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**EFFECTS OF MULTIPLE FLIGHTS FOLLOWING IMPACT-
AND BLAST- INDUCED TRAUMATIC BRAIN INJURY ON
LONG-TERM OUTCOMES PART 1**

**Molly Goodfellow, Julie Proctor, Amanda Hrdlick, Boris Piskoun
Parisa Rangghran, Su Xu, Rao Gullapalli, Ulrich Leiste,
William Fourney, Gary Fiskum**

**Center for Shock, Trauma and Anesthesiology Research
University of Maryland, Baltimore**

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711TH HUMAN PERFORMANCE WING,
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WRIGHT-PATTERSON AIR FORCE BASE, OH 45433
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ALICIA BURKE

Program Manager
Product Development Branch
Airman Biosciences Division

//signature//

MATTHEW DALTON, DR-III

Product Area Lead, En Route Care Section
Product Development Branch
Airman Biosciences Division

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1.0 EXECUTIVE SUMMARY

Background: Under-vehicle blast (UVB)-induced traumatic brain injury (TBI), generated from improvised explosive devices (IEDs), is a unique etiological mechanism of TBI affecting warfighters present in vehicles targeted by blasts. The hyperacceleration load caused by an IED-generated UVB can directly result in a TBI independent of an impact event, however, a secondary head impact injury in the cabin can also occur. Rodent models of TBI recapitulate the cellular pathophysiology and neurobehavioral deficits seen in humans, however, successfully translating these findings to improved patient outcomes is challenging. One explanation could be the neuroanatomical differences between humans and rodents, with the latter lacking the gyri and sulci present in the former. To address this modeling difference, a ferret model of TBI was developed to evaluate the effects of Blast-alone, controlled cortical impact (CCI)-alone, and combined blast + CCI (BCCI).

Methods: The protocol was reviewed and approved by the University of Maryland, Baltimore Institutional Animal Care and Use Committee (0620009) and the U.S. Air Force Surgeon General's Office of Research Oversight and Compliance as protocol number FWR-2020-0015A. These studies were conducted in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), in accordance with the Guide for the Care and Use of Laboratory Animals (NRC, 2011) and were performed in compliance with DODI 3216.1. Adult male ferrets were either exposed to Blast-alone (n=13) or CCI-alone (n=8). Once the blast level (G force) and CCI depth were finalized, a third set of ferrets were exposed to that combination of BCCI (n=8) or sham injury (n=5). Neurobehavioral assays were optimized, and the final protocols were applied to a subset of these animals (n=6-8 BCCI and n=5 sham). Blast-alone (n=5) and BCCI (n=5) ferrets underwent magnetic resonance imaging (MRI)/ magnetic resonance spectroscopy (MRS) scans at baseline and 7 days post-injury. All ferrets were euthanized by terminal perfusion with paraformaldehyde on day 7 and brains histologically analyzed for blood-brain barrier (BBB) disruption (immunoglobulin G (IgG)), diffuse axonal injury (amyloid precursor protein (APP)) and necrotic divot/inflammatory penumbra volume (ionized calcium-binding adaptor molecule 1, Iba1).

Results: All animals survived until terminal perfusion. Using immunohistology, we showed that, compared to shams, the Blast-alone group exhibited BBB disruption, indicated by punctate IgG staining, primarily localized to the ventral aspect of the brain. A CCI-alone also produced BBB disruption, however, the pattern and distribution were different than the Blast-alone group, with diffuse staining localizing to the cortex, preferentially presenting in the white matter. Additionally, CCI-alone injury resulted in robust diffuse axonal injury compared to sham and blast groups, demonstrated by APP staining, near the site of impact and underlying gray matter. The combination of injuries did not exacerbate IgG or APP staining. In addition, BCCI did not increase the size of the necrotic divot or inflammatory penumbra, as measured by morphologic changes in microglia (Iba1 immunohistochemistry).

Cortical neurochemicals measured by MRS near the site of impact were affected by the Blast-alone and BCCI differently at 7 days post-injury. Although gamma aminobutyric-acid (GABA), glutamate (Glu), and total creatine (tCr) were significantly decreased in both models compared to their baseline, these biochemicals were lower in the BCCI group than those in the

Blast-alone animals. Reductions in these neurotransmitters (Glu and GABA) and energy buffer (tCr) in the BCCI group may indicate impaired neurotransmission and energy generation. In addition, there were significant changes in glutamine (Gln), N-acetylaspartate (NAA), glutathione (GSH), and total choline (tCho) in the BCCI group compared to its baseline. The elevated Gln suggests neurotoxicity in the impact area. Decreased NAA and increased tCho indicate increased neuronal injury and cell death. High levels of GSH reflect oxidative stress. Interestingly, myo-inositol, a marker for glial content, increased only in Blast-alone ferrets suggesting increased glial activation in this group. BCCI animals also showed significant edema ipsilateral to the injury relative to Blast-alone and baseline, as evidenced by increased areas of hyperintensity on MRI scans.

Neurobehavioral alterations were also apparent in BCCI animals relative to shams. On the hippocampus-dependent Object Location Test, BCCI animals exhibited a spatial memory deficit by showing no preference for an object in a novel location whereas shams explored this object significantly more than an object in a familiar location. BCCI ferrets also demonstrated hyperactivity, traveling significantly greater distances in the Open Field arena than shams. While no significant differences were noted in thigmotaxis (an assay of anxiety-like behavior), play behavior (mood), or slips on a horizontal ladder (motor function), data did show a clear trend toward impairment in BCCI animals, suggesting that larger group sizes may provide sufficient power to detect between-group differences. No significant impairments were noted in perirhinal cortex-dependent novel object recognition. Two assays (Light-Dark Box and Escape Test) did not provide compelling or reliable results.

Conclusions and future directions: To our knowledge this is the first ferret polytrauma model combining TBI with UVB. Results indicate that a single UVB is sufficient to induce acute BBB disruption. CCI-alone leads to both BBB disruption and cortical diffuse axonal injury but this is not exacerbated by combination with UVB. BCCI does, however, result in several alterations in key cortical metabolites indicative of increased neuronal injury, oxidative stress, and glial activation as well as impaired neurotransmission and energy generation. Additionally, we found evidence that BCCI in ferrets increases hyperactivity, as well as impairs spatial memory. Taken together, we provide a military, combat-relevant model of UVB in a gyrencephalic animal, the ferret. Our results are like that which is reported in service members with blast-associated TBI regarding the neuro- pathophysiology and behavioral sequela. The results presented here will be used to address military-relevant questions on the effects of aeromedical evacuation (hypobarica) on secondary injury development to determine protocols for safe transport of service members following TBI.

2.0 INTRODUCTION

Warfighter and civilian TBI can lead to significant neurologic morbidity, including delayed and chronic neuropsychiatric problems often labeled as chronic traumatic encephalopathy (CTE). Approximately twenty percent of all military personnel serving in the recent conflicts in the Middle East have suffered some form of TBI. Those who undergo moderate to severe TBI or mild TBI in combination with trauma to other organ systems are often air-evacuated for 5-10 hours (hrs) to Landstuhl Regional Medical Center within one day post-injury. Air Force flight surgeons and Critical Care Air Transport Team (CCAT) personnel have expressed concern that stressors associated with aeromedical evacuation (AE) might worsen clinical outcomes. Specifically, there is fear that prolonged exposure to the hypobaria that is normally present in airplane cabins (equivalent to 8000 feet (ft) altitude) could adversely affect damaged organs, such as the brain, lungs, gut, and kidneys.

Researchers at the University of Cincinnati, University of Maryland, and the Naval Medical Research Center (NMRC) have responded to these concerns by testing the effects of AE-relevant hypobaria in animal models of TBI.¹⁻⁶ Using four different TBI models and three different species (mouse, rat, and pig) these labs found that exposure of animals to 6 hrs of hypobaria equivalent to 8000 ft within hours to days after injury results in worse outcomes, including behavior, neuronal death, brain oxygenation, and inflammation. Depending on the outcome measure, these effects of hypobaria were observed within hours to four weeks following injury. One of the University of Maryland, School of Maryland paradigms is mild brain injury in rats caused by hyperacceleration (up to 2400G) associated with under-vehicle explosions. This unique blast TBI model is a highly combat-relevant model that can easily be used for long-term outcome measures.⁶⁻⁹ To maximize the clinical relevance of this blast TBI model, we combined the UVB-induced hyperacceleration with the CCI TBI model (hereafter referred to BCCI).

Ferrets and humans, but not rats, have gyrencephalic brains, characterized by extensive cerebrocortical convolutions. Histologic studies on the brains of humans diagnosed with CTE suggest a unique neuropathology characterized by hyper-phosphorylated Tau depositions localized at the base of cerebral sulci, which do not exist in the lissencephalic brains of rodents.^{10, 11} We therefore hypothesize that ferrets subjected to moderate, UVB induced TBI followed by multiple flights during the 6 months post-injury will exhibit histologic features of cortical sulci neuropathology and a delayed increase in abnormal MRI/MRS patterns and neurobehavioral alterations. The overall goal of the present study was to develop a ferret BCCI model that will be used in subsequent experiments to assess the long-term effects of multiple flights after blast polytrauma. Methodology was established for injury induction, MRI/MRS, neurobehavioral testing, and histopathologic analysis.

Gaps/Requirements: Various aspects of the ferret BCCI model needed to be established to proceed with the full multiple flights study. The following milestones/deliverables were addressed in the present project:

1. Conduct experiments using ferrets to reach an UVB level of acceleration that produces alterations in behavior and/or MRI measurements and/or neuropathology by 7 days post-injury
2. Determine which antibodies for diffuse axonal injury, inflammation, BBB dysfunction, etc. provide reliable, quantitative results for histologic outcome measures

3. Determine which neurologic tests provide reliable, quantitative assays for abnormal ferret behavior
4. Compare short-term neuropathology and behavioral alterations for ferrets exposed to blast alone, head impact (CCI) alone, and blast plus head impact (BCCI)

3.0 METHODS

3.1 Study Protocol

The protocol was reviewed and approved by the University of Maryland, Baltimore Institutional Animal Care and Use Committee (0620009) and the U.S. Air Force Surgeon General's Office of Research Oversight and Compliance as protocol number FWR-2020-0015A. These studies were conducted in a facility accredited by AAALAC, in accordance with the Guide for the Care and Use of Laboratory Animals (NRC, 2011) and were performed in compliance with DODI 3216.1.

3.2 CCI TBI Model

Using a well-characterized CCI model,¹²⁻¹⁴ isoflurane-anesthetized male ferrets (n=8) were subjected to a craniotomy followed by a single impact to the left parietal cortex at a depth of 4.0-6.0 millimeters (mm) with a Leica Impact One Stereotaxic Impactor (Leica Biosystems, IL). After injury, the bone flap was replaced, sealed with dental acrylic, and the scalp was sutured closed.

3.3 UVB-Induced TBI Model

Dexmedetomidine-sedated ferrets (n=12) were briefly anesthetized with isoflurane and loaded into individual, custom made restrainers affixed to the top blast plate, where they were permitted to awaken. Then, 1.75-2.5 grams (g) of pentaerythritol tetranitrate (PETN) explosive was detonated beneath the plate where sedated but conscious ferrets are secured, generating a peak acceleration of 2000-2400 G as previously published for rats.^{8,9} Ferrets were removed from the restraining device immediately following detonation.

3.4 UVB Plus CCI (BCCI) Model

Immediately following the exposure to UVB as above, ferrets (n=8) were re-anesthetized and received a secondary head injury using the CCI model as above.

3.5 Sham Injury

Isoflurane-anesthetized ferrets (n=5) received a craniotomy but no blast or impact.

3.6 Neurobehavioral Tests

Neurobehavioral tests were conducted with ferrets prior to injury and/or at 6 days post-injury. Ferrets were subjected to a combination of tests to assess mood (behavioral observation, open field), motor function (ladder walk, open field), and memory (object location and novel object recognition). AnyMaze (Stoelting, IL) and Behavioral Observation Research Interactive Software (BORIS)¹⁵ were used to analyze data. Assays were optimized throughout the experiment so results shown later in this report will only include data from the finalized protocols. These finalized protocols will be described in the results section.

3.7 MRI/MRS

MRI and MRS measurements were conducted with a subset of Blast-alone and BCCI ferrets (n=5 per group). There were two measurement times: a Baseline measurement, conducted 3 days prior to injury, and a Subacute Injury measurement, conducted 7 days post-injury.

3.8 Neuropathology

All ferrets were euthanized at 7 days post-injury followed by perfusion fixation. Specifically, per the animal protocol, the animals were deeply anesthetized with a cocktail of ketamine, xylazine, and acepromazine and then euthanized by transcardial perfusion. Brains were collected and processed in a manner permitting quantification of multiple neuropathological markers. Based on our recent publications, *rats* exposed to the uniquely military-relevant model of UVB demonstrate histopathological evidence of diffuse axonal injury, BBB dysfunction, and astrocyte/microglial activation which varies with increasing acceleration. We, therefore, focused on these histopathologic markers following BCCI.

