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14. ABSTRACT Because of sports injuries, automobile accidents, falls, etc., and with the escalation of the use of improvised explosion devices (IEDs) by our enemies as witnessed in the most recent military conflicts in Iraq and Afghanistan, there has been an increased awareness of closed head concussions, also commonly referred to as the mild TBI (mTBI) injury. The prevalence of this type of closed-head brain injury, estimated as afflicting over 300,000 deployed soldiers or approximately 30% of all deployed troops, has distinguished it as the "signature injury" of these military conflicts. Despite the enormity of this medical problem, and recognition of the importance for the need to quickly and accurately diagnose the event in the face of a limited clinical presentation (i.e. no obvious wounds to the head), objective diagnostic tools and knowledge about what occurs in the brain following this type of injury are limited. Of equal concern is our lack of understanding the impact of multiple concussions on the brain and its consequences on the long term health of individuals. In order to address this problem, the WRAIR projectile concussive impact (PCI) model was developed under directive of the Combat Casualty Care Research Program (CCCRP). Provided in this Year 2 Annual Report are the results of our Phase I studies focused on characterizing the neuropathologic, molecular and neurobehavioral changes following a single concussive impact (PCI) injury. Additionally, this Report also includes data comparing the effects of a single concussive impact to repeated concussive impacts using the PCI model. Phase I studies have been completed and these results set the foundation for Phase II studies designed to evaluate the effects of repeated concussions that occur prior to and after the resolution of the healing profile for a single concussion.

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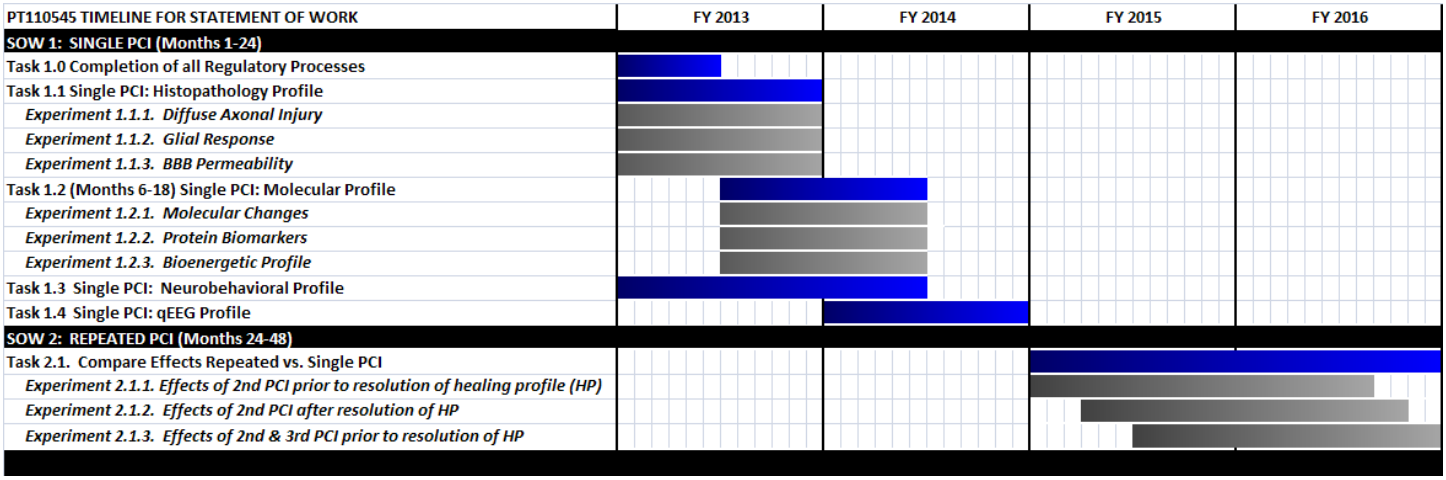
INTRODUCTION

Under the directive of the Combat Casualty Care Research Program (CCCRP) to establish a military-relevant model of concussive head injury, the proof-of-concept development of the WRAIR Projectile Concussive Impact (PCI) model of closed-head mTBI has been successfully completed. In addition, in collaboration with the Composites and Hybrid Materials Branch, Army Research Laboratory (Aberdeen) we have recently completed the development and implementation of custom-designed helmets combined with pressure sensor film analysis to detect the impact pressure distribution pattern both on the outer and inner helmet surface. The overall goal of the current proposal is to conduct longitudinal studies on the WRAIR PCI model following "SINGLE" or "REPEATED" PCI injuries in order to develop a more thorough understanding of the changes taking place at a cellular level following a single or multiple concussive events and to establish how those changes relate to clinically relevant mTBI behavioral and electrophysiological outcome metrics. Concussive head injury will be studied in the WRAIR PCI model using longitudinal and multi-modal designs to fully characterize the neuromotor, cognitive, emotional, and neuropathological evidence of brain injury. **Phase I (SOW 1) studies will fully characterize the neuropathological, molecular and neurobehavioral changes following a "SINGLE" PCI injury. Phase II (SOW 2) studies will evaluate the cumulative effects of "REPEATED" PCI injuries based on outcome metrics defined in SOW 1.**



Task 1.0 (Months 1-6) Regulatory review and approval processing for studies involving animal subjects. The following animal protocols have been approved by the WRAIR IACUC: WRAIR IACUC Protocol # 12-PN-18S and 13-PN-30S. ACURO approval has been obtained for each of the study protocols. All regulatory review/approval requirements have been completed.

During this timeframe, several engineering components of the PCI model were refined to provide optimal injury parameters. The original PCI device used dry ice sublimation to build up pressure inside a microcentrifuge tube and trigger the release of a small projectile (i.e. the microcentrifuge cap) targeted to impact a helmet-protected rat head. However, we subsequently identified several limitations to the dry ice sublimation/microcentrifuge tube method and these limitations have been addressed by modifications made to (A) the PCI device and more recently to (B) the projectile. In addition, two pilot projects were conducted to determine (C) the optimal angle of PCI injury and (D) to establish a positive PCI control group. These modifications and results are summarized below:

(A) PCI Device: Started during the past year and completed during 1st QTR (FY13 Q1) of this proposal, the PCI device was modified to use compressed gas (i.e. nitrogen) instead of dry ice sublimation as the trigger mechanism for launching the projectile. In addition, a computer control interface was implemented to control the operating pressure (Figure 1). The primary advantage of using compressed gas vs. dry ice sublimation is that the mechanical forces used to induce the injury are far more controllable, reproducible and quantifiable. In addition, the "pressure wave" generated by the release of compressed gas is of low magnitude and is not related

to the input pressure. Thus, the "pressure wave" effect is minimal and can be more effectively controlled. **Moreover, the intensity of the force can be titrated to produce a wider spectrum of closed-head concussive injury severities for study.** A patent application was submitted for this iteration of the device in August 2012 (U.S. Provisional Application Serial No. 61/521,446).

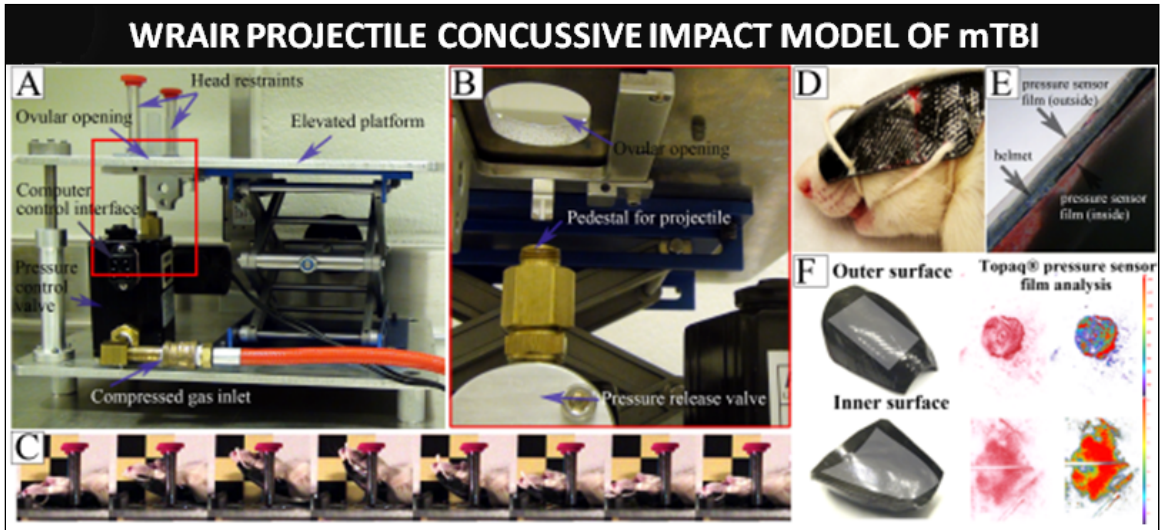


Figure 1. The WRAIR PCI Model of mTBI. (A) Elevated platform for holding the animal and aligning the projectile. (B) Instrument rig. (C) Head motion response to WRAIR PCI mTBI event. (D) Helmet made of woven glass-carbon. (E,F) Pressure sensor films are secured by adhesive to the inner and outer surfaces of the helmet. Pressure distribution is analyzed using the Topaq® system (Leung et al., 2012).

