in-vitro models leads to neurobiological changes in an overpressure dose-dependent and time-dependent manner. Lactate dehydrogenase was released significantly into the extracellular medium without cell death after blast exposure, indicating compromised cell membrane integrity. We further explored the integrity of cell membrane after blast exposure by fluorescent dye uptake/release techniques in SH-SY5Y human neuroblastoma cells. Our data indicate that blast exposure leads to an overpressure-dependent transient increase in the release of preloaded calcein AM into the culture medium with proportional intracellular decrease. Uptake of an extracellular nucleic acid-binding dye TO-PRO-3 iodide was also increased significantly

after blast exposure, indicating that the increased molecular transport is bidirectional and nuclear membrane integrity is also affected by blast exposure. These results suggest that blast exposure perturbs the integrity of the neuronal cell membrane, leading to increased bidirectional transport of molecules – a potential mechanism that can lead to traumatic brain injury. *NeuroReport* 23:342–346 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Keywords: blast exposure, fluorescence release, fluorescence uptake, membrane integrity, SH-SY5Y cells, traumatic brain injury

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Introduction

Blast traumatic brain injury and subsequent neurobehavioral abnormalities remain as major disabilities among service members [1–3]. Understanding the biochemical, molecular, and cellular mechanisms of blast traumatic brain injury and associated neuropathological and neurobehavioral deficits is still rudimentary but crucial for improved protection, treatment, and recovery. In-vitro brain models are powerful tools to investigate the cellular, biochemical, and molecular mechanisms of blast traumatic brain injury, discover putative biomarkers, and targeted therapeutics [4,5]. It has been reported that data from in-vitro models of traumatic brain injury are nearly 90% predictive of the in-vivo results, which strongly supports the utility of in-vitro models [4].

We have developed the first shock tube-based in-vitro blast traumatic brain injury model using brain cell lines [5]. Using this model, we reported that blast exposure results in a significant increase in the extracellular levels of lactate dehydrogenase without significant changes in cell mortality, and proposed that cellular membrane integrity is possibly compromised after blast exposure [5]. Here, utilizing fluorescence release and uptake techniques, we further investigated the effect of single and repeated blast exposures on neuronal cell membrane integrity using human neuroblastoma cells.

Methods

Cell line and culture condition

SH-SY5Y cells, Dulbecco's modified Eagle's medium, and fetal bovine serum were obtained from the American Type Culture Collection (Manassas, Virginia, USA). Cells were grown in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum and penicillin/streptomycin. The cells were kept at 37°C in a CO₂ incubator with 5% CO₂ and 95% air in a humidified atmosphere. Cells (4×10^4 cells/well) were grown in 96-well tissue culture plates 24 h before blast exposure. On the day of blast exposure, the medium was removed from the wells and 360 µl of fresh medium was added to fill the wells completely. The plates were then sealed with gas-permeable Mylar plate sealers (MP Biomedicals, Solon, Ohio, USA), and the edges of the plates and the sealer were secured using sterile tapes before blast exposure as described earlier [5].

Blast exposure

Culture plates containing the cells were exposed to single or repeated blasts as described earlier [5]. For single and repeated blast exposures, the blast overpressure (21.05 psi) that resulted in significant neurobiological changes in SH-SY5Y cells was used [5]. For repeated blast exposures, the plates containing cells were subjected to

three blasts (21.05 psi) with 2-min intervals between each blast [5]. For sham controls, cells were treated in the same way, except that the plates were not exposed to blast overpressure.

Fluorescence release and uptake studies

The fluorescent labeling dyes calcein AM and TO-PRO-3 iodide were purchased from Invitrogen Corporation (Carlsbad, California, USA). Calcein AM is a nonfluorescent cell-permeable dye, which is converted into green fluorescent calcein after acetoxymethyl ester hydrolysis by intracellular nonspecific esterases. Nonfluorescent TO-PRO-3 iodide is not easily permeable through the cell membrane of active cells. TO-PRO-3 iodide will enter the cells if the cell membrane integrity is compromised or the cells undergo apoptosis. Binding of TO-PRO-3 iodide to nucleic acids in the cells makes it far-red fluorescent.

