TITLE

Neuropathology and Neurobehavioral Alterations in a Rat Model of Traumatic Brain
Injury to Occupants of Vehicles Targeted by Underbody Blasts

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KEY WORDS: Acceleration, apoptosis, inflammation, anxiety, synapse

ACKNOWLEDGMENTS: This research was supported by a grant from the US Dept. of Defense W81-xWH-13-1-0016 and from the US Air Force Medical Service FA8650-11-2-6D04.

Abstract

Many victims of blast-induced traumatic brain injury are occupants of military vehicles targeted by land mines. Recently improved vehicle designs protect these individuals against blast overpressure, leaving acceleration as the main force potentially responsible for brain injury. We recently developed a unique rat model of under-vehicle blast-induced hyperacceleration where exposure to acceleration as low as 50G acceleration force results in histopathological evidence of diffuse axonal injury and astrocyte activation, with no evidence of neuronal cell death. This study investigated the effects of much higher blast-induced accelerations (1200 to 2800G) on neuronal cell death, neuro-inflammation, behavioral deficits and mortality. Adult male rats were subjected to this range of accelerations, in the absence of exposure to blast overpressure, and evaluated over 28 days for working memory (Y maze) and anxiety (elevated plus maze). In addition, brains obtained from rats at one and seven days post-injury were used for neuropathology and neurochemical assays. Sixty seven percent of rats died soon after being subjected to blasts resulting in 2800G acceleration. All rats exposed to 2400G acceleration survived and exhibited transient deficits in working memory and long-term anxiety like behaviors, while those exposed to 1200 acceleration G force only demonstrated increased anxiety. Behavioral deficits were associated with acute microglia/macrophage activation, increased hippocampal neuronal death, and reduced levels of tight junction- and synapse- associated proteins. Taken together, these results suggest that exposure of rats to high underbody blast-induced G forces results in neurologic injury accompanied by neuronal apoptosis, neuroinflammation and evidence for neurosynaptic alterations.

I. Introduction

Brain injuries resulting from direct or indirect exposure to explosions represent up to 68% of more than 300,000 U.S. military neurotrauma victims who served in the recent wars in Iraq and Afghanistan (Abdul-Muneer, et al., 2013, DePalma, et al., 2005, Hoge, et al., 2008, Okie, 2005, Owens, et al., 2008, Rosenfeld, et al., 2013). Blast-induced traumatic brain injuries (bTBI) are mainly classified in four sub-categories: 1. Primary injury, which results from blast overpressure. 2. Penetrating injuries caused by debris propelled by the explosion. 3. “Coup-counter coup” head injury resulting from secondary head impact 4. Exposure to intensive heat, toxic chemicals, and electromagnetic radiation (Cernak, et al., 2011, Mellor, 1988). Missing from these classifications is TBI induced by sudden, intense acceleration force without significant exposure to blast overpressure. Such injuries occur to occupants of vehicles targeted by land mines, where the V-shaped armored hulls deflect the bulk of blast overpressure laterally, away from the occupants. Nevertheless, the occupants are subjected to uniquely rapid and intense acceleration.

Clinical research and epidemiological studies suggest that the majority of all bTBI victims suffer from mild TBI (Hoge, et al., 2008, Lange, et al., 2013). Although most victims of mild-bTBI pass the Acute Concussion Evaluation (Brenner, et al., 2009, Elder, et al., 2010), many return to the hospital later, exhibiting cognitive and memory impairments, increased anxiety, and depression (Elder, et al., 2010, Jaffee and Meyer, 2009, Rosenfeld and Ford, 2010). Thus, a better knowledge of the pathophysiology of different forms of bTBI is needed to improve both short and long-term outcomes for bTBI casualties.

A number of different animal models of bTBI have been developed (Goldstein, et al., 2014, Kovacs, et al., 2014), with the goal of improving treatment for bTBI (Chen, et al., 2013, Schultz, et al., 2011). Such models have focused primarily on brain injuries caused by exposure to blast overpressure and shock waves, such as those experienced by “unmounted” warfighters on foot patrol. These models typically utilize rodents restrained within or just outside the end of a shock tube that exposes the animals to these forces. The results of these experiments demonstrate that exposure of laboratory animals to a single blast can cause significant pathological changes in the brain including the disruption of the blood brain barrier integrity (Abdul-Muneer, et al., 2013, Elder, et al., 2015), increased accumulation of inflammatory microglia/macrophages, neuronal cell death, and neurobehavioral deficits (Abdul-Muneer, et al., 2013, Kwon, et al., 2011, Vandevord, et al., 2012).

To better understand the pathology of blast-induced brain injury sustained by occupants of military and other vehicles, we recently developed a unique rat model of underbody blast-induced vertical hyperacceleration where relatively low blast intensity (50G acceleration force) revealed histopathological evidence of diffuse axonal injury and astrocyte activation in rats, with no neuronal cell loss or obvious behavioral deficits (Proctor, et al., 2014). The current study investigates the impact of much higher underbody blast-induced acceleration force of 1200 – 2800G on neuronal cell death, inflammation, levels of tight junction and synaptic proteins, behavioral impairments, and mortality at periods ranging from hours to 30 days post-injury.

II. Materials and Methods

II.1 Animals and their environment

All animal research protocols were approved by the University of Maryland, Baltimore Animal Use and Care Committee and the US Army Animal Care and Use Review Office. Male Sprague-Dawley rats (Harlan Laboratories, CA), 300-350 g were maintained under a controlled environment with an ambient temperature of $23 \pm 2^{\circ}\text{C}$, a 12 hr light/dark cycle, and continuous access to food and water *ad libitum*. Experimental groups consisted of 10 to 14 rats for behavioral studies, 4 to 8 rats for immunohistochemistry or biochemical analyses, and 8 – 14 rats for mortality incidence.