(B) PCI Projectile: In addition to intervals between repeated injuries; varying the intensity or severity of the mTBI insult is a critical factor to evaluate in preclinical mTBI studies (Fujito et al., 2012). In keeping with this, the modifications made to the PCI device also facilitate the use of small projectiles of different shapes/masses. Thus, during the FY13 Q1 of this project we collaborated with the Army Research Laboratory (ARL; Aberdeen MD) to test a number of small spherical (i.e. ball bearings) and cylindrical projectiles of different masses (ranging from 0.5 to 6g). **The steel ball bearings have produced the most desirable and consistent pressure distribution profile on the inner surface of the helmet while remaining within a range that meets the criteria for mTBI.**

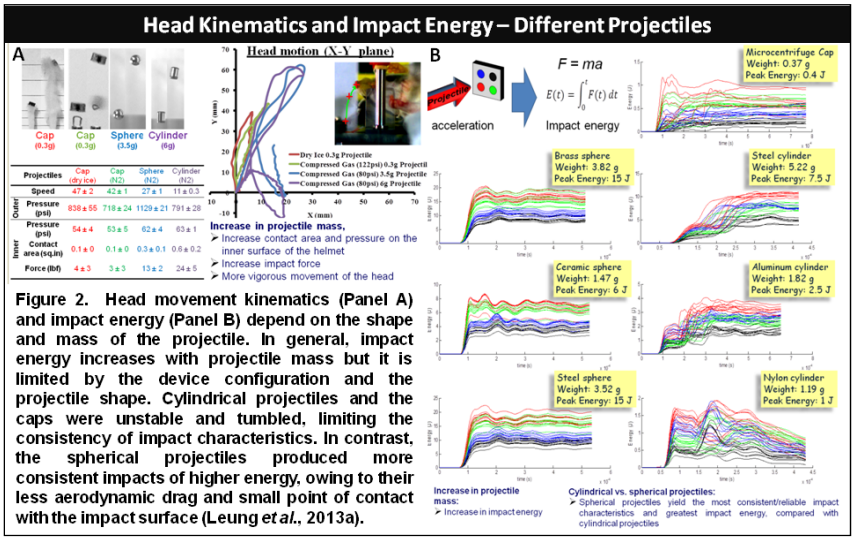


Figure 2. Head movement kinematics (Panel A) and impact energy (Panel B) depend on the shape and mass of the projectile. In general, impact energy increases with projectile mass but it is limited by the device configuration and the projectile shape. Cylindrical projectiles and the caps were unstable and tumbled, limiting the consistency of impact characteristics. In contrast, the spherical projectiles produced more consistent impacts of higher energy, owing to their less aerodynamic drag and small point of contact with the impact surface (Leung *et al.*, 2013a).

(C) Angle of PCI Injury: In an initial pilot experiment, we assessed PCI-induced injuries that were angled (A) 0° from the saggital plane (bilateral hit) or (B) either 45° or 90° from the saggital plane (unilateral hits). CatWalk automated gait analysis (Noldus, The Netherlands) was used to detect gait abnormalities at 2h, 1, 3, 7 days post-injury. Results showed that unilateral PCI produced a greater degree of gait alterations compared to bilateral PCI demonstrated by alterations in 46 or 32 (out of 210) gait parameters following the 45° and 90° hits respectively. In contrast, only 18 gait parameters were significantly altered following the bilateral (0°) PCI injury. Figure 3 provides a summary of the significant gait alterations detected in the three groups at different time points. Significant increases in mean intensities of both front and hind paw prints were observed in rats subjected

to unilateral hits (45° and 90°) at 1, 3 or 7 days post-injury ($p<.05$ vs. sham control). **Unilateral PCI angled at 45° produced the most robust gait abnormalities that are sustained under repeated testing conditions.**

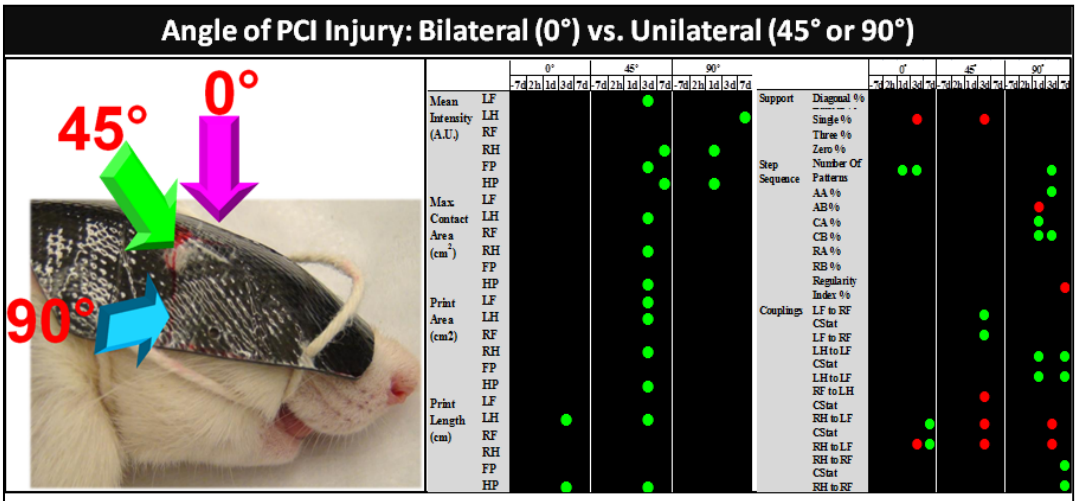


Figure 3. Adult rats were anesthetized with isoflurane, fitted with custom-designed helmets, and subjected to a single PCI angled at 0° (bilateral), 45° or 90° (unilateral) from sagittal plane. Sham controls received the same procedures without PCI. Gait performance was assessed using CatWalk automated gait analysis system at 7d prior to injury (baseline), 2h, 1d, 3d and 7d post-injury. Green (●) and red (●) dots represent significant increases or decreases vs. sham respectively (Leung et al., 2013a).

SUMMARY OF ADVANCED PCI MODEL: Carbon/glass fiber composite material is used for helmet fabrication; (2) the microcentrifuge cap in the original model has been replaced by a steel sphere (3.52 g) as the projectile; (3) pressure used to launch the projectile is set at 80 psi; and (4) the impact location is set at a 45° angle targeting the temporoparietal region (right hemisphere). These advancements have been presented at the Society for Neurotrauma Symposium in Nashville TN (Leung et al., 2013a) and are described in greater detail in Leung et al. (2014). **All aspects and components of the refined/advanced PCI model were approved in the current WRAIR IACUC Protocols 12-PN-18S and 13-PN-30S.**

PCI procedure (used for all tasks outlined below): The PCI injury apparatus consists of an elevated platform and a computer-controlled electro-pneumatic pressure release system used to launch a small projectile (3.52 g sphere) targeted at the rat's head. Following anesthetization with 5% isoflurane, a custom-designed helmet (Army Research Lab, Aberdeen Proving Ground, MD) is securely fastened onto the rat's head. Pressure sensor films (Fujifilm pre-scale pressure sensitive film) adhered to the inner and outer surfaces of the helmet are used to record the distribution and magnitude of pressure from the impact of the projectile. The anesthetized rat is placed on the elevated platform with its head positioned above an oval opening in the elevated platform such that the right hemisphere of the helmet-protected head is exposed to the projectile angled 45° from the saggital plane. A computer program is used to trigger the targeted release of the projectile at the rat's head. Immediately following PCI injury, the helmet is removed and the rat is returned to its home cage. Sham control animals receive the same procedures except the projectile impact.

The original study design called for the inclusion of a pressure wave (PW) control group to control for the potential effects of the PCI pressure wave. However, in the advanced PCI system, the need for a "pressure wave" (PW) control group has been negated by the refinements made to advanced PCI system because the "pressure wave" generated by the release of compressed gas is minimal. As a substitute for the PW group, we have included a positive PCI control group in the experimental design when needed to confirm that the outcome measures are capable of detecting injury signals. For this purpose, animals were subjected to 4 PCI-induced concussions (1 hour apart), representing a more severe concussion, yet remaining within the limits of the mTBI spectrum.

SOW 1 (Months 1-24): Fully characterize a "SINGLE" PCI head injury defining the acute temporal profile of histopathology, molecular (biomarkers/bioenergetics), neurobehavioral (motor/cognitive) dysfunction, and electrophysiological (EEG) changes following a single PCI.

Section II

Task 1.0 (Months 1-6): All regulatory review/approval requirements have been completed.

Task 1.1 (Months 1-12): Evaluate the regional and temporal profile of cellular changes following a single PCI injury. The effects of a single, lateral PCI on axonal injury using APP and CuAg staining are to be assessed at 6h, 24h, 72h, 7d, 14d and 28d post-PCI. The regional profile of the glial response to PCI injury are to be assessed at 6h, 24h, 72h, 7d and 14d post-injury using immunostaining markers for reactive astrocytes and activated microglia. The effects of PCI on BBB permeability will be examined at acute post-injury timepoints (6h, 24h and 72h) using biotin dextran amine (BDA; 3 kDA) and by IHC using antibodies for (1) Aquaporin 4 (AQ4) co-labeled with GFAP, and (2) tight junction and endothelial linkage proteins occluden, zonula occluden 1 (ZO-1), and claudin-5 (Cl-5).