Estimates of the volume/area of immunohistochemically stained regions of brain tissue as well as the necrotic divot were made using the Cavalieri method. Analysis was performed using a Axio Imager 2 motorized microscope (Zeiss, Germany) under 10x magnification by a microscopist blinded to animal treatment group. A randomly generated and placed 25 micrometers (μm) $\times$ 25 μm grid by Stereo Investigator software (MBF Biosciences, VT) was then used to calculate volumes (cubic millimeters - mm^3).

3.9 Data Analysis

Analysis of differences between groups was conducted using one-way, two-way, or repeated measures analysis of variance (ANOVA). *Post-hoc* multiple comparisons used the Fisher's least significant difference (LSD) test and differences between groups at $p < 0.05$ were considered significant. Assumptions of normality and equal variance were tested using the Shapiro–Wilks and Brown-Forsythe methods, respectively, prior to analyzing data. A one-sample *t* test was used to analyze preference in the object location/recognition tests. All analyses were conducted using SigmaPlot® statistical software (Systat, CA).

4.0 RESULTS

4.1 Injury Induction

Initial histopathological analyses of the CCI injury at a 4mm depth did not achieve sufficient severity to be considered a moderate injury. Therefore, the CCI depth was increased to 6mm. For the blast, n=9 animals received Blast-alone and n=2 animals received BCCI with a 1.75g PETN explosive. Unlike rats from previous studies, we did not note any immediate effects of the blast on the health of the animal (e.g. bleeding, labored breathing, mortality, etc.). Therefore, the explosive was increased to 2.5g (the maximum possible size for our facility) for an additional n=3 Blast-alone and n=6 BCCI. All animals survived until the designated terminal endpoint (7 days post-injury).

4.2 Neurobehavioral Outcomes

Neurobehavioral assay protocols were refined in the early stages of the experiment, leading to finalized methods for n=6-8 BCCI animals and n=5 sham injured animals. Data collected before methodologies were finalized is not shown.

4.2.1 Behavioral Observation (Mood)

Ferrets were released into the behavior testing suite for a period of exercise. The first five minutes of this period was filmed and analyzed for the latency to exit the carrier and to express species-specific play behavior according to the ethogram shown in Table 1.¹⁶

Table 1. Behavioral Observation Ethogram

Behavior	Description
Dooking	The animal emits a series of hoot/chuckle vocalizations. Excludes single or very short (<2 sec) strings of vocalizations.
Postural Change	The animal changes from a flat back to an arched back.
Piloerection	The animal's fur stands on end on its tail and/or back.
Weasel War Dance	The animal starts bouncing from side to side. They may shake their head or fall over. Excludes running or galloping.
Open Mouth	The animal opens its mouth. The upper teeth must be covered by the top lip.

An increased latency to exit and/or express these behaviors may be associated with changes in mood, putatively increases in either depressive-like or anxiety-like behavior (Figure 1).

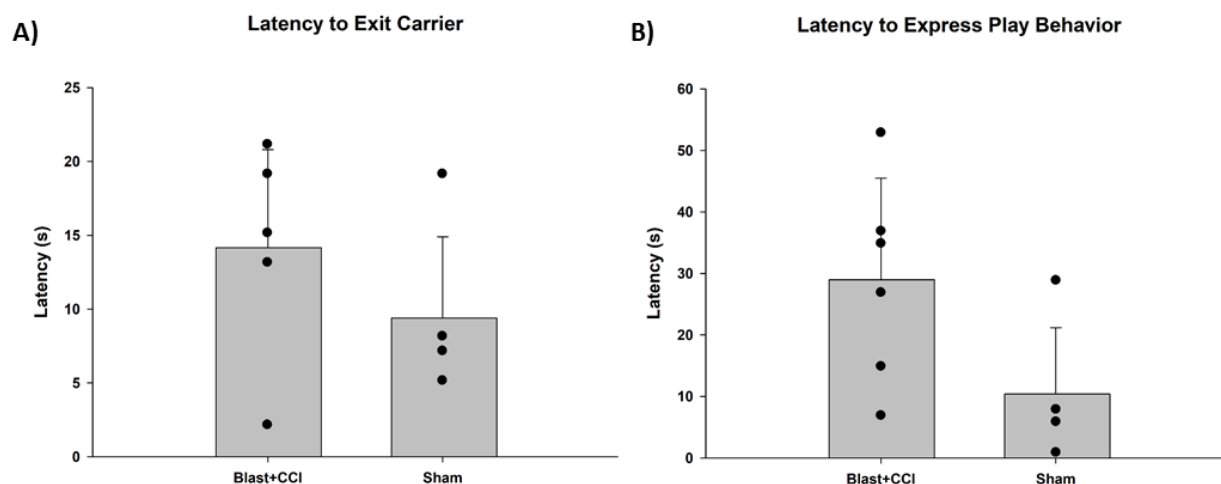


Figure 1. Summary of behavioral observation data.

A) Latency to exit the pet carrier (mean $\pm$ SE). No significant differences were noted between groups in latency to exit the carrier at 6dpi ($p=0.234$). B) Latency to express at least one play behavior (mean $\pm$ SE). The difference in latency was marginally significant ($p=0.059$). This suggests that, with increased n , it may be possible to detect changes in mood after BCCI.

4.2.2 Escape Test (Mood)

Ferrets were placed into an upright cuboid box for ten minutes. Their behavior was filmed and analyzed according to the ethogram shown in Table 2.

Table 2. Escape Test Ethogram

Behavior	Description
Resting	The animal lies down on the ground, keeping mostly still (may twitch or move slightly). They are not actively engaged with surroundings. Measured in frequency and duration. The latency to the final rest (after which no other behaviors occurred for the duration of the test) was noted.
Rearing	The animal stands on hind legs but does not put front paws on the wall. Measured in frequency.
Wall-Rearing	The animal stands on hind legs and puts front paw(s) on the wall of the enclosure. Measured in frequency.
Climbing	The animal digs at the wall of the enclosure but their hind legs do not leave the ground. Measured in frequency.
Digging	The animal digs into or toward the ground with front paws. Measured in frequency and duration.
Jumping	The animal jumps up toward the wall, hind legs leaving the ground. Their front paws are upward and steady. Measured in frequency.
Exploring	The animal moves minimally, most of the body is on the ground, explores the bottom/sides of the structure with their nose/mouth. They are not climbing, jumping, or rearing. Measured in duration.

Similar to the Porsolt Forced Swim Test in rodents,¹⁷ a decreased latency to immobility or to stop exploring/attempting to escape may be indicative of increased depressive-like behavior. Overall, the results on this test were not compelling. There was marked variability within groups and no trends were notable (Figure 2).

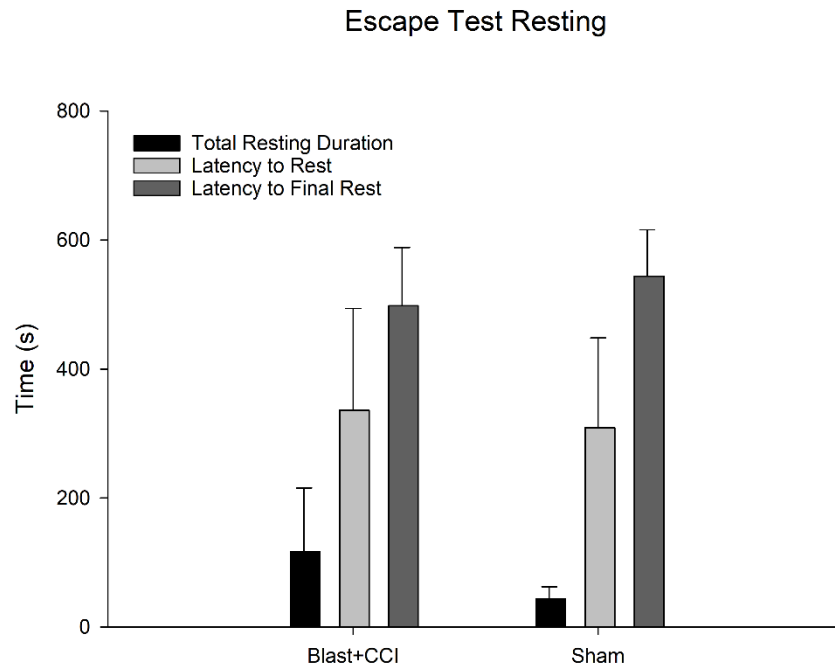


Figure 2. Escape test resting.

Resting behavior (mean $\pm$ SE) during the Escape Test. No trends were notable in the total resting duration, the latency to rest, or in the latency to final rest.

4.2.3 Open Field (Mood and Motor Function)

Animals were placed into an Open Field arena and allowed to explore for 10 minutes. Their total distance traveled as well as time spent in the center zone vs. the perimeter of the arena (also known as thigmotaxis, a behavior linked to anxiety in many species)¹⁸ was calculated using AnyMaze software (Figure 3).

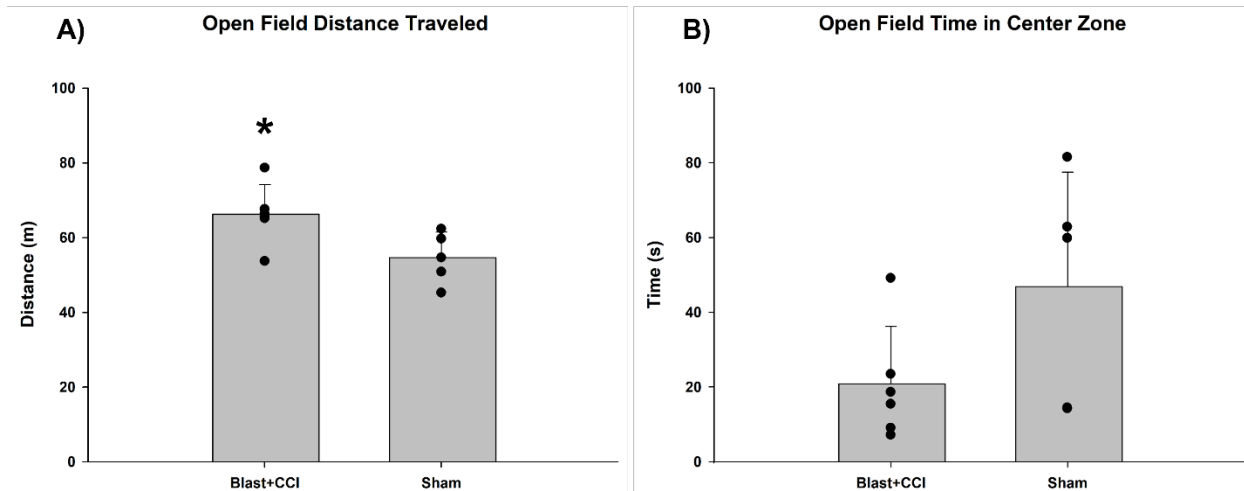


Figure 3. Open field analysis.

A) Distance traveled (mean ± SE) in the Open Field. BCCI animals traveled significantly further than shams ($F(1,9)=6.59$, $p<0.05$). This indicates the BCCI injury may induce acute hyperactivity. B) Time spent in the center zone (mean ± SE) of the Open Field. On average, sham animals spent more time in the center zone than BCCI animals. While not statistically significant ($p=0.098$), this trend suggests that, with increased n , elevated anxiety-like behavior in BCCI ferrets may be detectable.

4.2.4 Object Location Test (Memory)

After habituating to the arena during the Open Field test, animals were allowed to explore two identical objects in the arena for 10 minutes. After an hour's rest, animals were returned to the arena and allowed to explore the objects again. One of the objects was in the same location whereas the other was moved to a new location (Figure 4). The amount of time spent exploring the moved object relative to the total time spent exploring objects was calculated to determine the preference index (PI) (Figure 5). A $PI>50$ denotes a significant preference for the moved object and intact hippocampus-dependent spatial memory.¹⁹

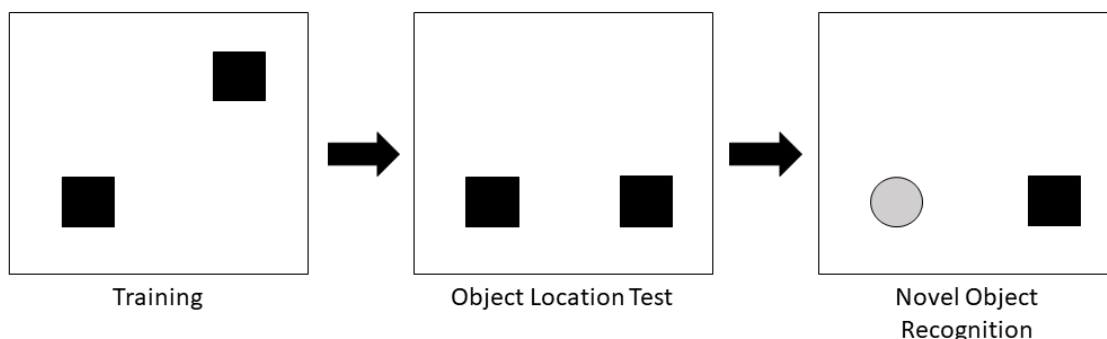


Figure 4. Schematic of the object location-novel object recognition test.