II.2 Exposure of rats to underbody blast-induced hyperacceleration force

Exposure of rats to underbody blast-induced hyperacceleration force was performed following scaling analysis to develop parameters to guide the design by a procedure modified from what we described previously (Proctor, et al., 2014, Zhao, et al., 2013). In brief, two rats were anesthetized concurrently with 4% isoflurane in room air for 5 min, allowing for placement within restraints without stress. Anesthesia was discontinued and the rats were secured, while still unconscious, within two fiberglass restrainers (Stoeling Inc., IL), with a custom addition of metallic cone to restrain the head and minimize secondary acceleration and head impact. The restrainers were bolted onto a 38 cm square and 2.5 cm thick aluminum platform. This platform was located immediately above a second 38 cm square aluminum platform that was either 5.0 cm thick (for 1200G acceleration force) or 2.5 cm thick (for 2800G acceleration). A 0.6 cm thick, hard rubber pad was present between the two platforms to dampen oscillatory acceleration forces. Both platforms had 2.5 cm wide holes located inside each of the four corners.

Aluminum rods, 2.0 cm wide and 90 cm high, were inserted through each of the holes and secured to a steel base on which the platforms rested, thus allowing for only direct movement of the platforms vertically following an underbody blast. The steel base was bolted to the edges of a steel tank filled with water to different stand-off distances from the bottom platform, resulting in relatively low acceleration force at large distances and relatively high acceleration force at short distances.

An explosive charge of pentaerythritol tetranitrate (PETN) was secured in the water at a fixed 5 cm depth of burial from the water line. The explosives weighing 0.75 g, for 1200G blasts, and 2.00 g, for 2400G and 2800G blasts, were detonated electromagnetically at exactly 5 min after anesthesia was discontinued. Parallel exposure of rats to 4% isoflurane for 5 min indicated that they were fully conscious 5 min after discontinuing the anesthesia. Explosion within the water ensured a non-compressible transfer of the explosive energy onto the bottom of the lower platform and subsequently to the upper platform where the rats were located. Accelerometers were placed on the top of the higher platform near the front of the rat restraints and the velocity, acceleration force, and JERK (first derivative of acceleration force) measured using UERD-Tools software (U.S NAVY). The peak acceleration force reached in these blast experiments was in the range of 0.30 to 0.50 msec. Pressure sensors were also placed near the head-end of the restraints during several experiments, verifying that the pressure changes experienced by the rats were less than one pound per square inch. Sham animals were also anesthetized with 4% isoflurane for 5 min, secured on the platform and removed 5 min later.

All blast and sham animals were returned to their cages immediately after the blast or sham procedure and examined for any physical injuries every 30 min for three hours. Necropsies were performed on all four animals that died immediately following the 2800G blasts.

II.3 Behavior

Y maze test

The Y maze is a Y-shaped platform that consists of three arms 50 cm long and 10 cm wide, each surrounded on three sides by 40 cm high walls. The open ends of the three arms are connected centrally and positioned radially at 120 degree angles (Stoelting Co., IL). The test was performed at approximately 1hr, and 6, 13 and 27 days post-injury, as previously described (Tchantchou and Zhang, 2013). Each rat was placed at the center of the maze and allowed to explore for 5 min. Movement was recorded using an overhead camera and analyzed using the Any-maze software (SD instruments, CA). Arm visit sequences and number of entries to each arm were recorded. Working memory was defined by the frequency of alternately exploring different arms and was determined using the formula: $\text{total number of alternations} / (\text{total number of arm entries} - 2) \times 100$.

Elevated Plus maze

This plus sign-shaped device consists of four perpendicular arms measuring 90 cm long and 10 cm wide, connected by a 10 cm x 10 cm central platform. Two of the arms are bordered on three sides by 20 cm high black plastic walls, while the other two arms together with the center of the maze are open. The arms are located 50 cm above the floor (Budde, et al., 2013). The test was performed on days 1, 8, 14 and 28 post-blast,

as previously described, with a minor modification (Budde, et al., 2013). In brief, rats were individually placed on the central area and allowed to explore the maze for 10 min. Movement was recorded by an overhead camera and the data were analyzed by Any-maze software (SD instruments, CA), to provide information including the time spent in each arm and the central area and the total distance travelled. Anxiety like behavior is inversely proportional to the time the rats spend in the open arms.

II.4 Tissue collection and processing

At different times following blasts or sham anesthesia (Fig. 1), rats were deeply anesthetized by intraperitoneal injection of a mixture of ketamine (160 mg / kg) and xylazine (120 mg / kg), and euthanized by exsanguination via transcardial perfusion. Anesthetized rats were initially perfused for 5 min with oxygenated artificial CSF containing 148 mM NaCl, 5 mM glucose, 3.0 mM KCl, 1.85 mM CaCl₂, 1.7 mM MgCl₂, 1.5 mM Na₂HPO₄, 0.14 mM NaH₂PO₄ (pH 7.4). Rats were then perfused with 500 ml fixative consisting of 4% paraformaldehyde in 50 mM K₂HPO₄ and 50 mM KH₂PO₄ (pH 7.4). The brains were removed, maintained in fixative for 24 hr, and transferred to 30% sucrose. After a few days when the brains sank to the bottom of their containers, each was sectioned in serial sections of 40 µm, preserved in cryoprotectant (66 mM NaH₂PO₄, 190 mM Na₂HPO₄, 0.87M sucrose, 30% ethylene glycol, and 1.25 mM povidone), and stored at -20°C for histology and immunohistochemistry.

For western blot analysis, ketamine-anesthetized rats were decapitated with a guillotine. Their brains were rapidly removed and dissected to yield the hippocampus, cortex and cerebellum, which were immediately frozen on dry ice and stored at -80°C.

II.5 Immunostaining

Immunohistochemical analysis of fixed, frozen sections was performed to determine the expression of inflammation biomarkers, F4/80 and inducible nitric oxide synthase (iNOS), and the apoptosis biomarker, cleaved caspase 3. Visualization utilized either fluorescence immunostaining or nickel diaminobenzadine (DAB) immunostaining. The number of dead or dying cells was quantified using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL).

a) Immunofluorescence staining

Brain sections were co-stained for the expression of cleaved caspase 3 and neuronal nuclear antigen (NeuN) or F4/80 and iNOS as previously described (Tchantchou, et al., 2007). In brief, free floating sections were rinsed in phosphate buffered saline (PBS), then blocked in 1% horse serum in PBS containing 0.3% Triton X for 1 hr. Sections were then transferred in a solution containing rabbit anti-Cleaved caspase-3 polyclonal antibody (1:10,000; Millipore, Temecula, CA) and mouse anti-NeuN monoclonal antibody clone, A60 (1:2000; Millipore, Temecula, CA) or rat anti-F4/80 monoclonal antibody (1:500; eBioscience, San Diego, CA) and rabbit anti-iNOS polyclonal antibody (1:3000; Millipore, Temecula, CA) and incubated at 4° C overnight. The sections were washed in PBS and incubated for 1 hr at room temperature in a mixture of corresponding secondary antibodies Alexa Fluor 488 or Alexa Fluor594 (1:1500; Invitrogen, NY). Sections were washed with PBS and counterstained with 4', 6-diamidino-2-phenylindole (DAPI).