Experiment 1.1.1 Diffuse Axonal Injury (DAI): DAI is a hallmark pathologic feature of TBI and has been consistently detected across the spectrum of TBI severities, including mTBI. Our proposed study will focus on the expression of beta-amyloid precursor protein (APP) and amino cupric silver (CuAg) expression as markers for acute axonal injury. The effects of PCI on axonal injury using APP and CuAg staining will be assessed at 6h, 24h, 72h, 7d, 14d and 28d post-PCI. **STATUS: Completed. Final results were included in Y2 Annual Report.**

Experiment 1.1.2. Glial Response: We previously reported significant increases in hippocampal expression of GFAP (glial fibrillary acidic protein; a marker for reactive astrocytes) in the PCI model at 24h post-injury. In the proposed study, the glial response to PCI injury will be examined in different brain regions (cerebral cortex, hippocampus, corpus callosum, thalamus, striatum and cerebellum) at 6h, 24h, 72h, 7d and 14d post-injury using immunostaining markers for reactive astrocytes and activated microglia**. STATUS: Completed. Final results were presented in Y2 Annual Report and Y3Q1 Quarterly Report.**

Experiment 1.1.3. Blood-Brain Barrier (BBB) Permeability: The effects of PCI on BBB permeability will be examined at discrete post-injury time points (i.e. 6h, 24h and 72h) using biotin dextran amine (BDA; 3 kDA) to detect more subtle BBB disruption that may not be apparent using Evan's blue extravasation or serum albumin IgG methods. In addition, the involvement of astrocytes and/or tight junctions in the BBB breakdown process will be examined by IHC using antibodies for (1) Aquaporin 4 (AQ4) co-labeled with GFAP, and (2) tight junction and endothelial linkage proteins occluden, zonula occluden 1 (ZO-1), and claudin-5 (Cl-5). **STATUS: Completed. Final results were included in Y2 Annual Report.**

Task 1.2 (Months 6-18): Evaluate the regional and temporal profile of molecular/bionergetic changes in brain tissue following a single PCI. Exp. 1.2.1: Changes in messenger ribonucleic acid (mRNA) levels will be evaluated following a single PCI injury in brain lysate by real-time polymerase chain reaction (PCR) with primers specific for known markers of cellular injury (i.e. GFAP, UCH-L1, Alpha-II spectrin, and APP). Exp. 1.2.2: Changes detected in mRNA expression will be correlated with changes in protein expression. Exp. 1.2.3: Changes in metabolic activity levels will be assessed using ultra-performance liquid chromatography (UPLC) measurements of adenosine triphosphate (ATP), adenosine diphosphate (ADP), creatine, phosphocreatine and N-acetylaspartate (NAA) levels to establish a profile of metabolic vulnerability/recovery in the PCI model.

Experiment 1.2.1. Molecular Changes: Changes in messenger ribonucleic acid (mRNA) levels will be evaluated following a single PCI injury in brain lysate by real-time polymerase chain reaction (PCR) with primers specific for known markers of cellular injury (i.e. GFAP, UCH-L1, Alpha-II spectrin, and APP) at 2h, 6h, 24h, 72h, and 7d post-PCI in comparison to sham and PW controls. We will correlate changes in mRNA expression with changes in protein expression (Exp. 1.2.2) to determine the precise mechanism of injury (i.e. gene regulation vs. protein

modification). **STATUS: Completed. Final results were included in Y2 Annual Report and Y3 Q1 Quarterly Report.**

Experiment 1.2.2. Protein Biomarkers: Changes in protein abundance for known markers of cellular injury (i.e. GFAP and its BDPs, UCH-L1, SBDPs, and c-APP) will be evaluated following a single PCI injury in brain tissue, cerebral spinal fluid (CSF) and serum by Western blot or enzyme-linked immunosorbent assays (ELISAs) following a single PCI injury at 2h, 6h, 24h, 72h, and 7d post-injury in comparison to sham and PW controls**. STATUS: Completed. Final results were included in Y2 Annual Report and Y3 Q1 Quarterly Report.**

Experiment 1.2.3. Bioenergetic Profile: Changes in metabolic activity levels will be assessed following a single PCI injury using the electromagnetic tissue fixation method to prepare brains for ultra-performance liquid chromatography (UPLC) measurements of adenosine triphosphate (ATP), adenosine diphosphate (ADP), creatine, phosphocreatine and N-acetylaspartate (NAA) levels to establish a profile of metabolic vulnerability/recovery in the PCI model. **STATUS: Completed. Final results were included in Y2 Annual Report.**

Experiment 1.2.4. microRNA Biomarker Profile (serum): microRNA Profiling as a novel biomarker for mTBI was added to this study in Y2. The microRNA profile in serum will be evaluated following a single PCI injury will be evaluated at 4h, 24h, 3d, and 7d. 4h. STATUS**: Completed. Final results were included in Y3 Annual Report.**

Task 1.3 (Months 1-18). Evaluate the neurobehavioral (motor, cognitive, and affective) profile following PCI injury. The goal of these experiments is to establish a comprehensive longitudinal neurobehavioral assessment of a single PCI injury on motor, cognitive, and affective (i.e. depression/learned helplessness) abnormalities. The key outcome metrics effects will be the degree of functional deficits on (1) a computer-assisted gait task, (2) a rotarod task, (3) a Morris water maze task, (4) a novel object recognition (NOR) task, and (5) a forced swim task. Groups will consist of sham, PW controls, and PCI (n=15/group) and separate groups of animals will be assessed for functional impairment on the respective tasks at both acute (< 3 days) and chronic (7-28 days) time points as outlined in tables provided in the SOW. **STATUS: Completed. Final results were included in Y2 Annual Report and Y3 Q1 Quarterly Report.**

Task 1.4 (Months 12-24). Evaluate quantitative electrophysiological (qEEG) profile of PCI-induced abnormalities in brain wave patterns: EEG power spectrum analysis was used to examine EEG power shift and altered EEG coherence following PCI, through continuous EEG recording out to 72h post-PCI, followed by a 2-h recording on post-injury Days 5, 7, and 14. Experimental groups consisted of sham, PW controls, and PCI (n=15/group; N=45). **STATUS: Completed. Final results were included in Y2 Annual Report and Y3 Q1 Quarterly Report.**

SOW 1 Summary and Conclusions: Results for each Task (i.e. Neuropathology, Molecular and Functional Outcomes) are summarized below:

Neuropathological Results: A single concussion produced significant bilateral increases in accumulation of β-APP indicative of axonal damage that peaked at 6h post-injury and were resolved by 72h. Corresponding hippocampal GFAP levels were slightly upregulated at 6h following and were significantly higher than sham at 24h in both hemispheres, indicative of progressive astrocyte activation. Significant microglial activation, indicated by Iba-1, was evident at 6h and 72h in the hippocampus following a single concussion (vs. sham) that was resolved at 7 days post-injury. Additionally, a significant increase in neuronal cell death (flourojade) was detected at 24h post-injury. Of these measures, β-APP, Iba-1 and flourojade were significantly higher following repeated vs. single concussion for at least one post-injury time point.

Molecular Results: Western blot results showed no changes in brain tissue protein levels for GFAP, UCH-L1, SBPD, β-APP, Tau or p-Tau following a single concussion. However, Tau and p-Tau were significantly elevated in the hippocampus at 72h post-injury following repeated concussion. CSF biomarker results showed significant

increases in GFAP and UCH-L1 at 1h post-injury and Tau at 24h post-injury following single and repeated concussion. Additionally, inflammatory cytokines were significantly upregulated in CSF at 1h and 24h post-injury following repeated PCI. Similar results were detected in serum biomarkers with inflammatory miRNAs (4h, 24h, and 3d) and inflammatory cytokines (1h) showing significant increases following a single concussion and GFAP showing a significant increase at 1h post-injury following repeated PCI. Of these results, miRNA measures appear to provide the most promising therapeutic target in serum**)** whereas Tau and inflammatory cytokines may provide additional targets in CSF

Functional Outcomes: PCI produces acute (≤ 4h) abnormalities in righting reflex (not shown), NSSR and BBB scores following both single and repeated concussion that are resolved by 24h post-injury. **Decrements in rotarod and MWM performance were detected only following repeated concussion.** However, significant alterations in sensorimotor (gait) activity were detected on the CatWalk following a single concussion out to 72h post-injury (and again at 1 month post) that were significantly higher following repeat concussion**).** Overall, these results indicate that, while the righting reflex, BBB and NSSR scores provide a useful inclusion/exclusion criteria matrix, the CatWalk provides the most useful metric for evaluating putative therapeutic effects during the acute post-injury phase.

SOW 2 (Months 24 - 48): Evaluate the cumulative effects of "REPEATED" PCI longitudinally across outcome metrics defined in SOW 1. Recent studies have implied that there is a correlation between sustaining repetitive concussions and experiencing worse outcomes, and it has been suggested that repetitive concussions can lead to the development of chronic pathological and psychological changes in the concussed subject. However, very little is actually known or understood about repetitive mTBI events and what factors are associated with the debilitating outcomes. Currently it is not known what changes are taking place in the brain with a concussion, how long the changes last and what happens if a second (or third, fourth, etc.) concussion is experienced during the healing period. Therefore, the goal of this SOW is to determine the effects associated with repetitive concussive injuries.