Animals are first placed into the arena for the Training phase, where they explore two identical objects for 10 minutes. After a 1-hour delay, they are returned to the arena for the 10-minute Object Location Test (OLT) in which one of the objects from the Training phase has been moved to a new location. One hour after the OLT, animals are placed back into the arena for Novel Object Recognition (NOR) in which the unmoved object from OLT is replaced with a novel object.

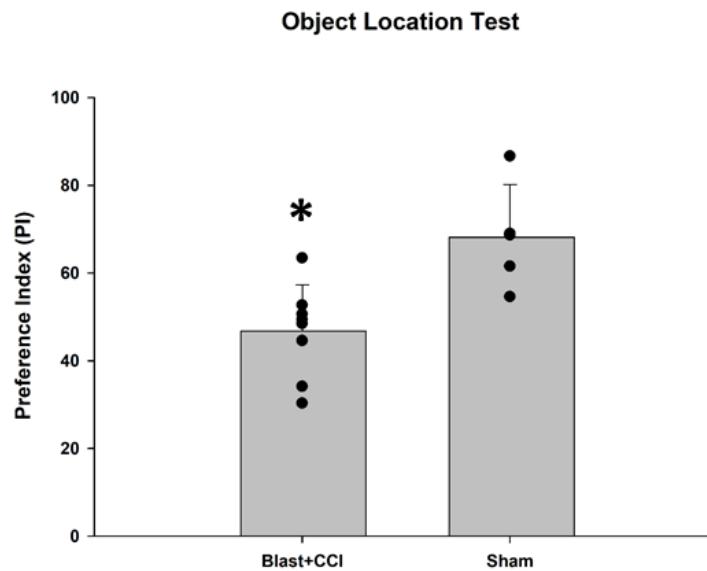


Figure 5. Preference index on the object location test.

BCCI animals spent significantly less time exploring the moved object than sham animals ($F(1,11)=11.53$, $p<0.01$), indicating an impairment in hippocampus-dependent spatial memory. Data are expressed as mean $\pm$ SE.

4.2.5 *Novel Object Recognition (Memory)*

One hour after the Object Location Test, animals were returned to the arena and allowed to explore two objects.²⁰ One of the objects is familiar and the other is novel (Figure 6). The amount of time spent exploring the novel object relative to the total time spent exploring objects was calculated to determine the PI. A PI>50 denotes a significant preference for the novel object and intact perirhinal-dependent novelty detection.¹⁹

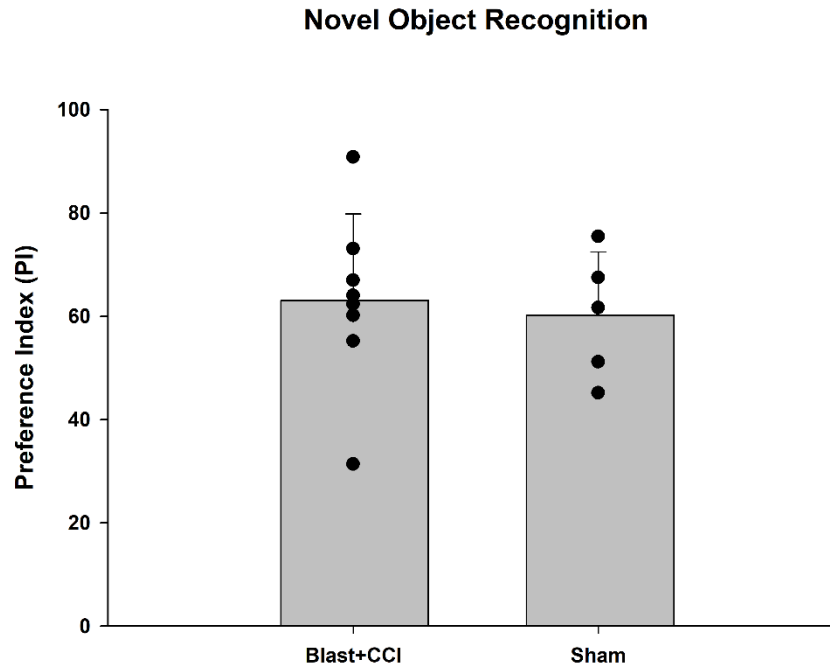


Figure 6. Preference index on novel object recognition.

No significant differences were noted between groups in time spent exploring the novel object ($p=0.75$) indicating intact perirhinal cortex-dependent novelty detection in BCCI animals. Data are expressed as mean $\pm$ SE.

4.2.6 Ladder Walk (Motor Function)

Animals were trained to walk across a horizontal ladder (dimensions) and were filmed crossing the ladder pre-injury (baseline) as well as 6 days post-injury. The footage was analyzed frame-by-frame using BORIS and the frequency and severity of slips were noted to calculate a slip score (Figure 7).²¹

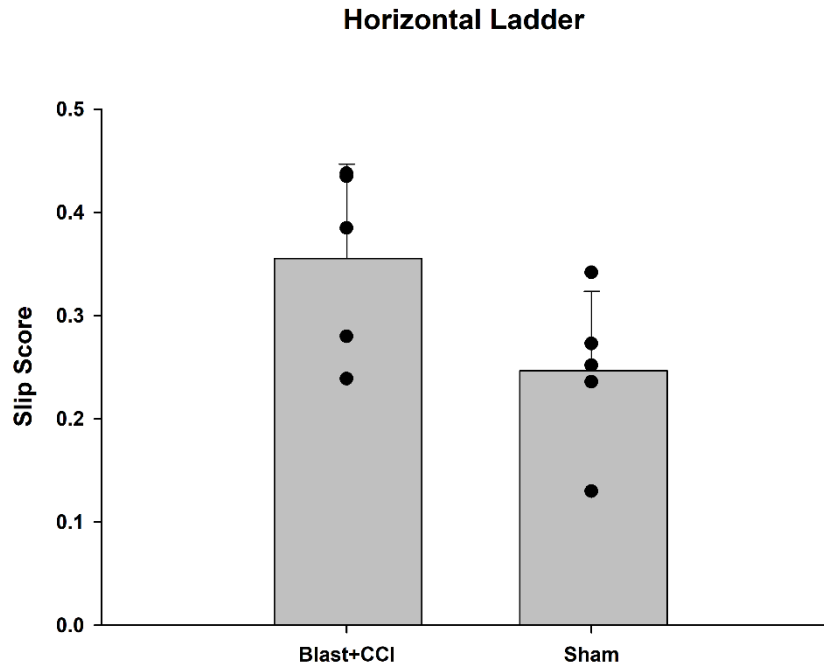


Figure 7. Slip score on the horizontal ladder walk.

The difference in slip score between groups was marginally significant ($p=0.075$) with BCCI animals, on average, performing worse than shams. This indicates that, with a greater n , between-group differences in motor function may be detectable. Data are expressed as mean $\pm$ SE.

4.2.7 Light-Dark Box Test

The Light-Dark Box test is a popular assay for anxiety-like behavior in rodents.²² An increased latency to exit from or increased time spent in the dark box into the light area of the arena is indicative of anxiety. While this assay was attempted with ferrets, it became clear early on that the animals treated the dark box as an object for exploration and not a refuge, leading to large variability within groups and making the test invalid for its intended purpose. As a result, this test was discontinued.

4.3 MRI/MRS

Neurometabolic analysis showed multiple changes following a blast injury and BCCI. Neurotransmitter's glutamate and GABA levels were significantly decreased at seven days in both the BCCI and blast-alone condition. Interestingly, only the BCCI group showed a concomitant increase in glutamine. Total creatine (tCr), an alternative fuel source, shared the same post-injury profile, with levels significantly decreasing in the BCCI and blast-alone group at seven days compared to baseline. BCCI animals also showed a significant increase in glutathione (GSH), which is linked to oxidative stress. N-acetylaspartate (NAA), a marker of neuronal integrity, was significantly decreased in the BCCI group after injury. The BCCI group showed a marked increase in tCho, a marker of cell membrane integrity. The blast-alone, but not BCCI, also showed a significant increase in inositol (Ins), which may indicate osmotic stress. No changes were noted in lactate (Lac), a marker of aerobic glycolysis, or tau, an osmoregulatory and glial cell marker. See Table 3.

Table 3. Magnetic resonance spectroscopy percent change in neurochemical expression.

Data were collected at 7 dpi, relative to baseline and expressed as mean $\pm$ SE.

Effect of treatment **p<0.01, effect of time #p<0.05, ##p<0.01. n=5 per group

	GABA	Gln**	Glu**	GSH**	Ins**	Lac	NAA**	Tau	tCho	tCr**
Blast	-12.6 $\pm$ 4.9 [#]	2.2 $\pm$ 1.8	-7.0 $\pm$ 2.9 [#]	-5.6 $\pm$ 5.8	11.3 $\pm$ 3.9 [#]	-53.8 $\pm$ 9.3	2.14 $\pm$ 1.5	-5.4 $\pm$ 4.7	5.5 $\pm$ 4.4	4.3 $\pm$ 1.5 [#]
BCCI	-22.9 $\pm$ 5.0 ^{##}	20.3 $\pm$ 6.9 ^{##}	-24.4 $\pm$ 1.2 ^{##}	24.2 $\pm$ 6.5 ^{##}	-5.4 $\pm$ 4.0	12.5 $\pm$ 102.7	-22.5 $\pm$ 1.3 ^{##}	5.3 $\pm$ 6.9	14.3 $\pm$ 6.5 [#]	-13.8 $\pm$ 1.2 ^{##}

Analysis of T2-weighted MRI scans 7 days post-injury found a significant increase in hyperintensity volume (indicative of edema) in BCCI brains ipsilateral to the injury relative to baseline and Blast-alone. No differences were noted in the hemisphere contralateral to injury (Figure 8).

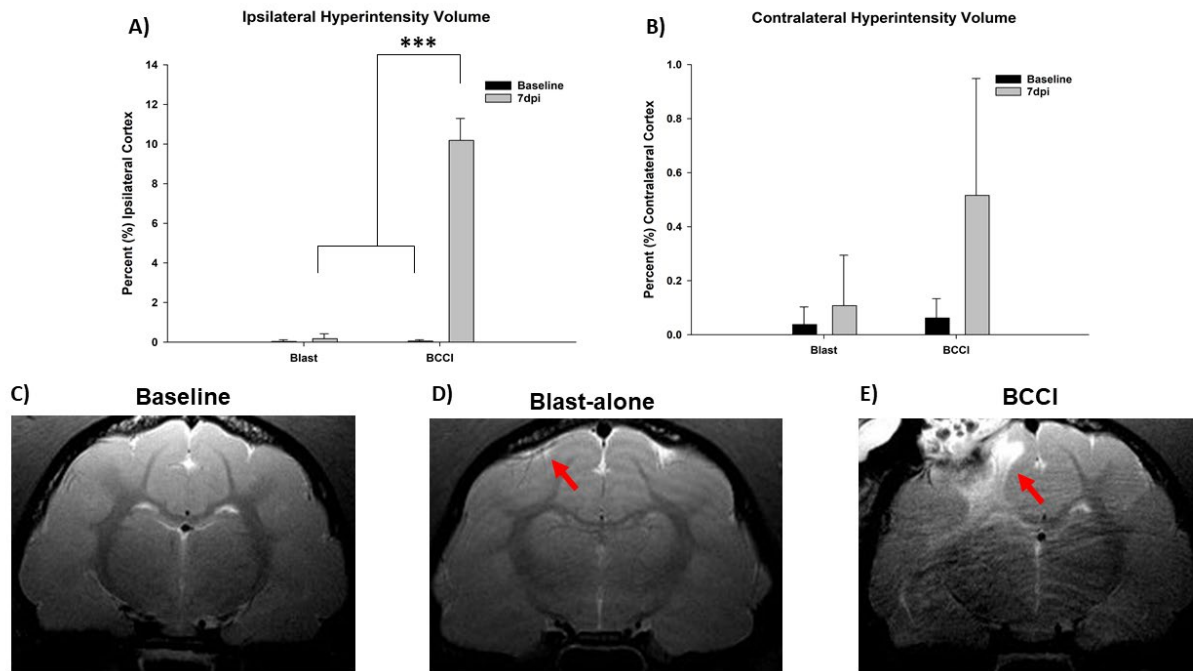


Figure 8. MRI scans of blast or BCCI animals.