b) Nickel DAB immunostaining

Brain sections were single-labelled for the expression of F4/80, using nickel DAB. Free floating sections were rinsed in PBS, blocked with 1% horse serum, and incubated with rat anti-F4/80 monoclonal antibody (1:15,000; eBioscience, CA) overnight at 4°C. Sections were washed and incubated at room temperature in **biotinylated goat anti-rat secondary antibody (1/2,000; Vector Laboratories Inc., CA)**, for 1 hr , followed by incubation in Vestastain solution and in nickel DAB and rinsed in PBS.

c) TUNEL assay

Floating sections were rinsed in PBS, transferred onto glass slides and allowed to air dry for 1 hr. They were rehydrated in PBS and stained to detect apoptotic cells marked by DNA fragmentation using a kit (Millipore, NY) and following manufacturer's instructions. DAPI used as a counterstain. All stained sections were mounted and examined under a Zeiss Axio-Imager microscope and images captured with x20 or x63 magnification objectives.

II.6 Western blot analysis

Western blotting was performed to determine the expression of Zona occluden (ZO-1), occludin, von Willebrand Factor (VWF), Bcl-2, heat shock proteins 70 (Hsp70), synaptophysin, PSD-95, spinophilin and beta-actin (used as loading control). The hippocampus, cortex and cerebellum were manually homogenized in lysis buffer containing 50 mM HEPES (pH 7.5), 6 mM MgCl₂, 1 mM EDTA, 75 mM sucrose, 1 mM dithiothreitol, 1% Triton X-100 and 1% protease/phosphatase inhibitor cocktail (Cell Signaling Technology, MA) and centrifuged at 10,000 G for 20 min. Proteins (20-30 µg) present in the supernatants were separated by electrophoresis on 4-12% SDS-polyacrylamide gels (Invitrogen, CA), then transferred to a nitrocellulose membrane.

Membranes were blocked with 1% BSA and incubated overnight at 4°C with rabbit polyclonal antibodies against VWF (1:400; Santa Cruz, CA), occludin (1:2000; Abcam, MA), spinophilin (1:500; Millipore, MA), synaptophysin (1:1000), PSD-95 (1:1000), Bcl-2 (1:750) all from Cell Signaling Technology, MA; a rat anti-ZO-1 monoclonal antibody (1:400, Millipore, MA) or mouse monoclonal antibodies against Hsp70 (1:750; Cayman chemical, MI) or β -actin (1:4000; Sigma, MO). Blots were washed in Tris-buffered saline with 0.1% TWEEN 20 and incubated for 1 hr at room temperature with corresponding HRP-conjugated secondary antibodies (1: 5000; Millipore, MA). Horseradish peroxidase labelled proteins was detected by enhanced chemiluminescence (ECL, Thermo Scientific, IL) and protein bands were visualized using a digital blot scanner (LI-COR, NE).

II.7 Quantification and statistical analysis

The optical fractionator method of stereology was employed to quantify cleaved caspase 3 or TUNEL positive cells in 40 μ M thick immune-stained sections, using the stereo-investigator software (MBF Bioscience, VT). A total of 6 sections selected to cover the entire hippocampus region, -1.60 mm to -6.3 mm from bregma, corresponding to every 12 serial sections were analyzed for each brain. For quantification, the dentate gyrus and the Cornu Ammonis 2/3 (CA2/3) sub-regions in the hippocampus of each brain hemisphere were demarcated. Using a grid spacing of 75 μ m x 75 μ m in the x and y-axis and guard zones of 2 μ m at the top and bottom of each section, immunopositive cell bodies were counted. The total number of cleaved caspase 3 or TUNEL positive cells in the hippocampal sub-region of interest was divided by the area to determine the cellular density per area unit, expressed as cells / mm².

Image J software (NIH) was used to measure immunoblot protein band signal intensity and the percent area of F4/80 immunostaining in brain tissue. Sections stained for F4/80 were optically segmented by threshold with the threshold greyscale value selected at 0.3 μm . These thresholded images were automatically measured for the relative percent containing positive F4/80 signal in ImageJ. This method is proportional to the percent area covered by F4/80 immuno-positive cells.

Statistical analyses were performed using GraphPad InStat 3 software (GraphPad Software, Inc., La Jolla, CA). Analysis of Variance (Chanda, et al.) together with Tukey-Kramer multiple comparisons post-test was used to compare differences among the multiple groups. Results are expressed as mean $\pm$ standard error of the mean (SEM). Statistical significance was defined as $p < 0.05$.

The individuals who performed the histologic, biochemical, and behavioral assays were blinded to animal group identifications, using codes that were revealed after data collection was completed.

III. Results

III.1 Blast-induced G force levels and mortality

Our previously reported underbody-blast experiments were conducted under conditions generating maximum acceleration forces in the range of 30 – 50G, with histologic evidence of TBI, but no obvious behavioral abnormalities, external injuries or mortality (Proctor, et al., 2014). In this study, the blast-induced G force was escalated to determine the maximum survivable underbody blast induced acceleration force and to characterize the effects of these higher blast levels on histologic and behavioral

measures of TBI. The acceleration force target for the first set of experiments was 1200G. Based on calibration curves generated with blasts using 0.75 g of PETN explosive present at different stand-off distances, we determined that a stand-off distance of 2.5 mm from the top of the water to the bottom of the lower platform would generate a peak acceleration force of approximately 1200G. The mean maximal force measured by accelerometers attached to the top plate near the head-end of the rat restraint was $1187 \pm 115\text{G}$ (SEM) for $n=10$ blasts. The change in acceleration force with time, referred to as JERK, was $0.48 \times 10^7 \text{ meters sec}^{-3}$. All rats survived these blast-induced G and JERK levels with no apparent external injury.