For fluorescence release studies, cells were incubated for 30 min with 100 μ l of medium containing 5 μ g/ml calcein AM. After incubation, the medium containing calcein AM was removed and the cells were rinsed with fresh medium without dye. The wells were then filled with medium, sealed, and exposed to single or repeated blasts. Calcein released after blast exposure was determined by transferring 100 μ l of medium into a 96-well black plate and measuring the fluorescence (excitation at 494 nm and emission at 517 nm) using a Spectramax M5 microplate reader (Molecular Devices, Sunnyvale, California, USA). Intracellular calcein levels remaining after the blast exposure were determined by removing the medium, rinsing the cell with fresh medium, and lysing the cells with 100 μ l water, followed by vigorous mixing. The fluorescence of the cell extract was measured as described above.

Fluorescence uptake studies were carried out by filling the wells containing SH-SY5Y cells with medium containing 5 μ M TO-PRO-3 iodide. Plates were sealed and exposed to single or repeated blasts. The intracellular fluorescence was visualized (excitation at 642 nm and emission at 661 nm) after 1 h using an inverted fluorescence microscope (Model IX70; Olympus, Center Valley, Pennsylvania, USA). The intensity of red fluorescence was quantified by densitometry using Image-Pro Plus software (Version 7.0; MediaCybernetics, Bethesda, Maryland, USA). The morphology of the cells after blast exposure was assessed by light microscopy.

Cell viability assay

The viability of SH-SY5Y cells after calcein AM incorporation and blast exposure was assessed using the Trypan Blue Exclusion Test as described earlier, with minor modifications [6]. Briefly, 15 min after single or repeated blast exposures, the cells were collected using 0.25% trypsin solution (Sigma-Aldrich, St Louis, Missouri,

USA). The cells were then centrifuged at 100g for 5 min, the supernatant was removed, and the cells were resuspended in PBS. Equal volumes of the cell suspension and 0.4% trypan blue solutions (Sigma-Aldrich) were mixed, and viable (unstained) and nonviable (stained) cells were counted after 5 min.

Statistical analysis

Statistical analysis was carried out by analysis of variance (ANOVA) using SAS software version 9.3 (SAS Institute Inc., Cary, North Carolina, USA). For single variance, one-way ANOVA, followed by a *t*-test was carried out. For multiple variance, two-way ANOVA, followed by Tukey's post-hoc test using HSD multiple comparisons were carried out. A *P*-value less than 0.01 was considered significant.