Analysis of hyperintensity volume in T2-weighted MRI scans A) ipsilateral and B) contralateral to the injury. BCCI animals had a significantly greater hyperintensity volume 7 days post-injury relative to Blast-alone as well as their own baseline, *** p<0.001. Representative MRI scans taken at C) baseline or 7 days post-injury in D) Blast-alone and E) BCCI animals. Red arrows indicate hyperintensities.

4.4 Histopathology

4.4.1 Inflammation

Microglia, a resident immune cell in the brain, can change their morphology from quiescent (with a small cell body and thin, branched processes) to activated (with a larger cell body and short, stout processes) when they detect a threat. The morphologic changes from quiescent to activated are accompanied by altered signaling, including increased expression of pro-inflammatory cytokines. Ionized calcium-binding adapter molecule 1 (Iba-1) is a commonly used microglial marker. When used in immunohistochemistry, microglial morphology is visible, including both the cell body and processes. The Fujifilm Wako anti-Iba-1 rabbit antibody (product number 019-19741) was found to work well in ferrets, allowing us to differentiate between the inflammatory penumbra (where microglial morphology takes on an activated phenotype) from healthy tissue (where microglia have a quiescent phenotype)(Figure 9).

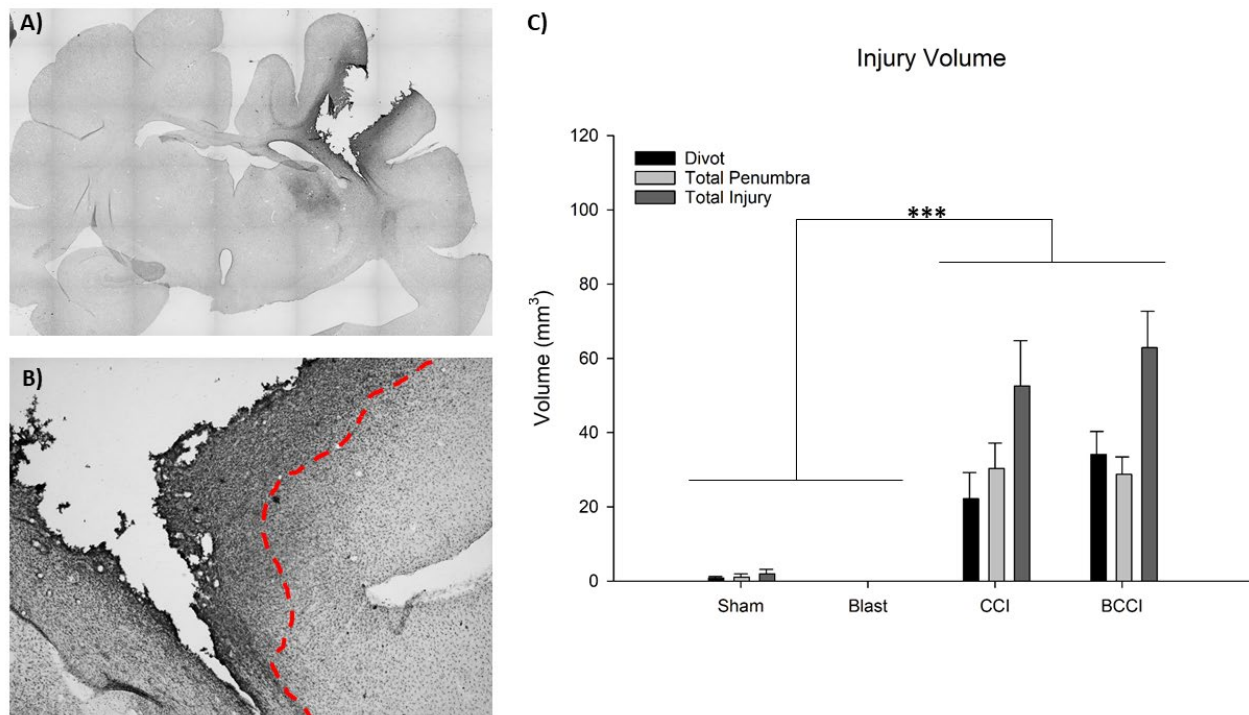


Figure 9. Histopathology of brains isolated from injured animals. (A) Montage image showing a ferret brain stained for Iba1 after CCI injury. (B) Demarcation of penumbra using Iba1 immunoreactivity. (C) Stereological analysis of injury volume based on Iba1 demonstrating that the injury volume is significantly greater in animals after CCI; blast does not have a significant effect on injury volume. An asterisk (*) denotes significance, *** $p < 0.001$.

4.4.2 BBB Dysfunction

BBB dysfunction can lead to disruptions in the function of the nervous system. Immunoglobulin G (IgG) is a common type of antibody found in blood. In a healthy brain, its size makes it difficult to cross the BBB. However, in the event of an injury, the BBB can become more permeable to large molecules and IgG becomes able to pass into brain tissue. The Novus goat anti-ferret IgG (NB7222) antibody produced clear and quantifiable staining in tissue, including gray matter, white matter, and effusions from blood vessels (Figure 10).

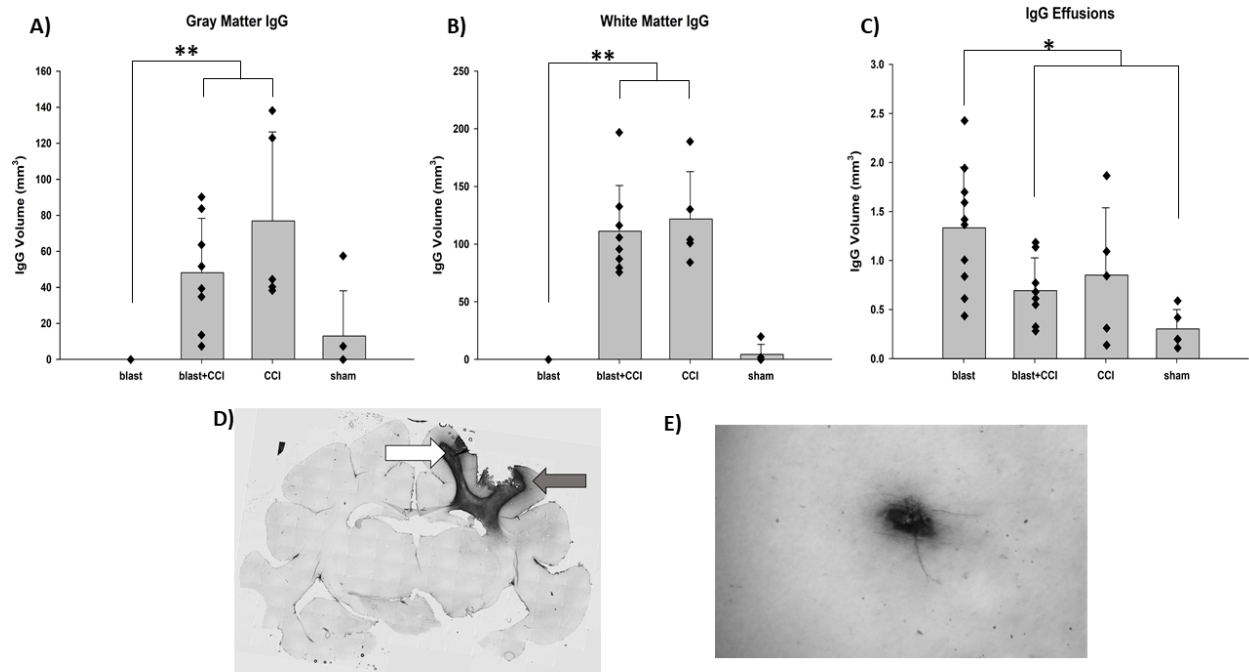


Figure 10. Evaluation of BBB integrity following injury.

Stereological analysis of IgG 7 dpi (A) in gray matter, (B) white matter, and (C) effusions from blood vessels. CCI-alone and BCCI brains show significantly more IgG in both white and gray matter than brains exposed to Blast-alone whereas exposure to Blast-alone increases IgG effusions relative to BCCI and Shams. An asterisk (*) denotes significance * $p < 0.05$, ** $p < 0.01$. Photomicrographs of (D) IgG in gray matter (gray arrow) and white matter (white arrow) and (E) IgG effusion surrounding a blood vessel.

4.4.3 Diffuse Axonal Injury

Diffuse axonal injury (DAI), including axotomy and stretching, results from rapid tissue deformation that occurs both during the primary insult as well as progresses during secondary injury processes. In addition to physical damage to the cell, DAI can also disrupt intracellular transport and ion channel function,²³ which further impairs intercellular communication. After TBI, increased APP is notable in white matter tracts as a marker of axonal damage (Figure 11). While the precise function of APP is unknown, it appears to play a role in cell growth and survival. The Invitrogen rabbit anti-beta APP antibody (51-2700) produced quantifiable staining in white matter.

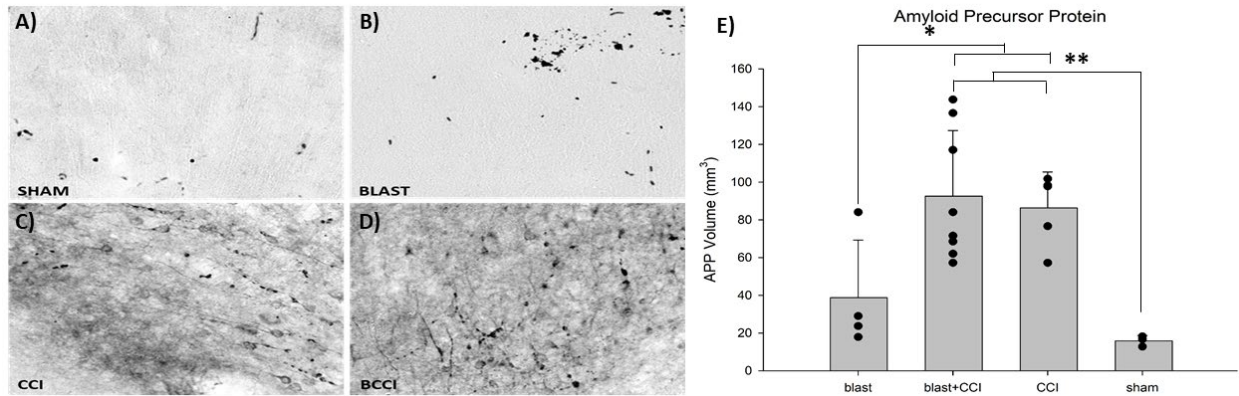


Figure 11. Analysis of diffuse axonal injury.

Photomicrographs of amyloid precursor protein (APP) in A) sham, B) Blast-alone, C) CCI-alone, and D) BCCI animals 7 days post-injury. E) Stereological analysis of APP found that BCCI and CCI-alone animals had significantly greater volumes of APP+ staining relative to Blast-alone and sham animals, * $p < 0.05$, ** $p < 0.01$.

4.4.4 Other Antibodies

We have found success with titrating several other antibodies that may be applied to the next phase of the project, including (Figure 12):

- Phosphorylated tau (Ser202, Thr205) mouse anti-human antibody (Invitrogen MN1020). Tau supports the formation and stabilization of microtubules in axons. In neurodegenerative disease, tau can become hyperphosphorylated and aggregate. Tauopathy is associated with cognitive decline.²⁴
- Glial fibrillary acidic protein (GFAP; polyclonal rabbit antibody, Dako Z0334), which is a marker for astrocytes. Reactive gliosis, characterized by proliferation and hypertrophy of astrocytes, can occur after injury and contribute to both injury progression and wound healing.²⁵
- Degenerating myelin basic protein (Dmbp; polyclonal rabbit antibody, Millipore AB5864). MBP is essential for the formation of myelin; the degenerated form is a marker of neurologic injury.
- Myeloperoxidase (MPO; polyclonal rabbit antibody, Dako A0398), an enzyme that is abundantly expressed in neutrophils, allowing for the detection of immune cell infiltration in tissues, including both lung and brain.