The second acceleration force target was 2400G. In order to reach this level, we used the same stand-off distance of 2.5 mm that generated 1200G forces, but increased the amount of PETN to 2.0 g. The mean maximal force generated under these conditions was $2460 \pm 76\text{G}$ for $n=6$ blasts. For these blasts, JERK was $1.09 \times 10^8 \text{ meters sec}^{-3}$. Although all animals survived these relatively high G forces, they exhibited lethargy and disorientation lasting several minutes immediately after the blast. These symptoms suggested that the 2400G force was close to the maximum survivable. We therefore set a third target of 2800G to test this hypothesis. In order to reach this level, we used the same 2.0 g of PETN and a stand-off distance of 2.5 mm but the mass of the two platforms was reduced by a net 33%, which increased the peak acceleration. The mean maximal force generated under these conditions was $2851 \pm 359\text{G}$ for $n=3$ blasts (6 rats). The JERK level was $1.77 \pm 0.33 \times 10^8 \text{ meters sec}^{-3}$. Four of the six rats subjected to this blast force died either immediately or within a few minutes after the blast. Necropsies performed on these animals revealed severe pulmonary

hemorrhaging and edema, without obvious damage to the heart, liver, or spleen. The two animals that survived this blast level were used for behavioral measurements through 28 days post injury.

III.2 Behavioral outcomes

Two different neurobehavioral tests were conducted to assess the effects of blast-induced acceleration on hippocampus-dependent memory and anxiety, respectively. The Y-maze test for memory was used on day zero, at one hour post-blast, and again on days 6, 13, and 27 (Fig 1). The plus maze test for anxiety was conducted on day 1, 8, 14, and 28. Each animal group consisted of n=10–14 rats.

a) Hippocampus-dependent working memory

The Y maze tests the innate ability of a rodent to continuously alternate visits in the three arms during the maze exploration. Rats with a good “working” memory ‘recognize’ the most recently explored arm of the maze and will successfully alternate arm visits approximately 70% of the time (Maurice, et al., 2009). The mean percent alternation for sham rats was only approximately 35% on day zero when tested one hour after brief anesthesia but improved to a normal range of between 65 and 75% on days 6, 13, and 27 days later (Fig. 2A). Rats subjected to 1200G blast force exhibited a similar alternation deficit on day zero and recovery on the subsequent test days to levels very similar to those of shams. In contrast, rats exposed to 2400G blast force displayed only 5% successful alternation on day zero, 30% on day 6, and between 50 and 65% on days 13 and 27. Spontaneous alternation by rats exposed to 2400G acceleration was significantly lower than that of shams on day zero ($p<0.05$) and day 6 ($p<0.001$) and lower than 1200G rats on both days zero and 6 ($p<0.05$) (Fig. 2A). The

two rats that survived the blasts that generated 2800G force exhibited spontaneous alternations of between 72% on day 13 and 80% on day 27.

b) Anxiety-like behavior

The elevated plus maze was used to test anxiety-like behaviors in rats subjected to blast-induced acceleration forces of 1200G and 2400G. Normal rats spend most of their time in the plus maze in the closed arms but display significant periods of inquisitive venture into the open arms. Rats that are interpreted as 'fearful and anxious' generally spend much less time in the open arms in comparison with normal rats. Sham rats spent more than twice as much time in the open arms on days 8, 14, and 28 compared to day 1 ($p < 0.05$) (Fig 2B). Animals that experienced either 1200G or 2400G acceleration spent between 10 - 30% as much time as shams in the open arms on days 8, 14, and 28 ($p < 0.05$) and did not improve over this period. There was also no significant differences in time spent in open arms by rats in the 1200 and 2400G acceleration groups.

In this paradigm, the decision to explore either the open arms or to enter a closed arm can only be made in the central zone where the open and closed arms cross. Rats subjected to both blast levels spent the same relative time in the central area (Fig. 2C). Total distance traveled was identical for sham and 1200G blasts; however 2400G blast rats traveled significantly less than both shams and 1200G rats on day 1 ($p < 0.05$) (Fig. 2D). The finding that the distance traveled by rats in the 2400G group was not significantly different than that of shams and rats in the 1200G group on days 8, 14, and 28 indicates that the deficit in time spent by the 1200 and 2400G groups in the open arms was not due to impaired motor performance. One of the two rats that survived the

2800G blasts spent essentially no time in the open arms on any of the test days but also exhibited total distance traveled that was less than any of the rats in the other groups. The other rat that survived the 2800G blast spent times in the open arms ranging from 26-38 sec during each of the four days of testing, which is similar to what was spent by rats in the 2400G group.

III.2 Accumulation of inflammatory cells

Activated microglia, macrophages, and astrocytes are commonly observed in other animal models of TBI (Ramlackhansingh, et al., 2011). To test whether our blast-induced hyperacceleration force TBI model results in neuroinflammation, we immunostained for activated microglia/macrophages present at one and seven days post-blast, using antibodies to the F4/80 marker protein. F4/80 immunostaining was observed primarily in perivascular and periventricular regions throughout the cortex (Fig. 3A) and hippocampus (not shown). Staining was quantified by expressing the total stained area as a percentage of the total area present in each coronal section. The number of animals per group ranged from n=4-8. Compared to shams that exhibited F4/80 immunostaining equivalent to $1.65 \pm 0.47\%$ of total brain area, rats subjected to underbody blasts that generated 1200G acceleration exhibited almost five-times greater F4/80 immunostaining at 24 hr post-blast ($7.62 \pm 1.35\%$; $p<0.05$) (Fig. 3B). Rats subjected to 2400G blasts exhibited 9 times as much staining for activated microglia/macrophages as shams ($15.07 \pm 1.28\%$; $p<0.01$). These results indicate a rapid extensive microglial / macrophage inflammatory response. However, by seven days post-injury, neuroinflammation measured by F4/80 subsided in both the 1200G

and 2400G blast groups ($3.58 \pm 0.88\%$ and $2.83 \pm 1.52\%$). Double labeling with antibodies to both F4/80 and inducible nitric oxide synthase (iNOS) confirm the pro-inflammatory identify of these cells (Fig. 3C) as iNOS is highly specific for pro-inflammatory cells, e.g., activated microglia and macrophages (Loane, et al., 2014, Tchantchou, et al., 2014). Secondary antibody specificity control measurements were performed by incubating brain sections from all experimental groups with the secondary antibody used in the nickel DAB or fluorescence staining, respectively. No significant immunoreactivity was observed in the presence of secondary antibody alone.