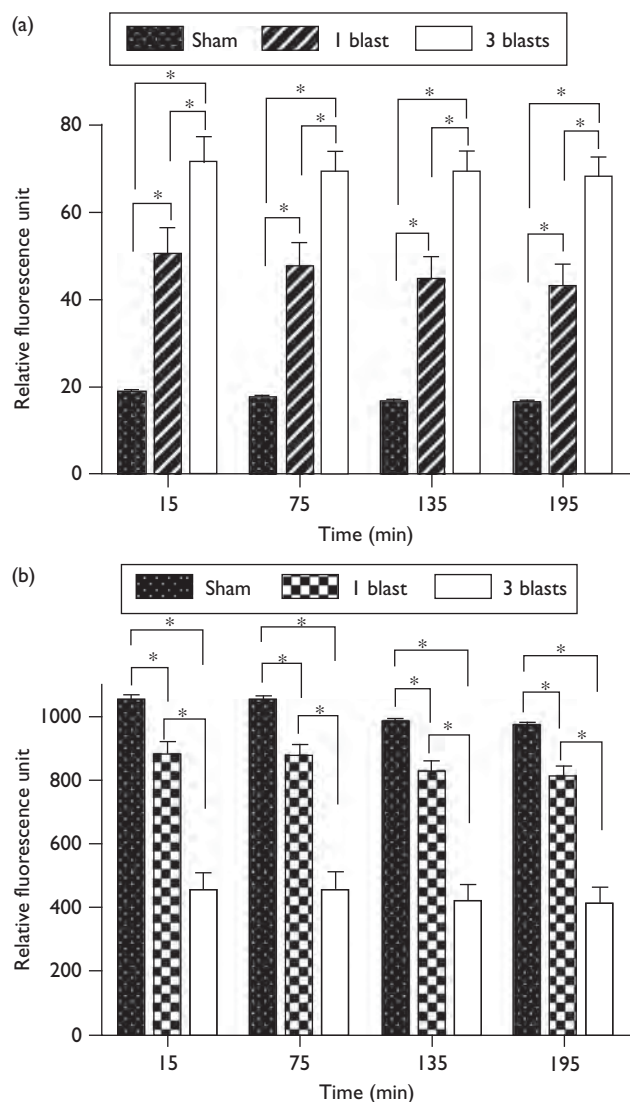
Results

Effect of blast exposure on the release of preloaded calcein

The results shown in Fig. 1a indicate that blast exposure led to a significant increase in the release of calcein into the culture medium, suggesting compromised neuronal plasma membrane integrity after blast exposure. The fluorescence in the culture medium increased from 18.78 ± 2.29 to 50.27 ± 15.36 ($P = 5.67 \times 10^{-4}$) after single blast exposure and increased further to 71.60 ± 13.65 ($P = 2.95 \times 10^{-6}$) after repeated blast exposures in 15 min. The release of dye was significantly higher ($P = 9.1 \times 10^{-3}$) in the repeated blast-exposed cells compared with single blast exposure (Fig. 1a). The intracellular fluorescence decreased from 1053.36 ± 42.33 to 877.16 ± 109.70 ($P = 4.31 \times 10^{-3}$) after single blast exposure and decreased further to 449.77 ± 144.46 ($P = 1.87 \times 10^{-6}$) after repeated blast exposures in 15 min (Fig. 1b).

Effect of blast exposure on the uptake of TO-PRO-3 iodide

Blast exposure in the presence of TO-PRO-3 iodide resulted in a significant increase in the infiltration of the dye from the culture medium into SH-SY5Y cells (Fig. 2a). No apparent changes in the morphology of cells occurred after single or repeated blasts (Fig. 2b). Densitometry analysis of the fluorescence generated by TO-PRO-3 iodide after binding with nucleic acids indicates that the infiltration of the dye increases with the number of blasts (Fig. 2c). The intracellular fluorescence intensity (expressed in arbitrary unit) increased from 352.50 ± 47.86 to 656.66 ± 58.06 ($P = 3.98 \times 10^{-6}$) after single blast exposure and increased further to 1181.16 ± 54.39 ($P = 1.91 \times 10^{-10}$) after repeated blast exposures. Repeated blast-exposed cells showed significantly higher ($P = 4.13 \times 10^{-8}$) intracellular fluorescence compared with the cells exposed to single blast.

Fig. 1

Release of calcein after blast exposure. Calcein fluorescence in the extracellular medium (a) and in SH-SY5Y cells (b) after single and triple blast exposures, indicating the release of the dye from the cells after blast exposure. Statistical analysis was carried out by two-way analysis of variance, followed by Tukey's post-hoc test using HSD multiple comparisons. **P*-value of less than 0.01 (*n* = 6).

Effect of blast exposures on cell viability

The percentages of nonviable cells in the sham, single blast-exposed, and repeated blast-exposed cells were 7.23 ± 1.31 , 6.97 ± 1.67 , and 7.48 ± 1.81 , respectively. The results analyzed by one-way ANOVA indicate no significant changes in the number of nonviable cells after single or repeated blasts (*P* > 0.05).