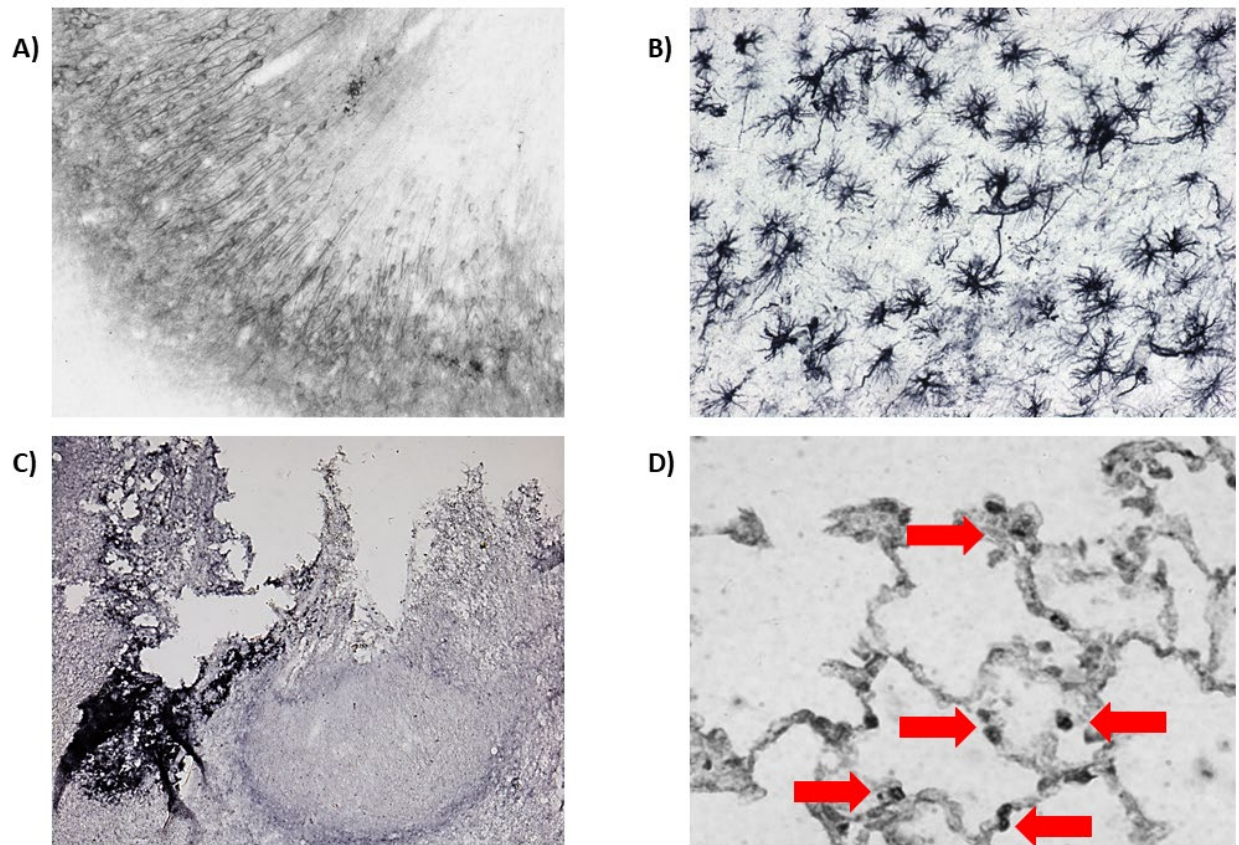


Figure 12. Histology analysis using antibodies specific to proteins of interest. Representative immunohistochemistry photomicrographs of A) phosphorylated tau, B) glial fibrillary acidic protein (GFAP), C) degenerating myelin basic protein (Dmbp), and D) myeloperoxidase; red arrows indicate infiltrating neutrophils.

Other histologic stains also work well in ferret tissue, including (Figure 13):

- Perls' stain, which detects iron deposits from microhemorrhages and degenerating tissue.
- Nissl stain, which labels the nucleus of neurons and allows for the detection of neuronal loss.
- Hematoxylin and eosin, which allows for the visualization of tissue structure.
- Fluoro Jade B, which stains degenerating neurons.

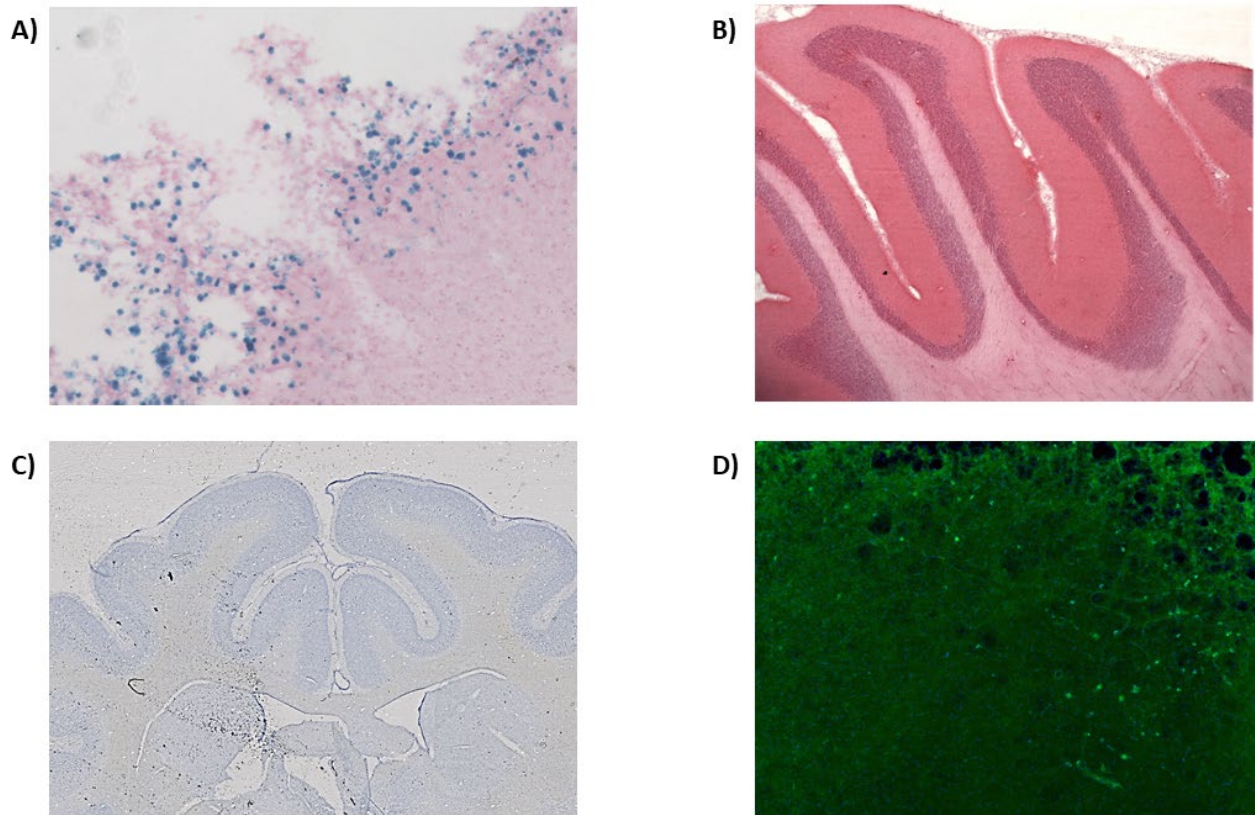


Figure 13. Histopathology analysis using stained tissues.
Representative histology photomicrographs of **A)** Perls' stain, **B)** Nissl stain, **C)** hematoxylin and eosin stain, and **D)** fluoro jade B.

5.0 CONCLUSIONS

We have successfully completed the aims of this project, including achieving all our original milestones. Our conclusions are detailed in the following subsections.

5.1 Ferret Model of UVB

We conducted experiments using ferrets to reach an UVB level of acceleration that produces alterations in behavior and/or MRI measurements and/or neuropathology by 7 days post-injury. We optimized experimental protocols to induce an UVB + CCI injury in ferrets that results in alterations in behavior, MRI measurements, and neuropathology 7 days post-injury. This model system will be critical in the second phase of the study, which examines the effect of AE on BCCI animals.

5.2 Histological Analysis of Blast Injured Ferrets

We determined the specific antibodies to evaluate diffuse axonal injury, inflammation, BBB dysfunction, etc. provide reliable, quantitative results for histologic outcome measures. We have

titrated antibodies and optimized histologic staining procedures to provide reliable, quantitative results for analyses of alterations in neuroanatomy after euthanasia. The antibodies/staining procedures that are now ready for use in Part II of this project include markers of inflammation (Iba1, GFAP, and MPO), BBB dysfunction and/or hemorrhage (IgG, Perls' stain, and Hematoxylin & eosin), and neurodegeneration (APP, Phosphorylated tau, dMBP, Nissl, and Fluoro jade B).

5.3 Neurobiological Analysis of Blast Injured Ferrets

In the present study, we determined which neurologic tests provide reliable, quantitative assays for abnormal ferret behavior. Specifically, we developed a comprehensive battery that produces reliable, quantitative analyses of neurobehavioral outcomes in ferrets, including those that evaluate mood (behavioral observation including latency to exit from the pet carrier into the open space of the room and latency to express species-specific play behaviors and open field including thigmotaxis), memory (object location test and novel object recognition), and motor function/learning (ladder walk and open field distance traveled). This analysis will be used in phase II, to examine changes in the neurobiology of BCCI animals that undergo AE.

5.4 Neuropathology of Blast Injured Ferrets

We compared short-term neuropathology and behavioral alterations for ferrets exposed to Blast-alone, head impact (CCI)-alone, and blast plus head impact (BCCI). Importantly, the results of this pilot experiment demonstrate that BCCI induces a unique injury, resulting in significant and quantifiable behavioral, neurochemical, and neurostructural changes that are measurable one-week post-injury.

We observed behavioral changes, including a trend toward altered mood in BCCI animals relative to shams, which approached statistical significance ($p < 0.10$) on both observation and open field tasks. This occurred even with relatively small sample sizes. BCCI animals were also impaired relative to shams on the hippocampus-dependent object location test but showed no impairment in the perirhinal cortex-dependent novel object recognition test. BCCI animals were hyperactive compared to sham animals and demonstrated marginally significant motor impairment on the horizontal ladder.

Neuroimaging analysis showed significantly more edema ipsilateral to injury after BCCI relative to blast-alone, which was evidenced by greater hyperintensity area on T2-weighted MRI scans. Additionally, several neurometabolic changes were noted. Relative to baseline, both blast-alone and BCCI lead to reductions in glutamate, GABA, and tCr, suggesting altered neurotransmission and metabolic capacity. BCCI (but not blast-alone) led to increased glutamine, GSH, and tCho, as well as decreased NAA. Collectively, these changes suggest altered neurotransmission, oxidative stress, impaired neuronal integrity, and cell membrane integrity in this injury model. Finally, blast-alone (and not BCCI) led to increased Ins, which is indicative of oxidative stress.

We also observed histopathological changes that support the presence of a unique injury following BCCI. Specifically, CCI-alone and BCCI induce significant tissue loss and inflammatory penumbra relative to Blast-alone and sham, as evidenced by increased volumes of activated microglia (i.e. increased Iba1). Further, CCI-alone and BCCI induced significant BBB dysfunction

in both white and gray matter relative to blast-alone and sham, as evidenced by increased IgG+ staining. Blast-alone induced a significant increase in IgG effusions from blood vessels relative to BCCI and sham. Finally, CCI-alone and BCCI induce significantly more diffuse axonal injury than blast-alone or sham, as evidenced by increased APP+ staining.

In conclusion, the procedures developed during this pilot project are currently being applied to the full experiment, which investigates the effects of exposure to 0-5 simulated aeromedical evacuations following BCCI. They will provide a means by which to evaluate any structural or functional changes induced by injury and/or hypobaric exposure in a novel ferret TBI model.

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APPENDIX A. POSTER: NATIONAL NEUROTRAUMA SOCIETY ANNUAL MEETING

Characterization of a ferret model of under-vehicle blast-induced traumatic brain injury

Molly Goodfellow¹, Mike Shaughness¹, Boris Piskoun¹, Amanda Hrdlick¹, Julie L Proctor¹, Su Xu¹, Rao Gullapalli¹, Ulrich Leiste², William Fourney², Catriona Miller¹, and Gary Fiskum¹

¹*University of Maryland School of Medicine, Baltimore, MD*, ²*University of Maryland, College Park*

Under-vehicle blast (UVB) causes a unique TBI in warfighters targeted by improvised explosive devices. UVB hyperacceleration produces an impact independent TBI; however, an impact injury can also occur. While rodent UVB studies are useful, translating findings into improved patient outcomes is challenging, perhaps because rodents lack human-like brain gyrification. Thus, a UVB model was developed in gyrencephalic ferrets.

Adult male ferrets were exposed to UVB via pentaerythritol tetranitrate detonation (2000 – 2500G), controlled cortical impact (CCI; 4 – 6mm depth), combined UVB+CCI (BCCI), or sham injury (craniotomy). Six days post-injury (dpi), mood (play behavior), motor dysfunction (ladder walk, open field), and memory (novel object recognition and location) were assessed. A subset of UVB-alone and BCCI ferrets received magnetic resonance spectroscopy (MRS) scans at baseline and 7dpi. Ferrets were euthanized 7dpi for brain histologic analysis.