III.3 Tight junction proteins and vascular endothelial cell damage

Brain injuries, including those caused by models of open field explosions, have been associated with the alteration of vascular proteins, which play important roles in the maintenance of the blood brain barrier integrity (Abdul-Muneer, et al., 2013, Walls, et al., 2015). To determine if vascular protein levels are altered in response to underbody blasts, we performed western blot analyses of the tight junction proteins occludin and ZO-1 and the vascular endothelial damage marker von Willebrand Factor (VWF) in different regions of the brains of rats at 24 hr following blast or sham procedures. This time was chosen to coincide with the peak of neuroinflammatory cellular responses occurring primarily at perivascular and periventricular regions, as seen with F4/80 immunohistochemistry. Representative immunoblots for proteins present in the hippocampus, cortex, and cerebellum are shown in Fig. 4A, B, and C, respectively. The number of animals used for these assays ranged from n=4-8. Compared to sham animals, the expression of occludin was reduced by ~40% in the hippocampus ($p < 0.01$)

and cortex ($p<0.001$) of rats subjected to 2400G acceleration force intensity (Fig. 4D), with no significant change in the cerebellum (Fig. 4D). Blasts that generated 1200G force levels did not result in significant alterations of occludin expression. Furthermore, the expression of ZO-1 was decreased in the cortex ($p<0.01$) and hippocampus ($p<0.05$) of 2400G blast rats compared to sham rats (Fig. 4E). Similar to occludin, ZO-1 expression levels were not affected in the cerebellum or after 1200G blasts (Fig. 4E). von Willebrand Factor is a protein produced and released from vascular endothelial cells in response to stress. VWF expression was significantly increased in the hippocampus following 2400G blast ($p<0.05$) and in the cerebellum following 2400 and 1200G blasts (Fig. 4F; $p<0.05$).

III.4 Expression of the anti-apoptotic protein Bcl-2 and heat shock protein 70

In many models of TBI, neuroinflammation is accompanied by oxidative stress, which contributes to cell death cascades. We therefore examined the expression levels of the oxidative stress response protein Hsp70 and the anti-apoptotic protein Bcl-2 in different brain regions 24 hr following blast exposure. Representative immunoblots for these proteins present at 24 hr post-blast in the hippocampus, cortex, and cerebellum are shown in Fig. 5 A, B, and C, respectively. The number of animals used for these assays ranged from $n=4-8$. Following both 1200G and 2400G blasts, there was a 50% increase in Hsp70 expression in the hippocampus ($p<0.01$) and 30% increase in the cerebellum ($p<0.05$), compared to sham animals (Fig. 5E). In contrast, exposure to blast-induced acceleration forces of 1200 and 2400G dramatically reduced Bcl-2 immunoreactive protein levels in both the hippocampus, by 40% ($p<0.001$), and the

cerebellum, by 50% ($p < 0.001$) (Fig. 5D). Hsp70 and Bcl-2 levels in the cortex were unaffected by underbody blasts. Since a reduction in Bcl-2 can increase susceptibility to cell death, we quantified cell death in the hippocampus, using TUNEL staining, and immunohistostaining for active (cleaved) caspase 3.

III.5 Hippocampal cell death

Blast exposure at 1200G and 2400G acceleration forces increased the appearance of cleaved caspase 3 (C-casp-3) immunoreactive cells in the dentate gyrus (Fig. 6A) and the CA2/CA3 areas (not shown) of the hippocampus; however, very sparse staining for either biomarker was observed in the CA1 area (not shown). The number of animals used for these assays ranged from $n=4-8$. The number of cleaved caspase 3 positive cells present in the dentate gyrus was 11 ± 2 per mm^2 for rats exposed to 1200G acceleration force and 13 ± 3 per mm^2 for 2400G blast rats, in comparison to 2 ± 1 per mm^2 for sham animals ($p < 0.01$) (Fig. 6B). In the CA2/CA3 area of the hippocampus, the cell count was 7 ± 1 per mm^2 for 1200G blast rats ($p < 0.05$) and 11 ± 2 per mm^2 for 2400G blast rats ($p < 0.01$), as opposed to 1 ± 1 per mm^2 for sham rats (Fig. 6B). After counting 350 cleaved caspase 3 immunoreactive cells in the DG using a 63 x magnification objective, we found that 92% of these cells co-labeled with the neuronal marker NeuN (Fig. 6A).

Cleaved caspase 3 immunoreactivity reflects the presence of a caspase 3 dependent apoptotic pathway but is not a direct indicator of cell death. Additional evidence that exposure of rats to blast-induced acceleration forces of 1200 – 2400G came from quantification of TUNEL positive cells (Fig. 7A), which is a marker of DNA

fragmentation and late stage cell death. The number of TUNNEL-positive cells present in the DG at 24 hr post-injury was 8 ± 1 per mm^2 for rats exposed to 1200G blast intensity ($p < 0.001$) and 11 ± 2 per mm^2 for 2400G blast rats ($p < 0.001$) in comparison to 2 ± 1 per mm^2 for sham animals (Fig. 7B). Similar results were observed on the Ca2/Ca3 area where the count was 4 ± 1 per mm^2 for 1200G blast rats ($p < 0.05$) and 8 ± 1 per mm^2 for 2400G blast rats ($p < 0.001$), compared to 2 ± 1 per mm^2 for shams (Fig. 6B).

III.6 Expression of synaptic proteins

In addition to cerebrovascular damage, inflammation, and neuronal death, significant disruption to synaptic connectivity is a hallmark of even mild forms of TBI (Gao, et al., 2011, Przekwas, et al., 2016). Evidence suggestive of synaptic disruptions following exposure of rats to underbody blasts came from immunoblot measurements of the synaptic proteins PSD95, synaptophysin, and spinophilin, as shown in Fig. 8A. The number of animals used for these assays ranged from $n=4-8$. These analyses revealed significant, 25–50% reductions in the expression levels of synaptic markers PSD95, synaptophysin and of the dendritic spine marker spinophilin in different brain regions 24 hr following exposure to underbody blast that produced either 1200 or 2400G acceleration forces (Fig. 8 D-F). PSD95 levels were reduced in the hippocampus, cortex, and cerebellum ($p < 0.01$). Synaptophysin was reduced in the hippocampus ($p < 0.01$) and cerebellum ($p < 0.001$) but not the cortex. Spinophilin immunoreactivity was reduced in the hippocampus ($p < 0.05$) but not the cortex and was not detected in the cerebellum.