Task 2. Determine the effect of "REPEATED" PCI injuries compared to a "SINGLE" PCI injury based on the healing profile. The experimental design and specific time points for repeated PCI injuries for SOW 2 will be based on the optimal outcome metrics and time points identified in SOW 1. More specifically, the data generated in SOW 1 will fully characterize the injury profile in the brain after a single PCI injury. Based on this data, a "healing profile" will be developed that demonstrates the time at which the brain has essentially returned to normal based on the associated neuropathological, molecular and neurobehavioral changes. We will evaluate the effects of repeated PCI injuries occurring either before or after resolution of the healing profile. For example, if the data from a single PCI injury in SOW 1 demonstrates that the healing profile has resolved by 72h post-PCI, then separate groups of animals will be subjected to additional PCI injuries at either (A) 24h and/or 48h post-PCI (during the healing period) or (B) 24h after resolution of the healing profile (i.e. 4 days post-PCI).

Notably, during our early Phase I studies, we reported that a single PCI injury is extremely mild - specifically developed by our team to cause no skull fracture and no bleed, to best mimic the "invisible wounds" of war suffered by our troops, and by civilians involved in sports or other activities where there is risk of concussion. Because the single PCI is so mild, we included a repeated PCI (r-PCI) "positive control" group for each outcome metric in order to ensure that our individual outcome metrics were sensitive to the different levels of injury severity. These positive control groups consisted of exposing animals to 4 PCIs spaced 1 hour apart (i.e. r-PCI4x1h). This r-PCI4x1h control group was developed based on pilot experiments showing that 4 hits was the threshold for inducing injuries that did not result in any skull fractures or bleeds, which started showing up after 5 hits and were more prevalent with 6 hits. The 1-hour interval between r-PCI injuries was selected to better control/monitor exposure to anesthesia between cohorts. This also had the added benefit of allowing us to

conduct righting reflex assessments following each successive r-PCI across a number of experiments. Those results have all been previously reported as part of our initial single vs. repeated PCI comparisons, and have been incorporated into guiding the experimental design for our Phase II studies.

Task 2.1. Regional and temporal profile of neuropathological alterations following repeated concussion: Phase I (SOW I) results indicates that the healing profiles (i.e. when significant alterations induced by the injury are no longer detectable) for axonal injury and blood brain barrier permeability are ≤24h whereas the healing profile for the neuroinflammatory response (GFAP and Iba-1) is approximately 72h. Based on those results, Phase II animals were exposed to multiple PCIs spaced 1h, 24h apart or 3 days apart depending on their healing profile. As before, separate groups of animals were used for blood brain barrier markers of tight junction proteins (AQ4, ZO-1 and CL-5) due to the need to use fresh frozen tissue. For all markers, animals were euthanized at 24h, 72h and 7d following the last PCI (additional post-injury time points may be added as indicated by the results).

Experiment 2.1.1 Diffuse Axonal Injury (DAI) – 24h healing profile: Based on the results of Phase I experiments, we chose to expose separate cohorts of animals to will be exposed to either 2 or 3 PCIs spaced 8h apart (prior to resolution of the healing profile) or 2 PCIs spaced 48h apart (after resolution of the healing profile). Animals will be euthanized at 24h, 72h and 14d post-injury and brain tissues will be stained for Beta-amyloid precursor protein (β-APP) and amino cupric silver (CuAg) expression as markers for acute (<7 days post-injury) and subacute (> 7 days post-injury) axonal pathology respectively. **STATUS: All brain samples have been collected and are currently being processed/analyzed. No additional results to report at this time.**

Experiment 2.1.2. Glial Response (72h healing profile): Based on the results of Phase I experiments, we chose to expose separate cohorts of animals to either 2 or 3 PCIs spaced 24h apart (prior to resolution of the healing profile) or 2 PCIs spaced 7 days apart (after resolution of the healing profile). Animals will be euthanized at 24h, 72h, and 14d post-injury and brain tissues will be stained for glial fibrillary acidic protein (GFAP) and for ionized calcium binding adaptor molecule (Iba1; microglia/macrophage marker). **STATUS: All brain samples have been collected and are currently being processed/analyzed. No additional results to report at this time.**

Experiment 2.1.3. Blood-Brain Barrier (BBB) Permeability (24h healing profile): Based on the results of Phase I studies, we chose to expose separate cohorts of animals to either 2 or 3 PCIs spaced 8h apart (prior to resolution of the healing profile) or 2 PCIs spaced 48h apart (after resolution of the healing profile). Animals will be euthanized at 24h and 72h post-injury and brain tissues will be processed using markers for tight junction proteins involved in the BBB breakdown process including antibodies for tight junction and endothelial linkage proteins occluden, zonula occluden 1 (ZO-1), and claudin-5 (Cl-5). **STATUS: All samples have been collected and processed and are currently being analyzed at this time. No additional results to report at this time.**

Experiment 2.1.4. Microglial Phenotype: Based on the phase-I neuropathological results, we expanded the neuroinflammatory study by characterizing microglial phenotypes during the peak activation time points following repeat injury. To understand the microglial phenotype changes following concussive brain injury, single and repeat PCI (4 hits; 1h interval) injuries were conducted. Two endpoints (6h and 72h) were selected based on earlier experiments that showed increased Iba-1 (microglial marker) staining following PCI in rats. Following intra-cardiac perfusion, brains were harvested and processed for immunostaining. Coronal sections (30μm) were immunostained with MHC-II/Iba-1 (M-1 phenotype) or CD163/Iba-1(M-2 phenotype) antibodies. M-1 (co-labelled for MHC-II and Iba-1) and M-2 (co-labelled for CD163 and Iba-1) microglia in different brain regions (cortex, hippocampus and striatum) were counted manually at 20X magnification. M-1 or pro-inflammatory phenotype was mostly located in the cortex whereas the anti-inflammatory M-2 phenotype was predominate in cortex, striatum and hippocampus. Repeat PCI increased M-1 type expression bilaterally compared to single PCI, and concomitantly downregulated M-2 type microglia at both time points studied. At 6h, following repeated PCI, the increase in M-1 signal was 39% and 62% respectively for contralateral and ipsilateral cortex, whereas much

higher increases in M-1 expression were detected at 72h post-injury (647% and 1500% respectively for contra and ipsilateral cortex). M-2 microglial counts were decreased by 26% and 14% at 6h respectively in contralateral and ipsilateral cortex. At 72h, the decline in M-2 phenotype was more pronounced with 50% and 30% respectively for contralateral and ipsilateral cortex. Moreover, repeat PCI increased M-1/M-2 ratio bilaterally compared to single PCI (89% increase at 6h compared to single PCI) indicating a shift towards inflammatory phenotype. These findings indicate microglial polarization following concussive brain injury. Following multiple concussions, the microglial phenotype showed a shift towards proinflammatory M-1 type, a response that may serve as a novel therapeutic target for TBI. **STATUS: Complete. Results were included in Year 3 Annual Report.**

Task 2.2. Regional and temporal profile of molecular changes following single and repeated PCI injury:

Experiment 2.2.1. Protein Biomarkers (brain tissue): UCHL1 has been eliminated as a target and replaced with the more relevant tau protein targets. Phase I studies showed acute (4h) transient reductions in GFAP and GFAPbdp levels following a single PCI in the cortex and hippocampus (injured hemisphere). In addition evidence of increased levels of total tau and tau phosphorylation levels was observed at 72h following repeated PCI (i.e. 4 hits spaced 1h apart), but not single PCI. Phase II research will look at 24 and 7 day intervals and at later time points post injury (7 days) to determine whether significant alterations in total tau and tau phosphorylation levels are evident under these conditions. We previously reported the results of repeated PCI injuries (4 injuries) conducted using 1 hour (PCI4x1H), 24 hour (PCI4x24H), or 7 day (PCI4x7D) inter-injury intervals [Experiment 2.2.1b]. Overall, the results of these experiments indicated that tau and tau phosphorylation are more robustly affected by repeated injuries that occur in close temporal proximity whereas GFAP alterations appeared more prevalent with longer inter-injury intervals and thus may be more valuable in evaluated therapeutic interventions. **STATUS: All samples have been collected, processed and analyzed. A manuscript is being prepared for publication based on these experiments.**

Experiment 2.2.2. Biomarkers (CSF and serum): Phase I studies showed significant increases in GFAP in CSF 1h following a single PCI. In Phase II studies, animals will be exposed to either 2 or 3 PCIs spaced 24h apart or 2 hits spaced 7 days apart. Recent research led us to expand our investigation of tissue pathology markers and biomarkers to include Cathepsin B. The purpose of these experiments was to determine if brain cathepsin B is up-regulated following repeated projectile concussive impact (rPCI) in rodent models of TBI. The preliminary study has been completed and the results, showing Cathepsin B is upregulated following repeated PCI in correlation impaired righting reflex were included in the FY15 end-of-year Annual Report. **STATUS: All samples have been collected, processed analyzed. A manuscript has been submitted for publication based on these experiments.**

Experiment 2.2.3. Bioenergetic Profile (brain tissue): Phase 1 studies showed significant alterations in metabolic activity levels following a single PCI that were evident from 30 min. to 6h post-injury (peak level) that were primarily resolved by 24h post-injury. For Phase II experiments, rats will be exposed to 2 PCIs spaced 6h apart, 24h apart and 7 days apart. All animals will be euthanized at 2h following the last PCI. **During the past year, we conducted additional analyses on serum-based alterations in metabolite profiles using blood samples collected from the same cohorts as listed above.** The preliminary results (Figure 4 below) show a biphasic profile of metabolic dysregulation that is evident up to 7 days (165 hours) post-injury following a single PCI (Figure 4A). These changes detected in serum samples directly correspond with the biphasic profile of metabolic dysregulation that we previously reported in brain tissue and CSF in the same animals. In addition, significant alterations following repeated PCI at varied intervals (Figure 4B) shows a similar pattern of metabolic dysregulation, which also corresponds to what was previously observed in brain tissue and CSF for the same animals. STATUS: Sample collection and processing has been completed. Results of metabolic dysregulation in brain tissue and CSF were reported in Y3, Q2 and summarized in the Year 3 Annual Report. A manuscript is currently being prepared for submission based on the collective results of Phase I and Phase II experiments.