Relative to shams, BCCI animals demonstrated impaired spatial memory, hyperactivity, and putative elevations in anxiety-like behavior. MRS showed altered neurochemical concentrations following BCCI, suggesting impaired neurotransmission and energy generation, neuronal injury/death, and oxidative stress. Finally, compared to shams, UVB- and CCI-alone generated elevated immunoglobulin (IgG) staining, indicative of BBB disruption. CCI-alone caused robust diffuse axonal injury compared to sham and UVB-alone, demonstrated by beta-amyloid precursor protein (β APP) staining. BCCI did not exacerbate IgG or β APP. Results were significant $p < 0.05$. Neuropathological outcomes in this model closely mirror those of human blast survivors. The model will next be applied to evaluate secondary injury following aeromedical evacuation (hypobaria) to inform protocols for safe transport of injured personnel.

Funding: US Air Force FA8650-20-2-6H20.

Characterization of a ferret model of under-vehicle blast-induced traumatic brain injury



Molly Goodfellow¹, Michael Shaughness¹, Boris Piskoun¹, Amanda Hrdlick¹, Julie Proctor¹, Su Xu², Rao Gullapalli², Ulrich Leiste³, William Fournery³, Catriona Miller¹, Gary Fiskum¹



¹ University of Maryland School of Medicine Departments of Anesthesiology and the Center for Shock, Trauma, and Anesthesiology Research (STAR) | ² University of Maryland School of Medicine Department of Diagnostic Radiology and Nuclear Medicine | ³ University of Maryland-College Park Department of Aerospace Engineering

Under-vehicle blast (UVB) causes a unique traumatic brain injury (TBI) in warfighters targeted by improvised explosive devices. UVB hyper-acceleration can produce a TBI independent of impact, however, an impact injury can also occur. Rodent studies investigating blast and/or impact-induced TBI are useful but translating findings to improved patient outcomes is challenging, perhaps due to the rodent lissencephalic brain. Thus, a model of UVB + impact TBI was developed in ferrets, who possess human-like gyrencephalic brains.

Methods: In this pilot study, male ferrets were sedated, secured to a metal plate "vehicle," and exposed to UVB via detonation of pentaerythritol tetranitrate (Blast) or anesthetized and exposed to controlled cortical impact (CCI), combined UVB+CCI (BCCI), or craniotomy-alone (Sham). Six days post-injury (dpi), mood (play behavior), motor function (ladder walk, open field), and memory (novel object recognition and object location) were assessed. A subset of Blast and BCCI ferrets received MRS scans pre-injury and 7dpi and were then euthanized for brain histologic analysis. As this was a pilot study, only data from finalized behavioral protocols are shown.

Open Field and Object Recognition: Anxiety, Locomotion, Memory

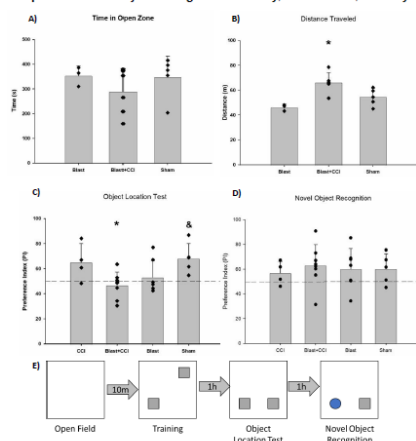


Figure 2. Six dpi, (A) no differences were noted in time spent in the open zone of the arena, suggesting comparable levels of anxiety-like behavior. (B) BCCI animals demonstrated hyperactivity, traveling significantly further than Blast and Sham animals. (C) BCCI animals were significantly impaired relative to Sham in the object location test, however only Sham showed a significant preference for the moved object. An asterisk (*) denotes different from Sham, an ampersand (&) denotes a significant preference, $p < 0.05$. (D) No animal group preferred the novel object, perhaps due to task fatigue. (E) A schematic of the test, $n = 4-8$ per group.

β-Amyloid Precursor Protein: Neurodegeneration

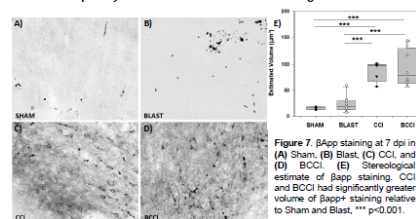


Figure 7. BAPP staining at 7 dpi in (A) Sham, (B) Blast, (C) CCI, and (D) BCCI. (E) Stereological estimate of BAPP staining. CCI and BCCI had significantly greater volume of BAPP staining relative to Sham and Blast, $*** p < 0.001$.

Ladder Walk: Motor Function

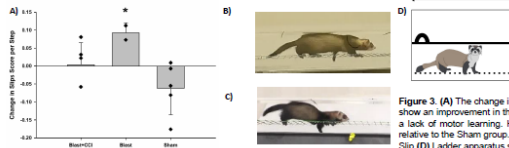


Figure 3. (A) The change in slips score on 6 dpi, normalized to baseline. On average, Sham appear to show an improvement in their slips score whereas Blast and BCCI animals did not improve, suggesting a lack of motor learning. However, only the Blast group showed a statistically significant improvement relative to the Sham group. An asterisk (*) signifies different from Sham, $p < 0.05$. (B) Slight slip (C) Deep Slip (D) Ladder apparatus schematic, $n = 2-5$ per group.

Behavior Observation: Anxiety and Mood

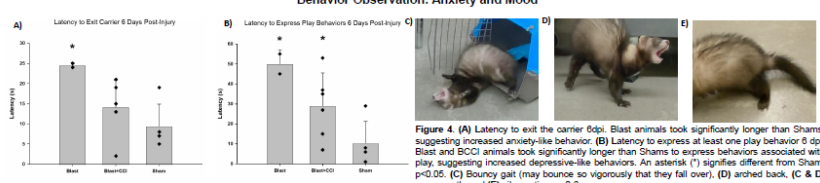


Figure 4. (A) Latency to exit the carrier 6dpi. Blast animals took significantly longer than Sham, suggesting increased anxiety-like behavior. (B) Latency to express at least one play behavior 6 dpi. Blast and BCCI animals took significantly longer than Sham to express behaviors associated with play, suggesting increased depressive-like behaviors. An asterisk (*) signifies different from Sham, $p < 0.05$. (C) Bouncy gait (may bounce so vigorously that they fall over), (D) arched back, (C & D) open mouth, and (E) piloerection, $n = 2-5$ per group.

Immunoglobulin G: Blood-Brain Barrier Permeability

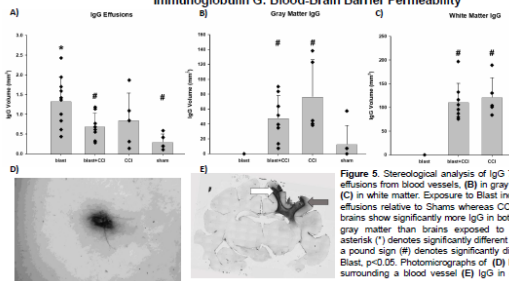


Figure 5. Stereological analysis of IgG 7 dpi (A) of effusions from blood vessels, (B) in gray matter, and (C) in white matter. Exposure to Blast increases IgG effusions relative to Sham whereas CCI and BCCI brains show significantly more IgG in both white and gray matter than brains exposed to Blast. An asterisk (*) denotes significantly different from Sham, a pound sign (#) denotes significantly different from Blast, $p < 0.05$. Photomicrographs of (D) IgG effusion surrounding a blood vessel (E) IgG in gray matter (gray arrow) and white matter (white arrow).

Iba1: Injury Volume

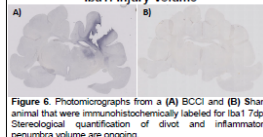


Figure 6. Photomicrographs from a (A) BCCI and (B) Sham animal that were immunohistochemically labeled for Iba1 7dpi. Stereological quantification of divot and inflammatory penumbra volume are ongoing.

Conclusions

- Combined BCCI injury induces a unique set of cognitive/behavioral impairments including hyperactivity, spatial memory deficits, and increased depressive-like behavior.
- Blast may also cause impaired motor coordination and increased anxiety and depressive-like behavior but experiments with increased group sizes are warranted.
- CCI and BCCI lead to significantly elevated markers of neurodegeneration (BAPP) and blood-brain barrier dysfunction in gray and white matter (IgG). Blast increases IgG, but only as effusions from blood vessels.
- Both Blast and BCCI cause significant changes in neurochemical expression.
- Ferrets have great potential as a gyrencephalic model for brain injury, allowing for the collection of neurobehavioral, in vivo imaging, and postmortem histopathological data¹⁴.

References

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	GABA	Glu ⁺	Glu ⁺	GSH ⁺	Iba1 ⁺	Lac	NAA ⁺	Tau	ICho	ICR ⁺
Blast	-12.6 ± 4.9 [*]	2.2 ± 1.8	-7.0 ± 2.6 [*]	-5.6 ± 5.8	11.3 ± 3.0 [*]	-53.8 ± 9.3	2.14 ± 1.5	-5.4 ± 4.7	5.5 ± 4.4	4.3 ± 1.5 [*]
BCCI	-22.9 ± 5.0 ^{##}	20.3 ± 6.0 ^{##}	-24.4 ± 1.2 ^{##}	24.2 ± 6.5 ^{##}	-5.4 ± 4.0	12.5 ± 102.7	-22.6 ± 1.3 ^{##}	5.3 ± 6.9	14.3 ± 6.5 [*]	-13.8 ± 1.2 ^{##}

Table 1. Magnetic resonance spectroscopy percent change (mean ± SE) at 7 dpi in ipsilateral cortex, relative to baseline. Results suggest both Blast and BCCI animals have neurotransmission changes (GABA, Glu, Gln) and impaired energy generation (ICR). BCCI animals also have oxidative stress (GSH), neuronal death (NAA), and cell membrane breakdown (ICho). Blast animals, and not BCCI animals, show osmotic stress or glial cell loss (Iba1). Effect of treatment $^{*}p < 0.01$, effect of time $^{*}p < 0.05$, $^{*}p < 0.01$, $n = 5$ /group.

APPENDIX B: ORAL PRESENTATION: MILITARY HEALTH SERVICES RESEARCH SYMPOSIUM

Characterization of a ferret model of under-vehicle blast-induced traumatic brain injury

Molly Goodfellow PhD¹, Mike Shaughness PhD¹, Boris Piskoun BS¹, Amanda Hrdlick BS¹, Julie L Proctor MS¹, Su Xu PhD¹, Rao Gullapalli PhD¹, Ulrich Leiste PhD², William Fourney PhD², Catriona Miller PhD¹, and Gary Fiskum PhD¹

¹*University of Maryland School of Medicine, Baltimore, MD,*

²*University of Maryland, College Park*

Background: Blast-induced traumatic brain injury (bTBI) is the signature injury for service members (SMs) with injured military personnel reporting neuropsychiatric symptoms and long-term cognitive disability² which are associated with significant neurometabolic changes³ and altered white matter integrity⁴. Post-mortem analysis of brains from veterans with a history of blast exposure showed neurovascular alterations⁵ and neuroinflammation⁶. Under-vehicle blast (UVB)-induced TBI, generated from improvised explosive devices (IEDs), is a unique etiological mechanism of TBI affecting warfighters present in vehicles targeted by blasts. The hyperacceleration load, caused by an IED-generated UVB, can directly result in a TBI independent of an impact event, however, a secondary head impact injury in the cabin can also occur.

We previously published a rat model of under-vehicle bTBI in which a blast-induced acceleration of 2460 ± 76 G resulted in 100% survival but caused cerebrovascular damage, neuroinflammation, pulmonary hemorrhage, neuronal death, and neurobehavioral alterations⁷. When blast is combined with controlled cortical impact (CCI), an acute increase in cortical myeloperoxidase and decrease in cerebellar calbindin occurs, indicative of cortical inflammation and cerebellar neuronal injury. Rodent models of TBI recapitulate the cellular pathophysiology and neurobehavioral deficits seen in humans, however, successfully translating these findings to improved patient outcomes is challenging. One explanation could be the neuroanatomical differences between humans and rodents, with the latter lacking the gyri and sulci present in the former. Therefore, there are multiple advantages to ferret models, including: 1) the presence of gyri and sulci, 2) similar location of the hippocampus to humans, and 3) a higher white to grey matter ratio than rats and mice. To address this modeling difference, a ferret model of TBI was developed to evaluate the effects of UVB-alone, CCI-alone, and combined blast + CCI (BCCI).

Methods: The protocol was reviewed and approved by the University of Maryland, Baltimore Institutional Animal Care and Use Committee (0620009) and the U.S. Air Force Surgeon General's Office of Research Oversight and Compliance as protocol number FWR-2020-0015A. These studies were conducted in a facility accredited by AAALAC, in accordance with the Guide for the Care and Use of Laboratory Animals (NRC, 2011) and were performed in compliance with DODI 3216.1. Adult male ferrets were either exposed to UVB-alone (n=13) or CCI-alone (n=8). Once the blast level (G force) and CCI depth were finalized, a third set of ferrets were exposed to that combination of BCCI (n=8) or sham injury (n=5). Neurobehavioral assays were optimized, and the final protocols were applied to a subset of these animals (n=6 BCCI and n=5 sham).