Table 1 provides a summary of the changes in levels of important proteins related to synaptic neurotransmission, cerebrovascular damage, and cell death that occur in different brain regions following exposure of rats to blast-induced accelerations of either 1200 or 2300G.

Discussion

Several conclusions can be drawn from our observations. First, high blast-induced acceleration loads result in a transient deficit in working memory and a chronic increase in anxiety, as measured using the Y maze and elevated plus maze, respectively. Second, a decrease in the levels of tight junction proteins taken together with an increase in VWF immunoreactivity suggest that high underbody blast loads result in cerebrovascular damage. Third, these blasts result in neuroinflammation, as reflected by increased perivascular and periventricular F4/80 immunopositive cells during the first week following injury. Fourth, underbody blasts cause significant neuronal death primarily within the hippocampus. Fifth, the significant reduction in both presynaptic and postsynaptic proteins that occurs within 24 hr following high blast loads presents the possibility that functional neuronal synapses are compromised. We previously reported significant axonal injury at lower underbody blast acceleration force (Proctor, et al., 2014).

Linear and rotational acceleration were two of the first forces postulated be primarily responsible for TBI caused by head impacts. While linear acceleration was found to produce mostly focal brain injuries caused by pressure gradient resulting in well-circumscribed intracerebral hematomas (Ommaya, et al., 1966, Zhang, et al.,

2001), rotational acceleration produced both focal and diffuse brain injuries caused by shear strain (Gennarelli, et al., 1982, Zhang, et al., 2001). In contrast, our underbody blast-induced vertical acceleration model does not produce any detectable focal lesions but rather diffuse damage to blood vessels, synapses, and hippocampal neurons. Previous work also provided qualitative evidence for diffuse axonal injury (Proctor, et al., 2014).

Impairments in Y and elevated maze tests could be impacted by motor deficits as well as by cognitive deficits. However, with the exception of the rats analyzed one day following 2400G blasts, there was no difference in the total distance traveled between sham and blast rats at 1 – 28 days post-blast in any of the behavioral metrics. Thus, motor impairments are unlikely to be complicating interpretation of behavioral alterations observed at 6 - 28 days after the blasts. The results obtained on day zero or one are possibly less reliable than those obtained at later times since the values in the shams for both tests were lower than the values obtained at later times. Most likely, relatively poor performance by shams on days zero or one is due to behavioral disruption from anesthesia or other stress during the first 24 hours.

The behavioral alterations apparent in our underbody blast model exhibit some of the characteristics observed in blast overpressure rodent models of TBI. For example, a single exposure of mice to open field overpressure blast causes significant deficits in cognition and spatial memory, both occurring seven days post-insult (Rubovitch, et al., 2011). In other studies, rodents subjected to overpressure as well as blast shock waves showed significant deficits in learning, spatial memory and increased anxiety at up to at least one month following blast exposure.(Cernak, et al., 2011, Kamnaksh, et al., 2012,

Vandevord, et al., 2012) Thus, either open field blast overpressure or under-vehicle blast-induced acceleration is sufficient to cause mild to moderate, long-term behavioral alterations. Thus, it may not be sufficient to protect from overpressure in vehicle design without considering the acceleration injury caused by transfer of kinetic energy to the vehicle.

Neurobehavioral impairments observed following TBI are often associated with markers of neuroinflammation. Neuroinflammatory mechanisms are emerging as a potential common effector of TBI damage to the brain, as well as markers of injury. In our model, we observed a blast dose-dependent increase in F4/80-positive, activated microglia and / or macrophages in perivascular and periventricular regions of the brain. F4/80 positive cells co-labeled with antibodies to inducible nitric oxide synthase (iNOS), which contributes nitrous oxide dependent exacerbation of brain damage caused by inflammation (Foley, et al., 2008). F4/80 immunostaining returned to nearly normal levels by 7 days post-blast suggesting the microglial phase of acceleration induced injury peaks rapidly following the blast.

A range of evidence supports the role of inflammation in the pathogenesis of open field blast-induced TBI. Observations include the increased expression of inflammatory mediators including macrophage inflammatory proteins, monocytes chemotactic proteins, IL-1 β , IL-6 TNF- α and INF- γ (Dalle Lucca, et al., 2012, Kamnaksh, et al., 2012, Kovesdi, et al., 2011, Tompkins, et al., 2013). cDNA microarray measurements indicate an increase in the mRNA levels of many pro-inflammatory genes including interleukins and TNF- α (Valiyaveettil, et al., 2013). At the cellular level, microglia activation and increase macrophage levels have been reported in the rodent

brains following blast exposure (Cho, et al., 2013, Dalle Lucca, et al., 2012, Sosa, et al., 2013, Tompkins, et al., 2013). In most of these studies, including ours, markers of inflammation responses from microglia occur acutely at 3-48 hours post-blast.

An elevation in the presence of inflammatory cells in the brain after TBI can be caused by endogenous microglial activation, migration, and proliferation. Systemically circulating macrophages can also invade the brain parenchyma, particularly if there is disruption in the blood brain barrier (BBB) and/or vascular endothelial cells. Two major components of tight junctions (Occludin and ZO-1), crucial in maintaining BBB integrity (Abdul-Muneer, et al., 2013, Armulik, et al., 2010), showed significant reductions in cortex and hippocampus. The mechanisms resulting in the reduction are unclear, but could be reduced synthesis rate or alterations in the proteolytic activity of enzymes such as the metalloproteinase-9, which is associated with effectors of BBB dysfunction following TBI (Higashida, et al., 2011). In contrast, an increase in VWF was observed primarily in the cerebellum after either 1200 or 2400G blasts. VWF activation may represent a compensatory mechanism to promote blood coagulation after vascular injury that occurs after TBI (De Oliveira, et al., 2007, Yokota, et al., 2002). The reduction in occludin and ZO-1 protein levels seen after underbody blasts is consistent with recent report by Abdul-Muneer and colleagues indicating that exposure of rats to a single blast-relevant shock wave caused a downregulation of tight junction protein expression, associated with increased levels of the oxidative stress markers 3-nitrotyrosine (3-NT) and 4-hydroxynonenal (4-HNE) (Abdul-Muneer, et al., 2013). In contrast to downregulation of occludin and ZO-1 proteins, an increase in the platelet adhesion regulating molecule VWF was only observed in the cerebellum. VWF

activation may represent a compensatory mechanism to promote blood coagulation after vascular injury (De Oliveira, et al., 2007, Yokota, et al., 2002) and be protective of this brain region. Additional studies are needed, however, to test whether these molecular changes are actually accompanied by BBB disruption.