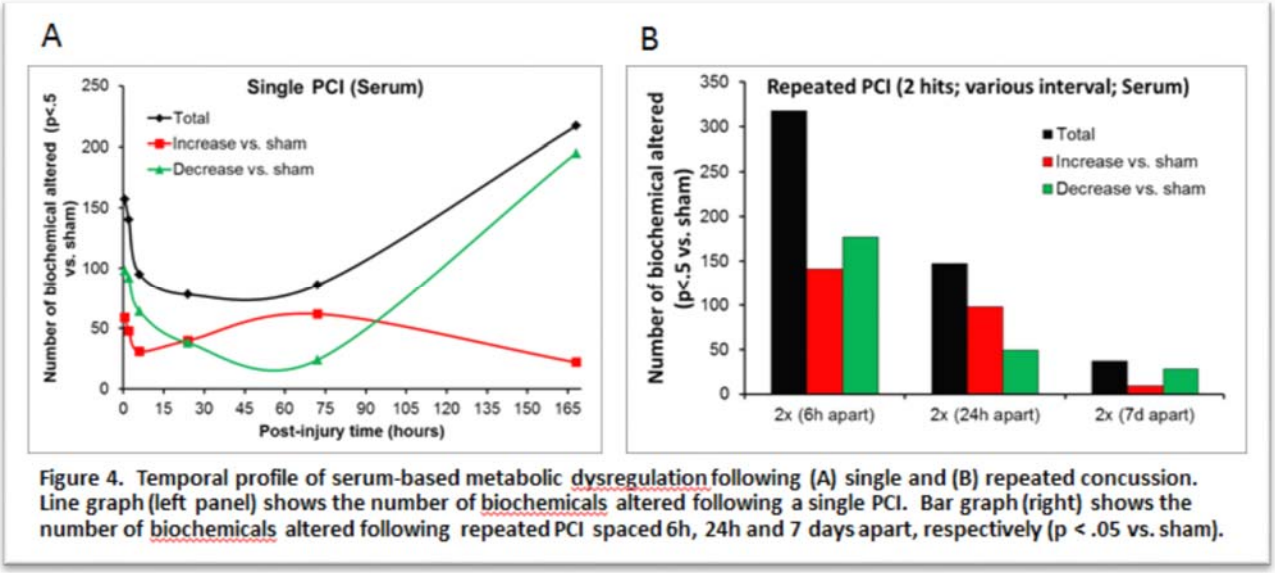


Figure 4. Temporal profile of serum-based metabolic dysregulation following (A) single and (B) repeated concussion. Line graph (left panel) shows the number of biochemicals altered following a single PCI. Bar graph (right) shows the number of biochemicals altered following repeated PCI spaced 6h, 24h and 7 days apart, respectively ($p < .05$ vs. sham).

Task 2.3. Evaluate the neurobehavioral (motor, cognitive, and affective) profile following PCI injury.

Experiment 2.3.1. Sensorimotor: In Phase I experiments; evidence of sensorimotor impairment was quantified using a number of outcome metrics including the NSSR (neurological severity scale revised) task, gaitwalk and rotarod tasks. Overall, the results indicated that a single PCI produced some acute alterations that were evident up to 4h post-injury. The effects of repeated concussion on gait abnormalities (CatWalk) and vestibular dysfunction (rotarod) with injuries a single PCI (one time point), spaced 24h apart (24PCI, 2x24h) and spaced 7 days (7dPCI, 2x7d) apart were examined. Results were reported in Year 3 Annual Report. A manuscript has been prepared based on the results of sensorimotor testing and EEG analysis (Experiment 2.3.3). This manuscript is current under review by the Journal of Neurotrauma.

Experiment 2.3.2. Cognitive: We previously reported Morris water maze results showing significant spatial learning deficits following repeated PCI at 1 month post-injury that were no longer evident at 6 months post-injury. In contrast, while animals exposed to repeated PCI failed to show a significant decrement on a working memory task at 1 month post-injury ($p>0.05$ vs. sham), their performance on the same task was significantly impaired at 6 months post-PCI ($p<0.05$ vs. sham). During the past year, we also completed experiments testing for cognitive impairment following repeated PCI on a novel object recognition (NOR) task. The NOR task taps into the rodent's innate foraging abilities. For this purpose, the animals are first exposed to a set of identical objects in an open field environment. After a delay, the animals are re-exposed to one of the original objects (i.e. familiar object) paired with a novel object. A significant preference for the novel object indicates a memory for the familiar object. However, in the case of our experiments, neither group showed a significant preference for the novel object indicating that the test failed to discriminate between injured and sham groups (Figure 5).

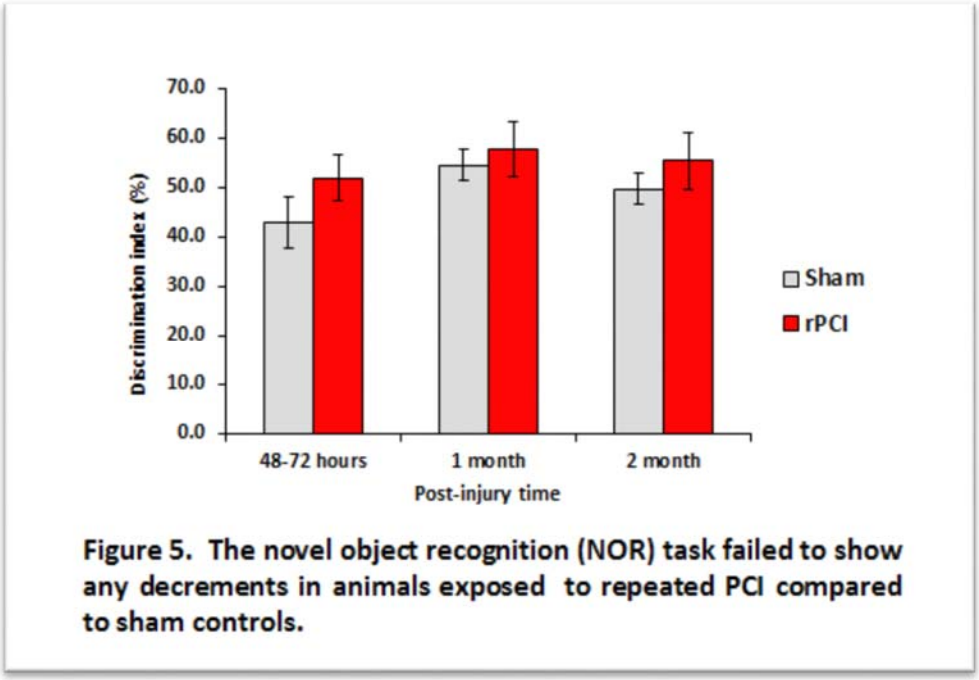


Figure 5. The novel object recognition (NOR) task failed to show any decrements in animals exposed to repeated PCI compared to sham controls.

Experiment 2.3.3. Affective: Phase I/II experiments failed to show any evidence of affective dysfunction on the forced swim task following a single or repeated PCI. Since this was our only affective measure, we sought to replace it with a different measure that would prove more sensitive and reliable for detecting affective behavioral measures (i.e. measures that would be akin to those observed in TBI patients with post-traumatic stress syndrome; PTSD). Thus we conducted additional, pilot experiments to evaluate the effects of repeated PCI on the elevated plus maze (affective measure of anxiety) and ultrasonic vocalization (affective measure of negative emotion). Results of elevated plus maze failed to detect any effects of repeated PCI and thus this test has been abandoned. However, the ultrasonic vocalization test proved to be very sensitive to repeated PCI. Further, we have reproduced these results across several time points, which indicate this test may provide a reliable measure for single and repeated concussion in rats (Figure 6). A manuscript outlining the initial proof of concept experiments was recently accepted for publication in the Journal of Neuroscience Methods (Browning et al., 2016).