In preparation for a UVB, ferrets were sedated with dexmedetomidine and briefly anesthetized with isoflurane and loaded into a restrainer affixed to the top blast plate where they awoke minutes later. Ferrets were exposed to a single underbody blast acceleration via detonation of pentaerythritol tetranitrate resulting in acceleration of 2000 – 2500 G. Mild traumatic brain injury was induced under 2-2.5% isoflurane anesthesia via CCI (4 – 6 mm depth, 6 m/s). A subset of ferrets in blast-alone and BCCI groups (n=5 each group) received an MRI scan at one week prior

to and 7 days after injury. Six days after injury, mood (play behavior observation), motor dysfunction (ladder walk, open field), and memory (novel object recognition and location) were assessed. All ferrets were euthanized by terminal perfusion with paraformaldehyde on day 7 and brains histologically analyzed for blood-brain barrier (BBB) disruption (immunoglobulin G, IgG) and diffuse axonal injury (beta-amyloid precursor protein, β APP).

Results: All animals survived until terminal perfusion. Using immunohistology, we showed that, compared to shams, the blast-alone group exhibited BBB disruption, indicated by punctate IgG staining, primarily localized to the ventral aspect of the brain. A CCI-alone also produced BBB disruption, however, the pattern and distribution were different than the blast-alone group, with diffuse staining localizing to the cortex, preferentially presenting in the white matter. Additionally, CCI-alone injury resulted in robust diffuse axonal injury compared to sham and blast groups, demonstrated by β APP staining, near the site of impact and underlying gray matter. The combination of injuries did not exacerbate IgG or β APP staining.

Cortical neurochemicals measured by magnetic resonance spectroscopy (MRS) near the site of impact were affected by the blast-alone and BCCI differently at 7 days post-injury. Although gamma aminobutyric-acid (GABA), glutamate (Glu), and total creatine (tCr) were significantly decreased in both models compared to their baseline, these biochemicals were lower in the BCCI group than those in the blast-alone animals. Reductions in these neurotransmitters (Glu and GABA) and energy buffer (tCr) in the BCCI group may indicate impaired neurotransmission and energy generation. In addition, there were significant changes in glutamine (Gln), *N*-acetylaspartate (NAA), glutathione (GSH), and total choline (tCho) in the BCCI group compared to its baseline. The elevated Gln suggests neurotoxicity in the impact area. Decreased NAA and increased tCho indicate increased neuronal injury and cell death. High levels of GSH reflect oxidative stress. Interestingly, *myo*-inositol, a marker for glial content, increased only in blast-alone ferrets suggesting increased glial activation in this group.

Neurobehavioral alterations were also apparent in BCCI animals relative to shams. On the hippocampus-dependent Object Location Test, BCCI animals exhibited a spatial memory deficit by showing no preference for an object in a novel location whereas shams explored this object significantly more than an object in a familiar location. BCCI ferrets also demonstrated hyperactivity, traveling significantly greater distances in the Open Field arena than shams. Finally, post-surgery shams exited an enclosure into an open area more quickly relative to their baseline whereas BCCI ferrets showed no change, suggesting increased anxiety-like behavior. No significant impairments were noted in play behavior, ladder walk performance, or novel object recognition.

Conclusions and future directions: To our knowledge this is the first ferret polytrauma model combining TBI with UVB. Results indicate that a single UVB is sufficient to induce acute BBB disruption. CCI-alone leads to both BBB disruption and cortical diffuse axonal injury but this is not exacerbated by combination with UVB. BCCI does, however, result in several alterations in key cortical metabolites indicative of increased neuronal injury, oxidative stress, and glial activation as well as impaired neurotransmission and energy generation. Additionally, we found evidence that BCCI in ferrets increases anxiety-like behavior and hyperactivity, as well as impairs spatial memory. Taken together, we provide a military, combat-relevant model of under-vehicle bTBI in a gyrencephalic animal, the ferret. Our results are similar to that which is reported in SMs with bTBI regarding the neuro- pathophysiology and behavioral sequela. The results presented here will be used to address military-relevant questions on the effects of aeromedical evacuation

(hypobaria) on secondary injury development to determine protocols for safe transport of SMS following TBI. Supported by US Air Force FA8650-20-2-6H20.

Disclaimers: The views expressed are those of the authors and do not reflect the official guidance or position of the United States Government, the Department of Defense or of the United States Air Force. The protocol was reviewed and approved by the U.S. Air Force Surgeon General's Office of Research Oversight and Compliance (AFOSR-2020-0015A) and by the University of Maryland, Baltimore Institutional Animal Care and Use Committee (0620009). Animal activities were conducted in compliance with all federal regulations governing the protection of animals and research.”

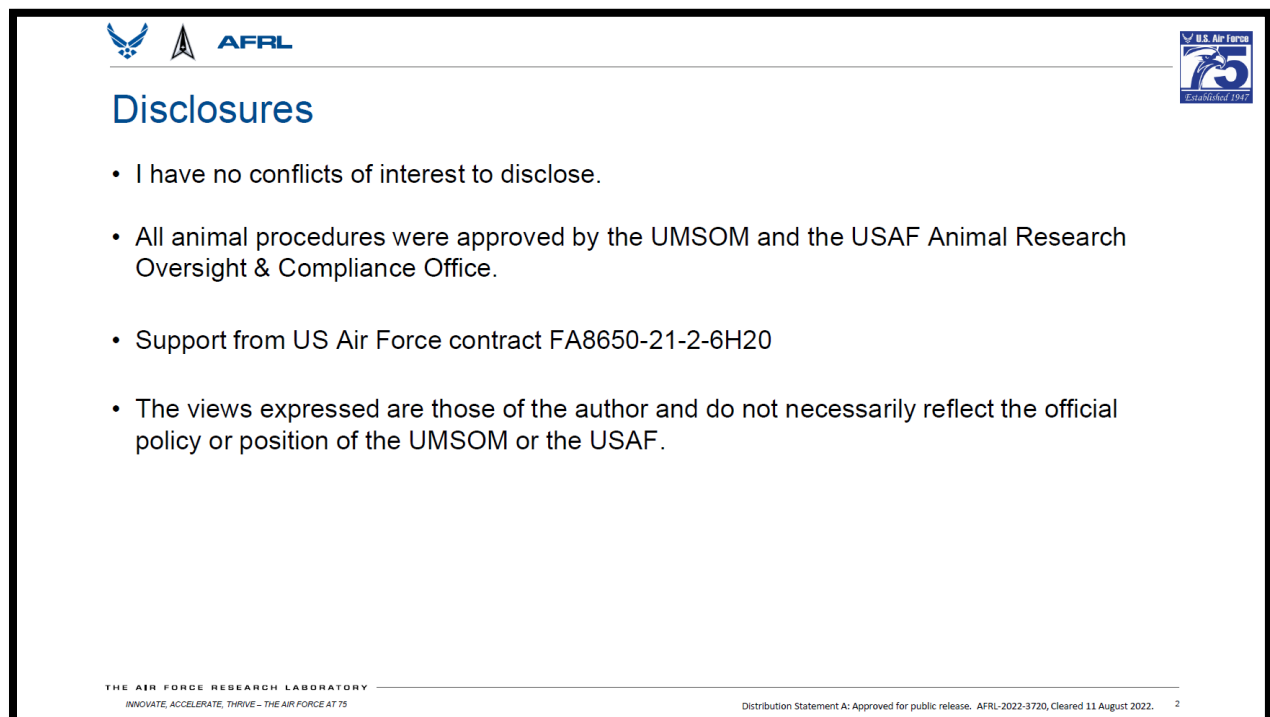
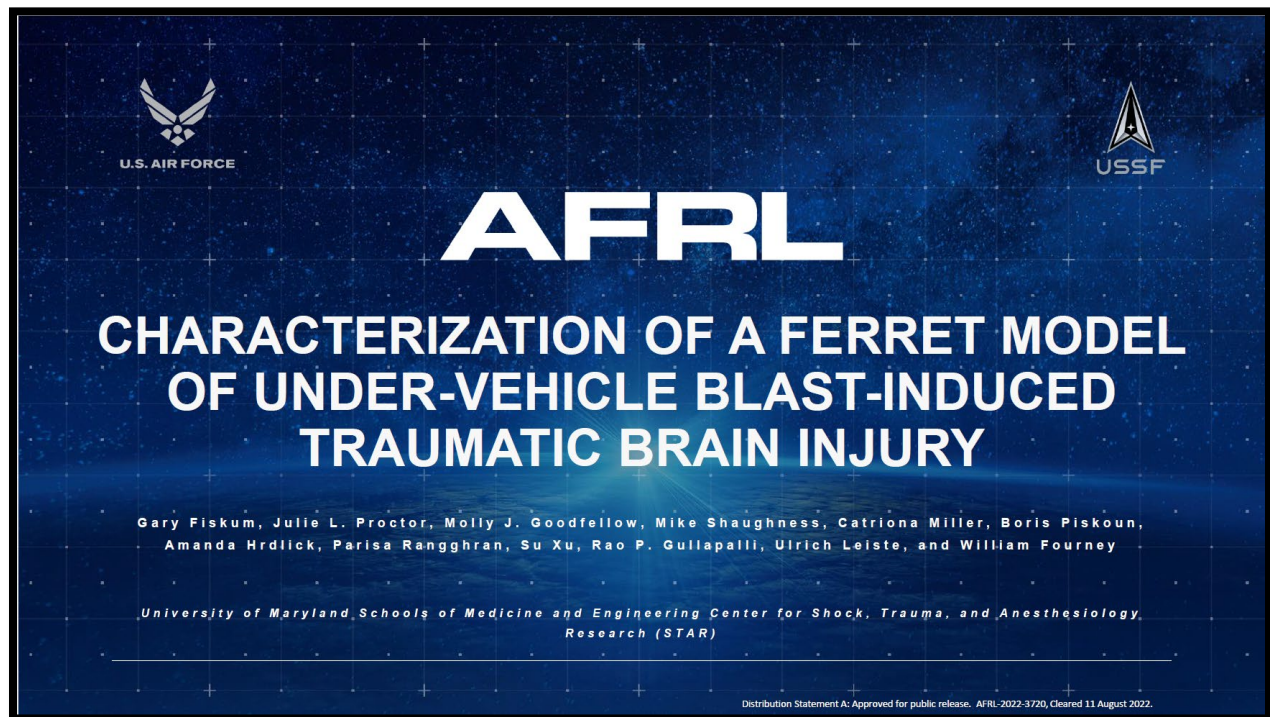
Learning objectives:

1. Discuss the advantages of a gyrencephalic model of bTBI.
2. Develop ideas for evaluating military relevant post-injury stressors
3. Compare similarities and differences between gyrencephalic and lissencephalic models of TBI.

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brain injury to occupants of vehicles targeted by underbody blasts. *Experimental Neurology*, 289, 9–20. <https://doi.org/10.1016/j.expneurol.2016.12.001>



Under-Vehicle Blast TBI Model



- "Free-field" blast TBI models are often used with rodents and exhibit features similar to what are experienced by unmounted warfighters.
- We developed a unique under-vehicle blast paradigm modeling injuries that occur to occupants present within military vehicles targeted by IEDs.
- TBI is detectable in rats following small-scale explosions at 100 G vertical acceleration and is survivable up to 2500 G, which is equivalent to a full-scale acceleration of 200 G.
- Rats may not be the best species to test in this model due to their lissencephalic brain, in contrast to the gyrencephalic brain of humans and other animals, including pigs and ferrets.
- We, therefore, also developed a ferret model of TBI caused by under-vehicle Blast in combination with controlled cortical impact (CCI).

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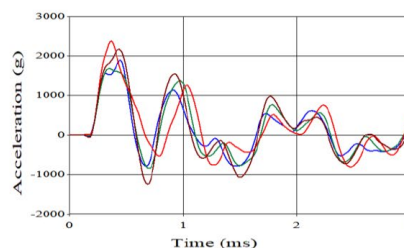
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Under-Vehicle Blast TBI Model

Ferret restraint on under-vehicle blast device



Accelerometry Results



Highly reproducible acceleration measured during under-vehicle blasts resulting in a peak force of 2000 G in less than 0.5 msec.