We previously reported the absence of significant cell death in the brains of rats following underbody blasts at low G forces (50G). However at the higher accelerations used in the present study (1200 – 2400G) there was significant cell death. Some studies have concluded apoptosis in different brain regions following exposure to blast that correlated with cognitive impairments associated with function of those regions (Kobeissy, et al., 2013, Kwon, et al., 2011, Vandevord, et al., 2012). We detected the presence of apoptotic cells in the hippocampus, which was associated with a decrease in Bcl-2 expression and an upregulation of the stress response protein Hsp70. Although the increased expression of Hsp70 may represent an attempt to protect against the deleterious effects of oxidative and other forms of stress (Adachi, et al., 2009, Kim, et al., 2015), the downregulation of the anti-apoptotic protein Bcl-2 is very likely to promote apoptotic and other forms of cell death. While loss of viable neurons in the hippocampus may contribute to deficits in working memory and anxiety like behavior, the effects of under-vehicle blasts on other factors, including axonal and synaptic neuroanatomy and neurophysiology may also contribute to behavioral alterations.

Adequate synaptic transmission is clearly required for the maintenance of neurobehavioral functions (Shankar and Walsh, 2009, Stancu, et al., 2014). Loss of synapses and dendritic spine has been reported in models of focal brain injury shortly after trauma and affect learning and memory performance (Gao, et al., 2011, Scheff, et

al., 2005). Interestingly, in our blast model, we observed decreased expression of synaptic markers synaptophysin and PSD-95 and that of the dendritic spine marker spinophilin, which all suggest a deficit in synaptic transmission. However, similar to changes seen with the expression of tight junction proteins between shams and blast rats, their expression was heterogeneous in different regions of the brain with a decrease observed in the hippocampus and little or no change in the cortex. This heterogeneous expression pattern could be due to anatomical differences in various brain regions and differences in sensitivity to excitotoxic insult (Geddes, et al., 2003). Prior studies in models of experimental brain trauma demonstrated increased vulnerability of hippocampal neurons and cerebellar purkinje cells to trauma (Anderson, et al., 2005, Igarashi, et al., 2007, Nawashiro, et al., 1995).

The degree to which our model applies to the brain injury suffered by humans present within vehicles targeted by underbody blasts is difficult to define, based on the complex array of forces that participate in these brain injuries and the tremendous difference in the mass of rats and humans. Based simply on the equation that force equals mass times acceleration, the 2400G experienced by our 350 g rats would be equivalent to 11G for a 75 kg human. Since the brain is the organ of interest in our study, the same scaling projects that the load of 2400G on a 2 g rat brain is equivalent to 4G on a 1300 g human brain. While most studies indicate that exposure to linear acceleration of less than or equal to 10G for a short period is unlikely to produce chronic or possibly even acute neuropsychologic symptoms, the conditions used to model linear acceleration are very different than those produced by an explosion under the belly of a vehicle. For example, the peak acceleration of 2400G induced by the underbody

explosion in our model was reached within 0.3 msec, which is at least two orders of magnitude shorter than duration when the maximum G force is reached during motor vehicle collisions. Thus, the rate at which the acceleration changes, known as JERK, may be an even more important factor than simply maximum G force. This hypothesis is supported by the fact that when we increased the peak G force by only 17% from 2400 to 2800G by reducing the weight of our “vehicle” the JERK rose by 62% and was followed immediately by death to four out of 6 rats and severe, chronic anxiety to one of the two surviving rats. Despite these scaling issues, we believe that insight into the pathophysiology of brain injury caused by underbody blast-induced acceleration is a very important addition to the knowledge gained from animal models of TBI caused by shock waves, which is more relevant to blast induced injury to the brains of unmounted warfighters.

Conclusions

In summary, our results demonstrate that high acceleration force induced brain injury altered the expression of proteins which function is crucial to maintain blood brain integrity, potentially leading to increased accumulation of inflammatory cells in the brain. Blast-induced brain trauma also caused neuronal cell loss in the hippocampus associated with reduced expression of proteins which modulate synaptic transmission. The neurologic consequences of these neuropathological alterations are a transient deficit in working memory and a chronic change in behavior interpreted as anxiety or depression. Overall, findings from our unique model of underbody blasts indicate that exposure to acceleration force alone is sufficient to cause at least mild TBI relevant to

what survivors of under-vehicle explosions often experience. Work is in progress to develop new vehicle hull designs that dramatically reduce the force that these blasts transfer to the occupants, thus reducing both mortality and injury to the brain and other vital organs.

Figure legends

Figure 1. Experimental design and timeline

Detailed timeline of behavioral tests and end points of experimental procedures starting on study day 0 (blast exposure). YM, Y-maze test; PM, Plus-maze test; H, Histology, WB, western blots.

Figure 2. Behavioral outcomes after underbody blast

(A) Y-maze test results demonstrating significantly lower spontaneous alternation among maze arms by 2400G blast rats than shams or rats exposed to 1200G acceleration force (*,[#]p < 0.05 for day 0-pi and [#]p < 0.05, **p < 0.01 for day 6 post-injury (pi) respectively). (B) Plus maze test demonstrating that rats exposed to either 1200 or 2400G blasts spent significantly less time in the open arms on days 8, 14 and 28-pi compared to shams (*p < 0.05, **p < 0.01 and ***p < 0.001). There were no significant differences in the time spent in the central area (C) and in the total distance travelled (D) among blast and sham rats (p > 0.05), except on day 1 post-injury when 2400G blast rats travelled significantly less distance than sham and 1200G blast animals (*,[#]p < 0.05). For both the Y maze and the Plus maze tests, the number of rats used in each experimental group was shams (n=10), Blast 1200 G (n=14) and Blast 2400 G (n=14)

Figure 3. Accumulation of inflammatory cells in the brain

(A) Representative microphotograph exhibiting F4/80 immuno-positive cells in the perivascular and periventricular regions of blast rats. (B) Quantification of the percent of F4/80 immuno-reactive area indicating a significant increase in microglia/macrophage

accumulation in the brain of 1200G and 2400G blast rats compared to shams 24 hr post-trauma (*p < 0.05 and **p < 0.01 respectively). (C) F4/80 immunopositive cells (Red) co-expressed the pro-inflammatory enzyme iNOS (Green); and DAPI used as counterstain. Experimental groups consisted of Sham (n=4), Blast 1200 G (n=8), Blast 2400 G (n=8) for each time point.