Discussion

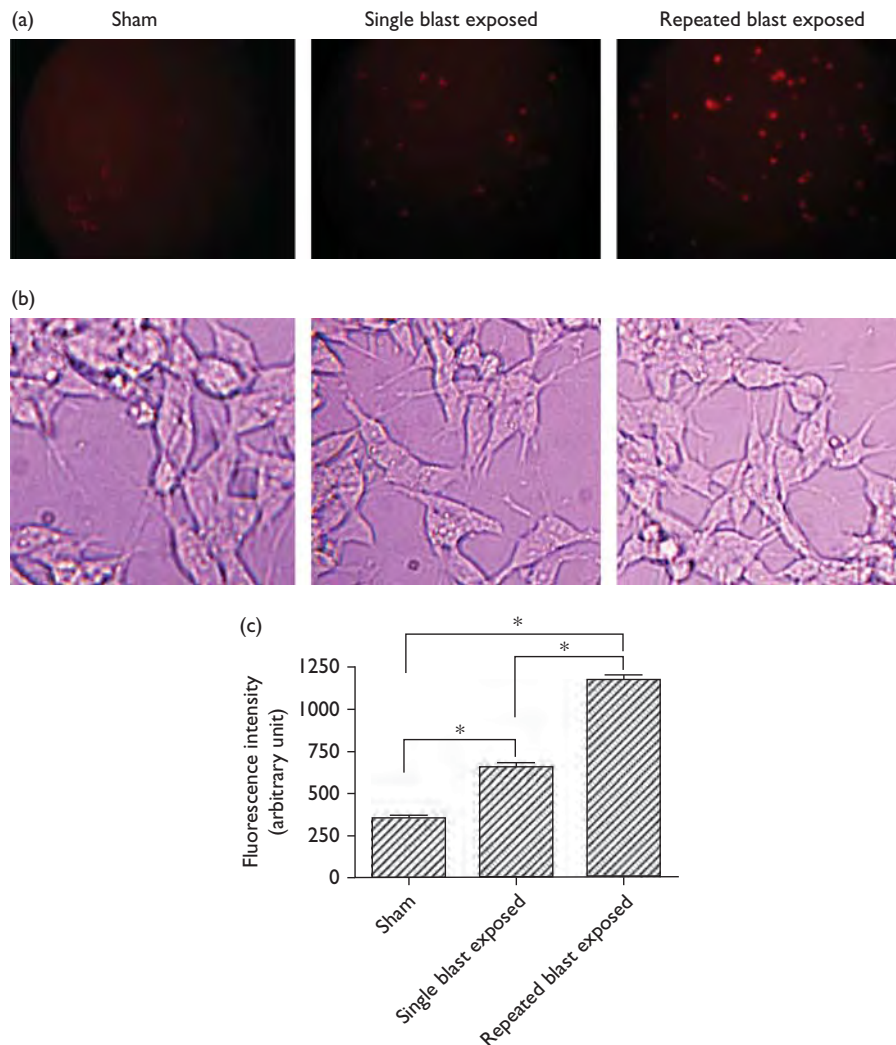
Our results suggest that blast exposure leads to a transient change in neuronal plasma and nuclear membrane integrity, resulting in rapid transport of molecules in and out of the cells without changes in cell mortality. These

results support our earlier hypothesis that plasma membrane damage is a potential mechanism for lactate dehydrogenase release into the culture medium after blast exposure [5]. It has been proposed that repeated blast exposures to SH-SY5Y cells may lead to the release of cyclophilin A into the culture medium and provide protection from the chronic effects of blast exposure [5], and our present results support this hypothesis. No previous studies have reported the disruption of neuronal nuclear or plasma membrane integrity after blast exposure using a shock tube.

The results shown in Fig. 1 indicate that both single and repeated blast exposures resulted in transient release of calcein from SH-SY5Y cells. As no changes in cell viability were observed, it is quite likely that the increase in fluorescence in the culture medium could have been due to the increased release of the dye. Moreover, the increase in fluorescence in the culture medium was accompanied by a corresponding intracellular decrease. The maximum release of calcein was observed at the earliest possible time point studied (15 min postblast exposure) and no further increase was observed at later time points, suggesting that the changes in the membrane permeability after blast exposure occur transiently, followed by rapid return to normal state.