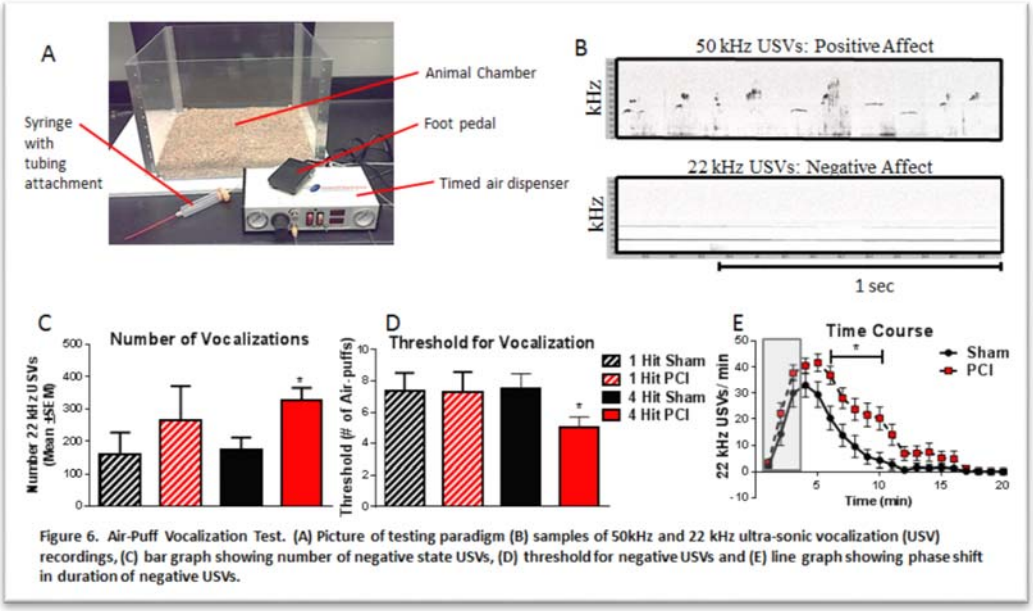


Figure 6. Air-Puff Vocalization Test. (A) Picture of testing paradigm (B) samples of 50kHz and 22 kHz ultra-sonic vocalization (USV) recordings, (C) bar graph showing number of negative state USVs, (D) threshold for negative USVs and (E) line graph showing phase shift in duration of negative USVs.

Experiment 2.3.4. EEG Analysis: Investigation of EEG functional changes in the repeated PCI model will focus on the magnitude of EEG slowing and persistency of the abnormality. The frequency of repeated hits and the time interval between each hit for this study will be determined based on the outcomes yielded from ongoing behavioral and molecular studies of repeated PCI. The results of qEEG power spectrum analysis on repeated

PCI were presented as a poster presentation at the June 2016 National Neurotrauma Meeting and those results are also part of a manuscript that is currently under review by the Journal of Neurotrauma (Figure 7).

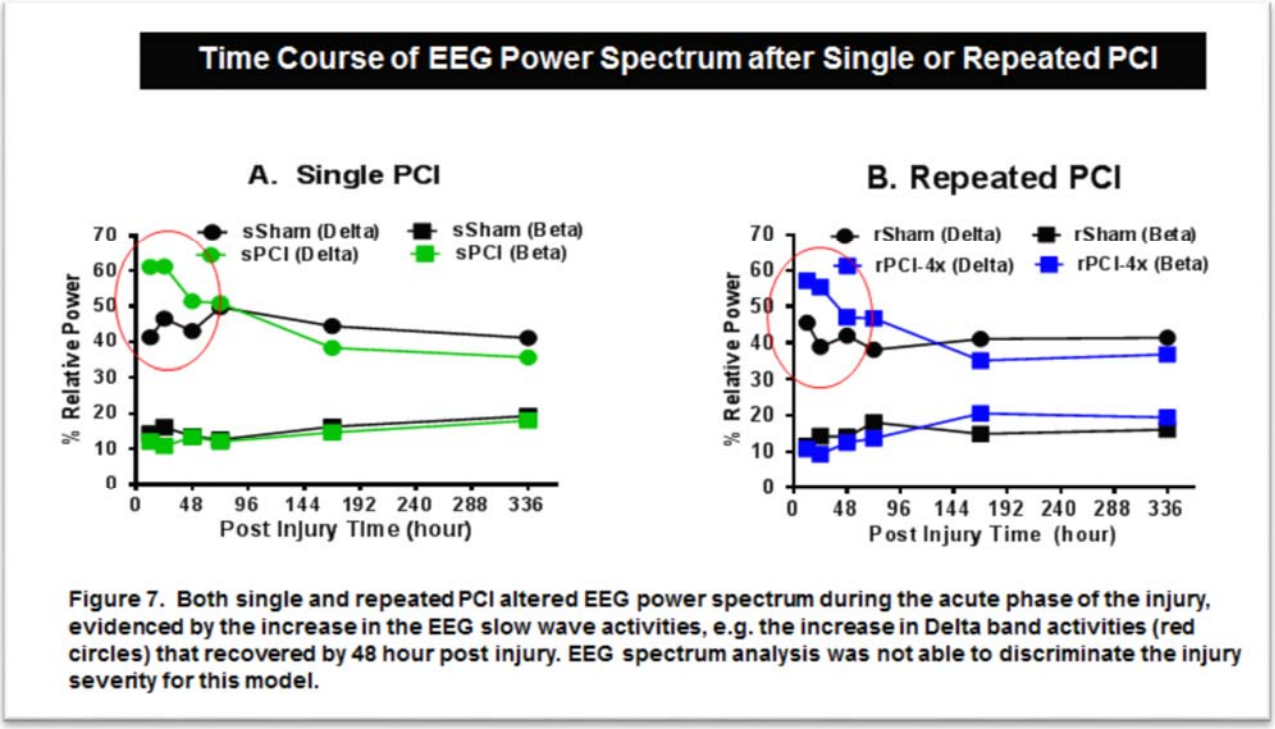


Figure 7. Both single and repeated PCI altered EEG power spectrum during the acute phase of the injury, evidenced by the increase in the EEG slow wave activities, e.g. the increase in Delta band activities (red circles) that recovered by 48 hour post injury. EEG spectrum analysis was not able to discriminate the injury severity for this model.

SUMMARY OF PHASE I/ PHASE II RESULTS TO DATE:

Histopathology Results:

1. Axonal Damage: A single concussion produced significant bilateral increases in accumulation of β-APP indicative of axonal damage that peaked at 6h post-injury and were resolved by 72h. Repeated concussion produced dramatically increases in β-APP expression that corresponded to delayed increases in silver staining.
2. Neuroinflammation: Corresponding hippocampal GFAP levels were slightly upregulated at 6h following and were significantly higher than sham at 24h in both hemispheres, indicative of progressive astrocyte activation. Significant microglial activation, indicated by Iba-1, was evident at 6h and 72h in the hippocampus following a single concussion (vs. sham) that was resolved at 7 days post-injury.
3. Blood Brain Barrier Permeability: No significant effects of single or repeated concussion were observed on aquaporin 4 (AQ4) expression (an indirect measure of edema). However, the pattern of delayed degradation of claudin 5 (CL5) and zona-occludin 1 (Z01) protein expression following single and repeated concussion suggests a progressive gradient in BBB disruption that is may be mediated by secondary causes, such as neuroinflammation and oxidative stress.

These results show significant alterations in histopathology following repeated concussion that remain evident even as outward symptoms are subsiding and provide further support for delayed return-to-duty guidelines following concussion.

Molecular Measures:

1. Western blot results showed no changes in brain tissue protein levels for GFAP, UCH-L1, SBPD, β-APP, Tau or p-Tau following a single concussion. However, Tau and p-Tau were significantly elevated in the hippocampus at 72h post-injury following repeated concussion.
2. CSF biomarker results showed significant increases in GFAP and UCH-L1 at 1h post-injury and Tau at 24h post-injury following single and repeated concussion. Additionally, inflammatory cytokines were significantly upregulated in CSF at 1h and 24h post-injury following repeated PCI.
3. Similar results were detected in serum biomarkers with inflammatory microRNAs (4h, 24h, and 3d) and inflammatory cytokines (1h) showing significant increases following a single concussion and GFAP showing a significant increase at 1h post-injury following repeated PCI.

Of these results, microRNA measures appear to provide the most promising therapeutic target in serum whereas Tau and inflammatory cytokines may provide additional targets in CSF

Metabolomic Measures:

1. Glucose utilization was significantly altered following both single and repeated concussion; indicative of energy crisis; increased lactate levels may indicate neuropath target (i.e. headache).
2. Neurotransmitters dysregulation may influence on behavior and recovery.
3. Reduced antioxidant level may be indicative of redox stress.
4. The levels of polyamine metabolites, e.g. putrescine, responded to the number of concussions, indicating its specificity to number of concussions.
5. Global metabolomic profile revealed an increased risk of disrupted metabolic homeostasis associated with repeated concussion versus a single concussion.
6. Random forest analysis resulted in a high predictive accuracy with top candidates, such as putrescine and associated metabolites, that were shared in multiple concussed injured groups.

The PCI injury generated significant metabolic changes that are relevant to clinical concussion. Further study of affected metabolic pathways may lead to novel, TBI-specific biomarker discovery.

Neurobehavioral Measures:

1. Sensorimotor: Acute (≤ 4h) abnormalities in righting reflex, NSSR and BBB scores following both single and repeated concussion. Decrements in rotarod performance were detected only following repeated concussion. Significant alterations in sensorimotor (gait) activity were detected on the CatWalk following a single concussion out to 72h post-injury (and again at 1 month post) that were significantly higher following repeat concussion.
2. Cognitive: Decrements in MWM spatial reference memory and working memory performance were evident as early as 1 month and out to 6 months, respectively, post-injury following repeated concussion. Switch from spatial learning to working memory deficits may be indicative of progressive deficits.
3. Affective: No decrements were detected following a single concussion on any measure of affective behavior (i.e. forced swim, plus maze). Effects of repeated concussion on the forced swim task were opposite what was expected, which may be indicative of increased anxiety. Ultra-sonic vocalization (USV) testing showed significant increase in negative state following repeated concussion.
4. Neurophysiology: Acute transient alterations in delta wave activity were evident following both single and repeated concussion but this measure was not sensitive to increased injury severity.