Figure 4. Vascular injury biomarkers

(A-C) Representative immunoblots illustrating the expression of Occludin, ZO-1, VWF and beta actin in the hippocampus, cortex and cerebellum 24 hr post-injury. Quantification of proteins bands indicating that (D) Occludin expression was significantly reduced in the hippocampus and cortex of 2400G blast rats compared to shams (**p < 0.01 and ***p < 0.001 respectively). (E) ZO-1 expression was significantly decreased in the cortex of 2400G blast rats compared to shams (**p < 0.01). In contrast, ZO-1 levels were significantly lower in the hippocampus of 2400G blast rats compared to 1200G blast rats (#p < 0.05). (F) Expression levels of VWF in the hippocampus were significantly higher in 2400G blast rats versus shams (*p < 0.05). In addition, VWF levels were also significantly higher in the cerebellum of 1200G and 2400G blast rats compared to shams (*p < 0.05). Experimental groups included Shams (n=6), Blast 1200 G (n=6) and Blast 2400 G (n=6).

Figure 5. Reciprocal changes in the levels of Hsp70 stress response protein and Bcl-2 anti-apoptotic protein in different brain regions following blast exposure

(A-C) Representative immunoblots describing the expression of Hsp70, Bcl-2 and beta actin in the hippocampus, cortex and cerebellum. Quantification of proteins bands signal intensity indicated that (D) Bcl-2 expression was significantly reduced in the hippocampus and cerebellum of rats exposed to 1200G blasts ($***p < 0.001$) as well as 2400G blasts ($***p < 0.001$), respectively, versus shams. (E) Conversely, the expression of Hsp70 was significantly increased in the hippocampus and cerebellum of 1200G blast rats ($**p < 0.01$) and 2400G blast rats ($**p < 0.01$ and $*p < 0.05$), respectively, compared to shams. Number of rats in each experimental group was Shams (n=6), Blast 1200 G (n=6) and Blast 2400 G (n=6).

Figure 6. Cleaved caspase-3 immunoreactive cells present in rat hippocampus

(A) Representative fluorescent image showing the expression of cleaved caspase-3 (Green) in DG cells that overlap with NeuN positive cells (Red) and DAPI. (B) Quantification of cleaved-caspase-3 immunopositive cells 24hr post-insult indicated a significant increase in positive cells in the DG and Ca2/Ca3 area of both 1200G ($**p < 0.01$ and $^{\#}p < 0.05$) and 2400G ($**p < 0.01$ and $^{\#\#}p < 0.01$) blast rats, respectively, compared to shams. Experimental groups included Shams (n=4), Blast 1200 G (n=8) and Blast 2400 G (n=8).

Figure 7. TUNEL positive cells in rat hippocampus

(A) Representative fluorescent image demonstrating the presence of TUNEL positive cells (Green) and DAPI-stained nuclei in the DG. (B) In comparison with shams, there

was a significant increase in TUNEL positive cells 24hr post-insult in the DG and Ca2/Ca3 areas of 1200G ($***p < 0.001$ and $^+p < 0.05$) and 2400G blast animals ($***p < 0.001$ and $^{+++}p < 0.001$) respectively. Experimental groups were Shams (n=4), Blast 1200 G (n=8) and Blast 2400 G (n=8).

Figure 8. Brain region selective reduction in the expression of synaptic proteins following exposure to blasts

(A-C) Representative immunoblots illustrating the expression of PSD-95, synaptophysin, and spinophilin in the hippocampus, cortex, and cerebellum. (Spinophilin was not detectable in the cerebellum). Quantification of proteins bands indicate that (D) PSD-95 expression was significantly reduced in the hippocampus, cortex and cerebellum of both 1200G and 2400G blast animals ($**p < 0.01$). Conversely, PSD-95 expression was only significantly affected in the hippocampus of rats exposed to 2400G blast ($**p < 0.01$). (E) Synaptophysin expression was dramatically reduced in the hippocampus and cerebellum of 1200G ($**p < 0.01$ and $^{###}p < 0.001$) and 2400G blast rats ($**p < 0.01$ and $^{###}p < 0.001$), respectively, in comparison with shams. (F) Spinophilin expression was only significantly affected in the hippocampus of 1200G and 2400G blast rats ($**p < 0.01$ and $^*p < 0.05$ respectively). Number of animals in each experimental group was Shams (n=6), Blast 1200 G (n=6) and Blast 2400 G (n=6).

Table 1: Recapitulation of changes in proteins expression in hippocampal, cortical and cerebellar tissues following exposure to underbody blast compared to shams.

(▼: significantly decreased; ▲: significantly increased; —: no significant change; **ND**: not detected).

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Proteins	Acceleration G force					
	1200G			2400G		
	Hippocampus	Cortex	Cerebellum	Hippocampus	Cortex	Cerebellum
ZO-1	—	—	—	—	▼	—
Occludin	—	—	—	▼	▼	—
VWF	▲	—	▲	—	—	▲
Hsp70	▲	—	▲	▲	—	▲
Bcl-2	▼	—	▼	▼	—	▼
PSD-95	▼	▼	▼	▼	—	—
Synaptophysin	▼	—	▼	▼	—	▼
Spinophilin	▼	—	ND	▼	—	ND

Table 1: Summary of changes in levels of proteins present in the hippocampus, cortex, and cerebellum 24 hr following exposure to underbody blast-induced accelerations of either 1200G or 1400G. (▼: significantly decreased; ▲: significantly increased; —: no significant change; ND: not detected).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8