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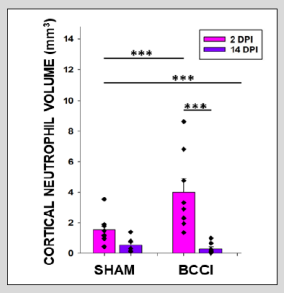
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4

Summary of Results Obtained with Rats Following the Combination of a Single 2000 g Blast plus Controlled Cortical Impact TBI (BCCI)

Neuroinflammation

Induces an increase in MPO positive neutrophils to the rim of the cortical divot

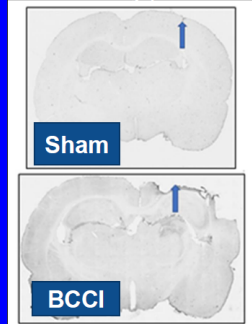


**Effects of hypobaria in this model presented at poster #7235 Tues AM*

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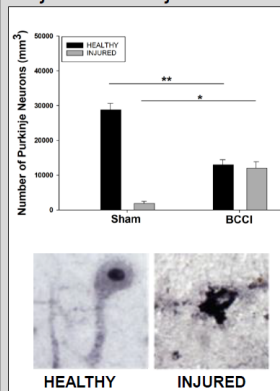
Cortical Pathology

Induces an acute and chronic cortical necrotic divot with underlying inflammatory penumbra



Cerebellar Injury

Increases number of injured Purkinje neurons



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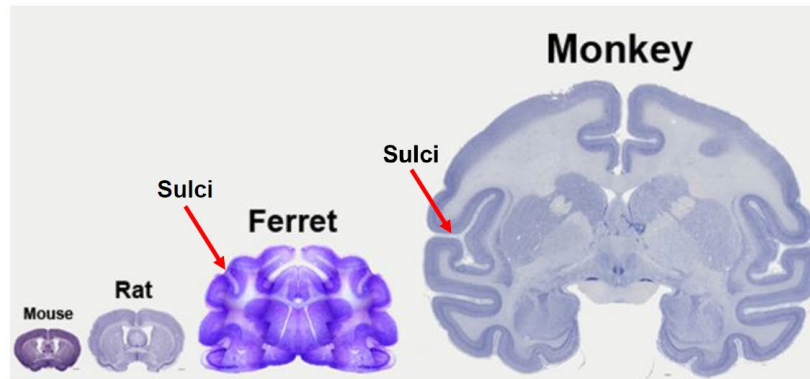
Use of Ferrets in Translational Models of Blast TBI

- Most animal TBI models use rats or mice, neuroanatomically characterized by a lissencephalic cerebral cortex that is devoid of gyri and sulci, in contrast to the gyrencephalic brain of humans and animals, such as pigs and ferrets.
- We recently published a study using ferrets subjected to controlled cortical impact TBI followed by hemorrhagic shock (Goodfellow et al., 2022). Results were similar to those obtained using rats except that all ferrets survived, whereas 60% of rats died immediately.
- The lab of Dr. Juliano at USUHS has published MRI and histopathology studies with ferrets in a closed head rotational brain injury model and in a repetitive blast TBI model (Schwerin et al, 2021; Hutchinson et al, 2022).

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Gyrencephalic Compared to Lissencephalic Brain Anatomy



In addition to brain size, the cortical topography and ratio of white to grey matter of ferrets are more similar to that of primates compared to rodents.

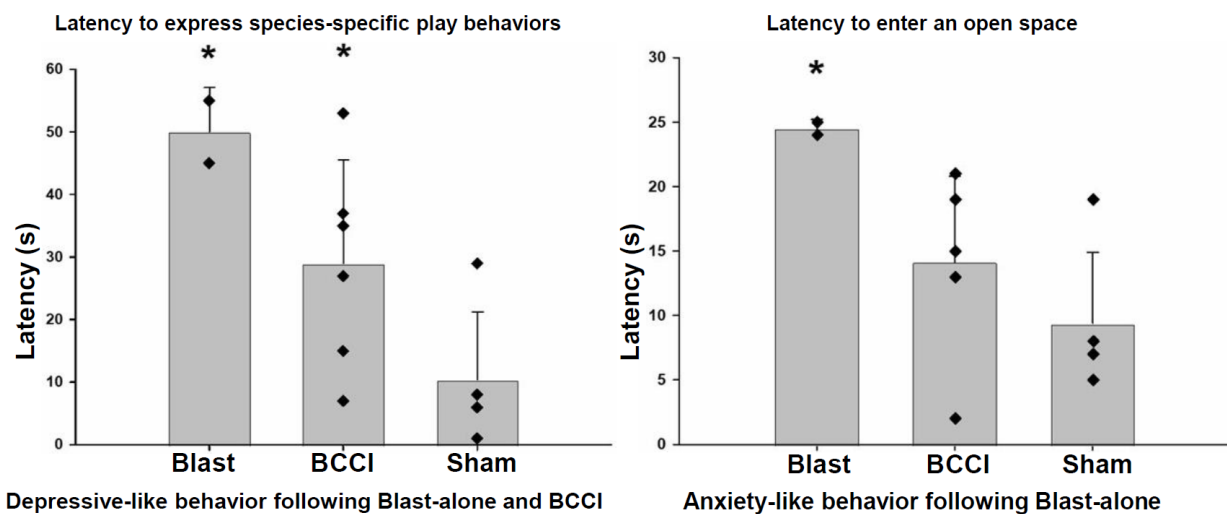
Methods

- Comparisons between Blast-alone, Controlled cortical impact (CCI)-alone and Blast plus Impact (BCCI)
- Behavior measured at baseline and 6 days post-injury (dpi)
- MRI/MRS at baseline and 7 dpi in Blast alone and BCCI
- 7 dpi histopathology

Timeline

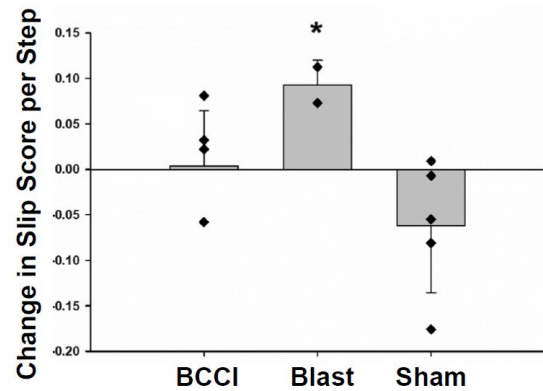


Altered Mood and Anxiety-Like Behavior

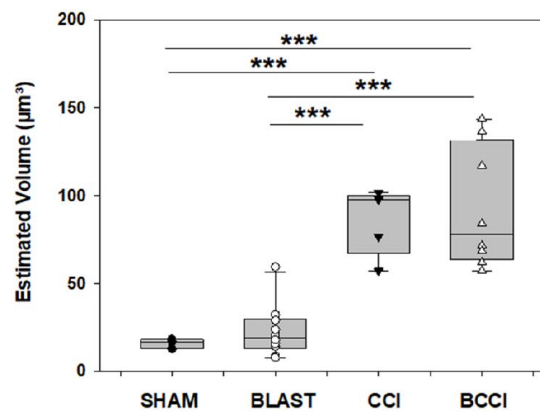
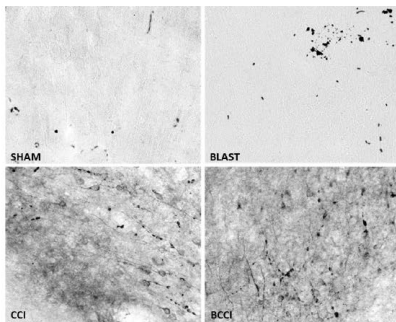


Impaired Motor Learning Following Blast Alone

FOOT SLIPS ON A HORIZONTAL LADDER

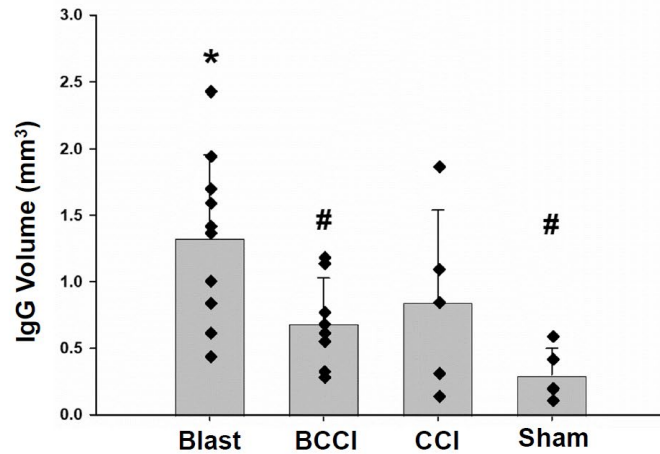
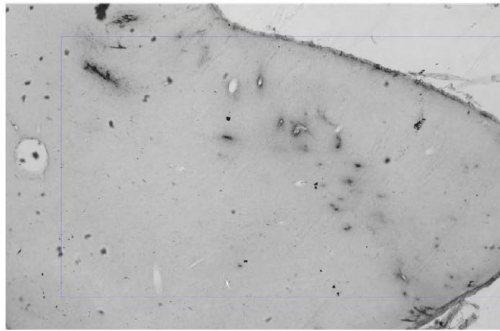


β -Amyloid Precursor Protein (β App) Neuropathology at 7dpi



β App immunostained brains indicate diffuse neuronal injury

Immunoglobulin G Histochemical Pathology Indicative of Vascular Injury and Breakdown of the Blood Brain Barrier



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T2-Weighted Magnetic Resonance Imaging (MRI)



Representative scans from 6dpi. Analysis of hyperintensity volume (indicative of edema) is ongoing.

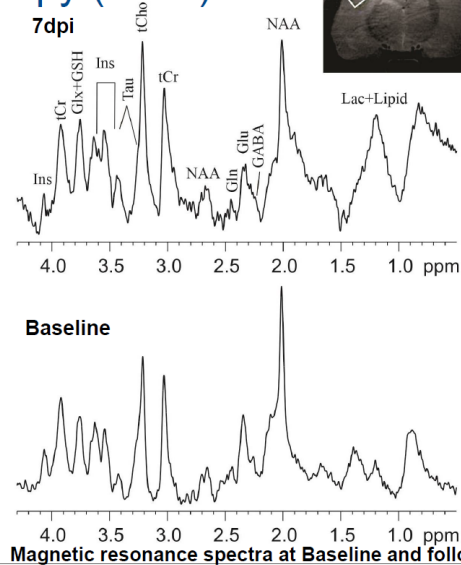
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Magnetic Resonance Spectroscopy (MRS)

Relative to baseline:

- Blast-alone and BCCI animals showed altered *neurotransmission* (GABA, Glu, Gln) and *energy generation* (tCr).
- BCCI, and not Blast-alone, animals also demonstrate *oxidative stress* (GSH), *neuronal death* (NAA), and *cell membrane breakdown* (tCho).
- Blast-alone, and not BCCI, animals show *osmotic stress or glial cell loss* (Ins).



Preliminary Conclusions and the Way Forward

- Ferrets can survive a 2000-G acceleration caused by an explosion under a small-scale model vehicle. Given a scaling factor of approximately 10, a full-scale explosion would exert a 200 G force on the occupants of the targeted vehicle.
- Both histologic and neurologic measurements that we have used with rats can be used with ferrets, with some modifications.
- Studies underway are extending final outcomes to 6 months, which are likely to detect abnormal neuroanatomic and neurochemical characteristics not seen in short-term outcome experiments.
- We are also testing the hypothesis that multiple exposures to flight-relevant hypobaria following TBI can exacerbate neuropathology and promote chronic abnormal behavior.

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

711 HPW/RHBAM	Air Force Research Laboratory, 711th Human Performance Wing, Airman Systems Directorate, Airman Biosciences Division, Product Development Branch, Enroute Care Section
AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
AE	Aeromedical evacuation
ANOVA	Analysis of variance
APP	Amyloid precursor protein
BBB	Blood-brain barrier
BCCI	Combined blast + controlled cortical impact
BORIS	Behavioral Observation Research Interactive Software
CCAT	Critical Care Air Transport Team
CCI	Controlled cortical impact
CTE	Chronic traumatic encephalopathy
DAI	Diffuse axonal injury
dMBP	Degenerating myelin basic protein
DODI	Department of Defense Issuances
dpi	Days post injury
ft	Feet
g	Grams
GABA	Gamma aminobutyric-acid
GFAP	Glial fibrillary acidic protein
Glu	Glutamate
GSH	Glutathione
hrs	Hours
Iba1	Ionized calcium-binding adaptor molecule 1
IEDs	Improvised explosive devices
IgG	Immunoglobulin G

Ins	Inositol
Lac	Lactate
LSD	Least significant difference
μm	Micrometers
mm	Millimeters
mm ³	Cubic millimeters
MPO	Myeloperoxidase
MRI	Magnetic resonance imaging
MRI/MRS	Magnetic resonance imaging and spectroscopy
MRS	Magnetic resonance spectroscopy
NAA	N-acetylaspartate
NMRC	Naval Medical Research Center
NOR	Novel object recognition
OLT	Object location test
PETN	Pentaerythritol tetranitrate
PI	Preference index
SE	Standard error
TBI	Traumatic brain injury
tCho	Total choline
tCr	Total creatine
UMSOM	University of Maryland School of Medicine
UVB	Under-vehicle blast