The transient increase in membrane permeability of SH-SY5Y cells increases with the number of blast exposures (Fig. 1). In a repetitive blast traumatic brain injury model, it has been shown that the neurobiological effects of blast exposure increase with the number of exposures [3]. Brain water content was significantly higher as early as 4 h postblast and no significant change was observed at 24 h [3], indicating the possibility of transient changes in cell membrane integrity as a mechanism for the rapid movement of extracellular water molecules into the brain cells, causing edema. In support of this, Nakagawa *et al.* [7] demonstrated the leakage of administered Evans blue dye in and around the blast-induced brain lesion and suggested acute changes in membrane permeability as a mechanism of brain edema. Exposure of SH-SY5Y cells to blast leads to neurobiological effects in an overpressure-dependent and time-dependent manner [5]. Thus, the results obtained in our present study suggest the possibility that transient changes in neuronal cell membrane integrity after blast exposure could be a potential mechanism for brain edema as well as acute neurobiological effects observed in blast traumatic brain injury. These findings point to the use of plasma membrane stabilizers as a potential therapeutic strategy against blast traumatic brain injury.

Repeated blast exposures in the presence of TO-PRO-3 iodide resulted in a significant increase in the infiltration of the dye into the cells, again supporting the above observation of compromised neuronal membrane integrity after blast exposure (Fig. 2). No overt changes in cell

Fig. 2

Uptake of TO-PRO-3 iodide after blast exposures. (a) Representative fluorescence microscopy figure from six different wells of the 96-well plate showing the penetration of TO-PRO-3 iodide into SH-SY5Y cells 1 h after single or repeated blast exposures. The figure shows the fluorescent-labeled cells present in an 80% confluent single well of 96-well plates. (b) Morphology of the cells in corresponding wells showing mostly intact axons and dendrites. (c) Densitometry analysis of the fluorescence intensity. Statistical analysis was carried out by one-way analysis of variance, followed by a *t*-test. **P*-value of less than 0.01 (*n*=6).

morphology occurred after single or repeated blast exposures (Fig. 2b). As the axons and dendrites were mostly intact after blasts, it is quite unlikely that the increased uptake of TO-PRO-3 iodide or the release of calcein is a result of broken axons or dendrites. Nonetheless, it is possible that blast exposure can affect the membranes of axons and dendrites in addition to the cell body, leading to unusual transport of molecules. As TO-PRO-3 iodide stains nucleic acids, the results additionally show compromised nuclear membrane integrity after blast exposure. This transient change in nuclear membrane integrity may be responsible for the phenomenon of enucleation of brain cells observed after blast exposure in different in-vivo blast traumatic brain injury models [8,9].

How does blast exposure lead to transient increase in neuronal plasma and nuclear membrane permeability? It is quite likely that blast exposure can cause shearing, stretching, and compression of neurons, resulting in porous plasma and nuclear membranes for a short time. Shockwave from lithotripter compresses tissues for 1 μ s, followed by tension for a few microseconds along with tissue shearing [7]. It has also been reported that the tensile stress caused by the lithotripter leads to cavitations [7,10]. Shearing or stretching of neuronal cell body, axons, and dendrites can influence the properties of receptors, which can affect the receptor-mediated transport of molecules [11,12]. Detailed studies are warranted to unravel the precise mechanism involved in the loss of membrane integrity after blast exposure.

Conclusion

Blast overpressure exposure results in a transient increase in the permeability of neuronal nuclear and plasma membranes, resulting in bidirectional transport of molecules. The increase in membrane permeability is cumulative with repeated blasts, occurs immediately after blast exposure, and exists only for a short time. The transient changes in nuclear and plasma membrane integrity are likely to be involved in acute brain edema, cellular injury, chronic neuropathological, and neurobehavioral deficits observed in blast traumatic brain injury.

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Conflicts of interest

The contents, opinions and assertions contained herein are private views of the authors and are not to be construed as official or reflecting the views of the Department of the Army or the Department of Defense.

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