Overall, these results indicate that, while the righting reflex, BBB and NSSR scores provide a useful inclusion/exclusion criteria matrix, the CatWalk and the USV test provides the most useful metric for evaluating putative therapeutic effects during the acute post-injury phase.

Summary of Key Deliverables and Way Forward

Purpose: Develop and characterize a novel animal model of mTBI simulating a concussive impact to the head

Products:

- ✓ A validated rodent model to study the effects of mild, concussive brain injury
- ✓ Knowledge about the effects of multiple concussive events
- ✓ A compact, inexpensive and high throughput device to produce a concussion

Payoff: A fully characterized, validated animal model of closed-head concussive mild TBI and a research tool to study treatment strategies

Way Forward

- **In Process: Proof-of-concept therapeutic studies on PCI model as part of core-funded program.**
- **In Process: Full characterization of neurobehavioral, neurophysiological and biochemical changes following closed-head concussion occurring in conjunction with polytrauma (PCI/Polytrauma Model).**
- **Planned: Future studies to evaluate neurotherapeutic resuscitation strategies and/or REBOA in PCI/Polytrauma Model.**

Section IV

All tasks are progressing on schedule.

KEY RESEARCH ACCOMPLISHMENTS

Year 1 Accomplishments

1. IACUC and ACURO approval completed for two active protocols (in vivo and molecular).
2. Completion of Advanced PCI Model and Parameters to include PCI Device (driven by compressed gas vs. dry ice sublimation); completion of helmet material and design testing; PCI projectile; angle/location of PCI injury on the rat head.
3. Completion of acute (6h – 7 days post-injury) histopathological studies of a single PCI. Analysis of chronic post-injury time points is in process.
4. Completed collection of all histopathological samples for evaluating blood brain barrier (BBB) permeability following a single PCI. Brain tissues are being processed and analysis is targeted for completion in the first Quarter of Year 2.
5. Completed all tissue collections for mRNA molecular and protein biomarker changes. Analysis of the effects of PCI on GFAP and GFAP breakdown products has been completed. Analysis of additional markers is ongoing.
6. Completed sample collection for changes in metabolic activity levels. Primary (2h) samples are currently being processed via contractual agreement by Metabolon for global analysis of over 4,000 metabolites.
7. Completed acute post-injury assessment of motor (i.e. gaitwalk) abnormalities following a single PCI. Chronic evaluations are ongoing.
8. Completed acute post-injury assessment of cognitive (MWM) function following a single PCI. Additional animals are currently being tested at chronic time points.
9. Added righting reflex measures to the neurobehavioral outcome parameters and reported results confirming the validity of the PCI model as a model of closed-head concussive mild TBI.

Year 2 Accomplishments

1. Completed longitudinal (6h – 28 days post-injury) histopathological analysis of diffuse axonal injury and glial activation following a single PCI and repeated (4x) PCI.
2. Completed histopathological evaluation of blood brain barrier (BBB) permeability following a single PCI and repeated (4x) PCI.
3. Completed all tissue processing and analyses for mRNA molecular and protein biomarker changes and repeated (up to 4x) PCI.
4. Completed additional evaluations of phosphorylated Tau and total Tau following a single and repeated (4x) PCI.
5. Completed additional evaluation of novel microRNA biomarker analysis in serum following a single PCI.
6. Completed metabolomics analysis of all tissue samples and identified post-injury metabolic profile following a single PCI and repeated (4x) PCI.
7. Completed acute and chronic post-injury assessment of neurological (i.e. righting reflex and NSSR score) dysfunction following a single PCI and repeated (up to 4x) PCI.
8. Completed chronic post-injury assessment of cognitive (MWM) function following repeated (4x) PCI.
9. Completed acute EEG measurements of brain wave activity following a single PCI.

Year 3 Accomplishments

1. Initiated experiments to determine the effects of rPCI delivered pre- and post- resolution of the healing profile on BBB permeability.

2. Added investigations to characterize microglial phenotypes following repeat injury paradigms; preliminary analysis demonstrates differential microglial activation following sPCI and rPCI.
3. Completed evaluation of protein biomarkers in brain tissue including Tau, pTau, GFAP and SBDP following (4x) PCI induced at different inter injury intervals.
4. Initiated investigations of CSF and serum biomarkers following single or (2-4x) PCI; results to date indicate elevations in serum GFAP, Tau and UCH-L1 with varying degrees of correlation to Righting Relfex and NSSR Scores.
5. Added studies examining the expression of Cathepsin B following rPCI.
6. Completed bioenergetic analyses of brain tissue and CSF following (2x) or (3x) PCI induced at different inter injury intervals.
7. Completed sample collection to investigate microRNA biomarker profiles from serum.
8. Completed sensorimotor analysis including performance on the CatWalk and Rotarod tasks following (2x) PCI induced at different inter injury intervals.
9. Completed acute and subacute assessment of cognitive (MWM) function following (4x) PCI at 1hr injury intervals.
10. Initiated studies of negative affect following (4x) PCI using the air-puff negative vocalization test, elevated plus maze test, and forced swim test; preliminary results indicate measures of negative affect using the air-puff negative vocalization test.
11. Completed EEG analysis following (4x) PCI at 1hr injury intervals.

Year 4 Accomplishments

1. Completed investigations to characterize microglial phenotypes following repeat injury paradigms; preliminary analysis demonstrates differential microglial activation following sPCI and rPCI.
2. Completed investigations of CSF and serum biomarkers following single or (2-4x) PCI; results to date indicate elevations in serum GFAP, Tau and UCH-L1 with varying degrees of correlation to Righting Relfex and NSSR Scores (Mountney et al., 2017; in press).
3. Completed studies examining the expression of Cathepsin B following repeated PCI.
4. Completed bioenergetic analyses of serum following (2x) or (3x) PCI induced at different inter injury intervals.
5. Completed acute and subacute assessment of cognitive function using novel object recognition task following (4x) PCI at 1hr injury intervals.
6. Completed studies of negative affect following single and repeated PCI using the air-puff negative vocalization test, elevated plus maze test, and forced swim test; Results indicate measures of negative affect using the air-puff negative vocalization test.
7. Completed EEG power spectrum analysis of single vs. repeated PCI at 1hr injury intervals.

REPORTABLE OUTCOMES

(All reportable outcomes since project inception are shown; those from the 2014-2015 funding year are shown in bold font):

Peer Reviewed Publications: 4

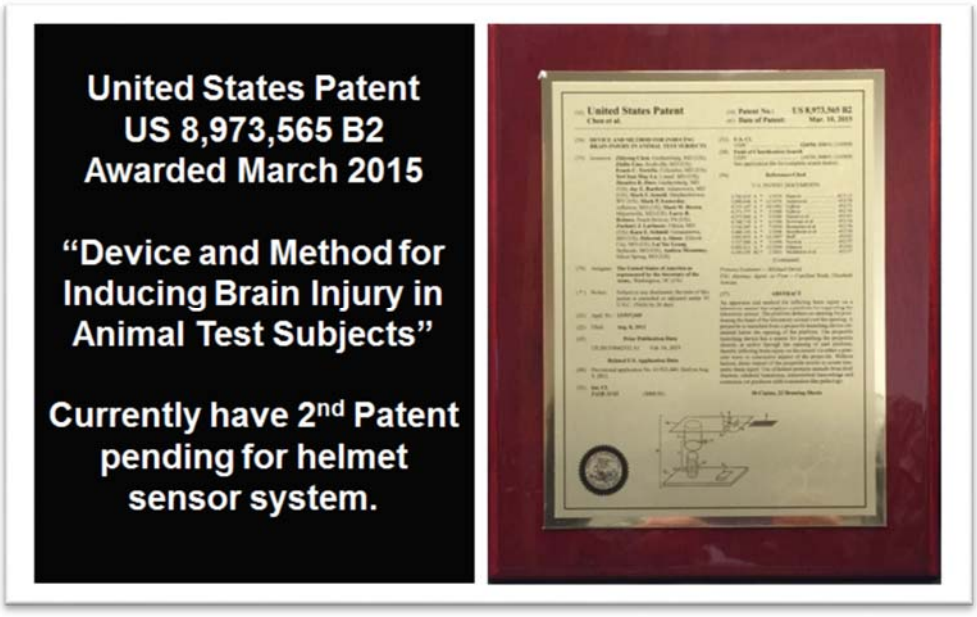
Patents: 1 and 1 pending

Abstracts: 21

List of Peer-reviewed Publications:

1. Chen Z, Leung LY, Mountney A, Liao Z, Yang W, Lu XC, Dave J, Deng-Bryant Y, Wei G, Schmid K, Shear DA, Tortella FC. A novel animal model of closed-head concussive-induced mild traumatic brain injury: development, implementation, and characterization. J Neurotrauma. 2012 Jan 20;29(2):268-80. doi: 10.1089/neu.2011.2057. PubMed PMID: 21988140.
2. Leung LY, Wei G, Shear DA, Tortella FC. The acute effects of hemorrhagic shock on cerebral blood flow, brain tissue oxygen tension, and spreading depolarization following penetrating ballistic-like brain injury. J Neurotrauma. 2013 Jul 15;30(14):1288-98. doi: 10.1089/neu.2012.2715. PubMed PMID: 23461630.
3. Browning JR, Whiteman AC, Leung LY, Lu XM, Shear DA. Air-puff induced vocalizations: A novel approach to detecting negative affective state following concussion in rats. J Neurosci Methods. 2016 Oct 29. pii: S0165-0270(16)30257-6. doi: 10.1016/j.jneumeth.2016.10.017. [Epub ahead of print] PubMed PMID: 27984100.
4. Mountney A, Boutte AM, Cartagena CM, Flerlage WF, Johnson WD, Rho C, Lu XCM, Yarnell A, Marcsisin, S, Sousa, Luong T, Zottig V, Leung LL, Deng-Bryant Y, Gilsdorf J, Tortella FC, Shear DA. Functional and molecular correlates following single and repeated rat closed-head concussion; indices of vulnerability following brain injury. J Neurotrauma. 2017 (accepted).

Patents:



List of Abstracts, Posters, Invited Talks

1. Leung L, Larimore Z, Holmes LR, McLoughlin S, Mountney A, Schmid K, Shear DA, Tortella FC (2012). WRAIR Projectile Concussive Impact (PCI) Model: Injury Device and Helmet Advance Development. 30th Annual Neurotrauma Symposium in Phoenix, AZ
2. Leung LY, Larimore Z, Holmes L, Cartagena C, McLoughlin S, Bustos F, Schmid K, Shear DA, Tortella FC. The WRAIR Projectile Concussive Impact Model: Effects of Impact Direction and Projectile Property. Military Health System Research Symposium 2013. Fort Lauderdale, Florida. August 2013. (Oral presentation)
3. Leung LY, Larimore Z, Holmes L, Cartagena C, McLoughlin S, Bustos F, Schmid K, Shear DA, Tortella FC. The WRAIR Projectile Concussive Impact Model: Effects of Impact Direction and Projectile Property. The 31st National Neurotrauma Symposium. Nashville, Tennessee, USA. August 2013.
4. Boutte AM, Mountney A, Johnson DW, Yarnell A, Tortella FC, Dave JR, Shear DA, Schmid KE (2014). Delayed consciousness, sensory-motor deficits and GFAP levels in repeated concussive impact. Journal of Neurotrauma 31:A-1-A-126; page A37.
5. Deng-Bryant Y, Leung LY, Readnower R, Yang W, Shear DA, Tortella FC (2014). Global metabolomics profiling reveals metabolic dysregulation, oxidative stress and neurotransmission alteration after concussion. Journal of Neurotrauma 31:A-1-A-126; page A10.
6. Leung LY, Deng-Bryant Y, Yang, W, Winter M, Tortella FC, Shear DA (2014). Regional and temporal histopathological changes following mild concussive brain injury. Journal of Neurotrauma 31:A-1-1-A-126; page A94.
7. Mountney A, Rho C, Yang W, Flerlage J, Yarnell A, Cartagena C, Schmid K, Bliese P, Tortella FC, Shear DA (2014). Longitudinal profile of sensorimotor deficits following single and repeated projectile concussive injury (PCI). Journal of Neurotrauma 31:A-1-A-126; page A119.
8. Leung LY, Deng-Bryant Y, Yang W, Winter M, Flerlage J, Tortella FC and Shear DA. (2014). Histopathological changes and cognitive deficits following closed-head concussive injury in rats. Military Health System Research Symposium; Fort Lauderdale, Florida. August 2014. (Oral presentation)
9. Leung LY, Larimore Z, Holmes L, Cartagena C, Mountney A, Deng-Bryant Y, Schmid K, Shear D, Tortella F (2014) The WRAIR projectile concussive impact model of mild traumatic brain injury: re-design, testing and preclinical validation. Annals of biomedical engineering 42:1618-1630.
10. Browning J, Deng-Bryant Y, Yang W, Yang X, Tortella F, Shear D, Leung LY (2015). Profile of blood brain barrier disruption following single and repeated closed head impact concussion in rats. Journal of Neurotrauma 32:A-1-A-152; page A26.
11. Boutte A, Mountney A, Abbatiello B, Grant S, Johnson D, Cartagena C, Tortella F, Shear D (2015). Multivariate biomarker profiling, sensorymotor deficits, consciousness after single and repeat projectile concussive injury. Journal of Neurotrauma 32:A-1-A-152; page A35.
12. Boutte A, Abbatiello B, Grant S, Hook G, Hook V, Tortella F, Shear S. (2015) Brain cathepsin B is elevated in both mild-closed and severe-penetrating traumatic brain injury models. Journal of Neurotrauma 32:A-1-A-152; page A49.
13. Johnson D, Boutte A, Schmid K, Shear D, Dave J, Tortella F, Cartagena C (2015). Investigation of putative acute serum diagnostic biomarkers in a projectile concussive impact injury. Journal of Neurotrauma 32:A-1-A-152; page A36.
14. Deng-Bryant Y, Leung LY, Yang Y, Tortella F, Shear D (2015). Discovering prognosticators for repeated concussion: A global metabolomics study. Journal of Neurotrauma 32:A-1-A-152; page A36.
15. Lu XM, Cao Y, Liao Z, Tortella F, Shear D (2015). Time-course profile of EEG abnormality detected by qEEG power spectral analysis following a single concussive brain injury in rats. Journal of Neurotrauma 32:A-1-A-152; page A86.

16. Deng-Bryant Y, Leung L-Y, Yang W, Tortella F and Shear D, "Discovering prognosticators for repeated concussions: a global metabolomic study", Military Health System Research Symposium; Fort Lauderdale, Florida. August 2015. (Oral presentation)

17. Johnson D, Boutte AM, Schmid KE, Shear DA, Dave JR, Cartagena CM, Tortella FC. Preclinical profile of putative microRNA biomarkers in mild vs. severe traumatic brain Injury. Military Health System Research Symposium; Fort Lauderdale, Florida. August 2015. (Oral presentation)

18. Deng-Bryant Y, Leung LY, Yang W, Gilsdorf J, Tortella F and Shear D. Increased number of concussions is associated with higher levels of metabolic dysregulation. Program No. 743.03 Neuroscience 2015 abstracts. Chicago, IL: Society for Neuroscience, 2015. Online. (Oral Presentation)

19. Cartagena CM, Boutte AM, Hwang H, Johnson D, Tortella FC, Shear DA. Differential profiles of molecular pathology following repeat concussion in an animal model of projectile concussive impact (PCI) injury. Program No. 43.01 Neuroscience 2015 abstracts. Chicago, IL: Society for Neuroscience, 2015. Online.

20. Johnson D, Cartagena CM, Boutte´ AM, Dave JR, Shear DA, Schmid KE, Tortella FC. Preclinical Profile of Putative MicroRNA Biomarkers in Mild vs. Severe Traumatic Brain, Military Health System Research Symposium, August 2015.

21. Ying Deng-Bryant, Lai Yee Leung, Jignesh Pandya, Weihong Yang, Janice Gilsdorf and Deborah Shear. Global metabolomics analysis in rats following penetrating ballistic-like brain injury. National Neurotrauma Society Symposium, June, 2016, Lexington, KY.

22. Xi-Chun May Lu, Ying Cao, Zhilin Liao, Ashley Whiteman, Frank C. Tortella, Deborah A. Shear. Time-Course Profile of EEG Abnormality Following Repeated Concussive Brain Injury Detected by qEEG Power Spectral Analysis in a Rat Model of Projectile Concussive Impact. National Neurotrauma Society Symposium, June, 2016, Lexington, KY.

CONCLUSIONS

Phase I (SOW 1) studies designed to evaluate the time course effects of a single concussion on clinically relevant outcome measures have been completed. Phase II (SOW2) studies designed to evaluate the effects of repeated concussions that occur prior to and after the resolution of the healing profile for a single concussion were continued in Y3. Some aspects Phase II studies are complete while others are ongoing. Already, many valuable insights into the effects of repeated concussive injuries have been obtained from these studies. We found that in the majority, but not all, of parameters evaluated repeated concussion induced a greater degree of deficit and greater number of deficits compared to a single injury (e.g. altered levels of conscious, gait abnormalities, working memory deficits, metabolomics dysregulation, microglial phenotype alterations, and tissue and biofluid markers such as GFAP and tau). In addition, we found that the healing profile was often specific to each parameter with some parameters resolving within a day (e.g. EEG functional changes), while in others there was a delayed in the appearance of pathology for several days (e.g. tissue tau GFAP, SBDP pathology). Some parameters showed significant deficits subacutely or chronically post injury (e.g. gait abnormalities, working memory deficits). Interestingly, a few parameters showed a biphasic pattern where deficits resolved initially but reappeared later (e.g. gait abnormalities and metabolomics dysregulation). Evaluation of inter-injury intervals indicated that while a 24h inter-injury interval lead to a reduction in the number of positive CSF biomarker findings, changes in some tissue pathology parameters were as severe or showed greater severity (e.g. metabolomics dysregulation, cortical GFAP/ SBDP pathology). In contrast, with a 7 day inter-injury interval there was substantially reduced pathology, although deficits and pathology remain (gait abnormalities and metabolomics dysregulation). Thus, the PCI model effectively recapitulates many of the characteristic features of mTBI/concussion. The establishment of clear pathology seen with both single and repetitive concussion in the PCI model provides the necessary groundwork for future studies of treatment paradigms designed ameliorate the deleterious effects of impact concussion.

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