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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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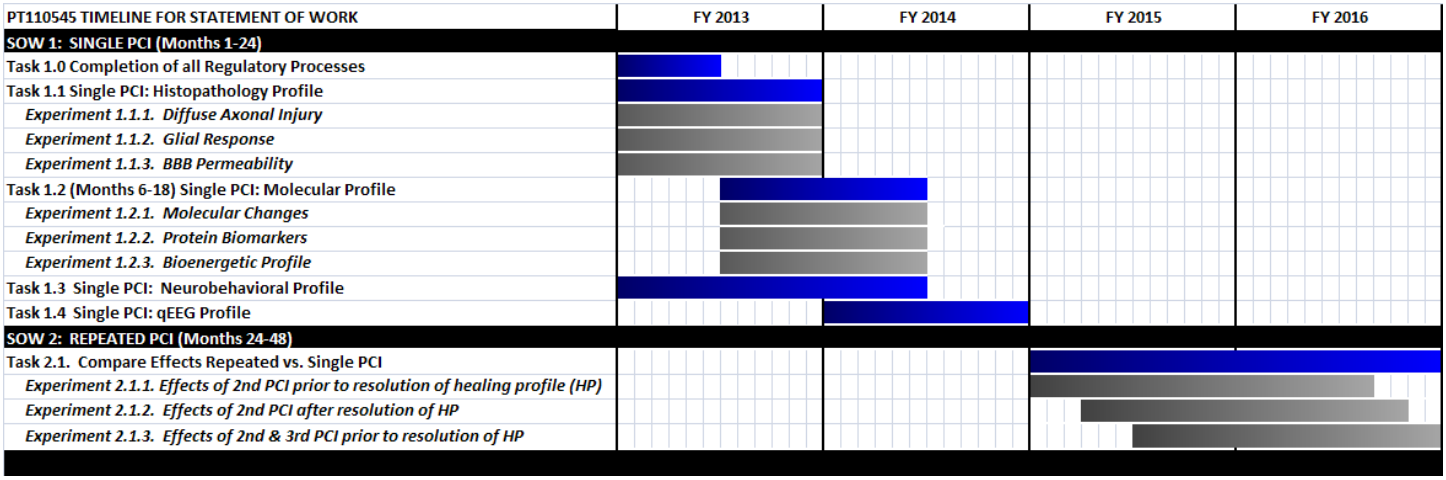
INTRODUCTION

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 a "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).

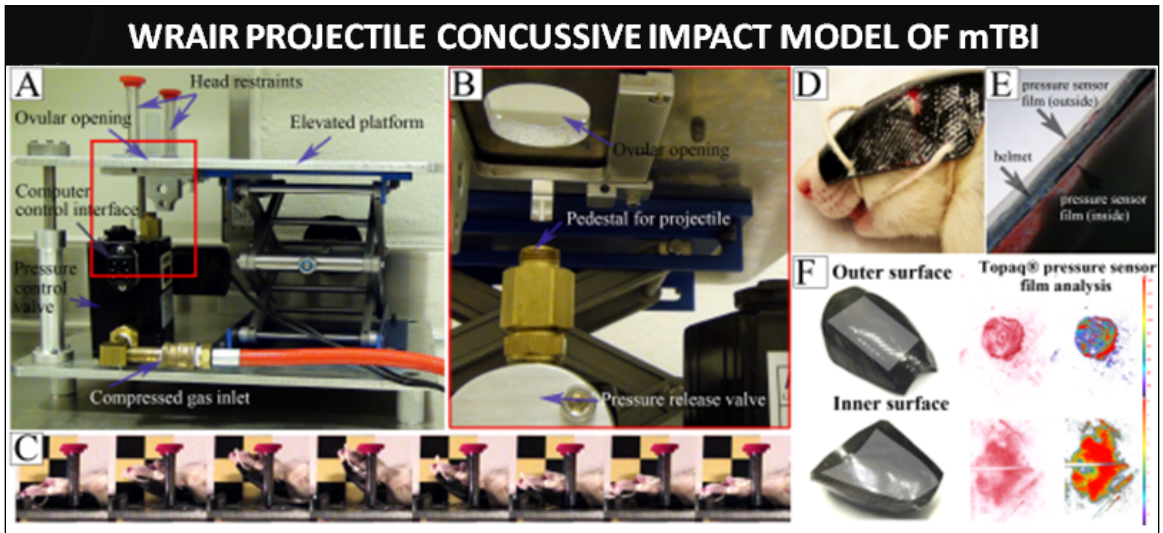


Figure 1. The WRAIR PCI Model of mTBI. (A) Elevated platform for holding the animal and aligning the projectile. (B) Instrument rig. (C) Head motion response to WRAIR PCI mTBI event. (D) Helmet made of woven glass-carbon. (E,F) Pressure sensor films are secured by adhesive to the inner and outer surfaces 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.***

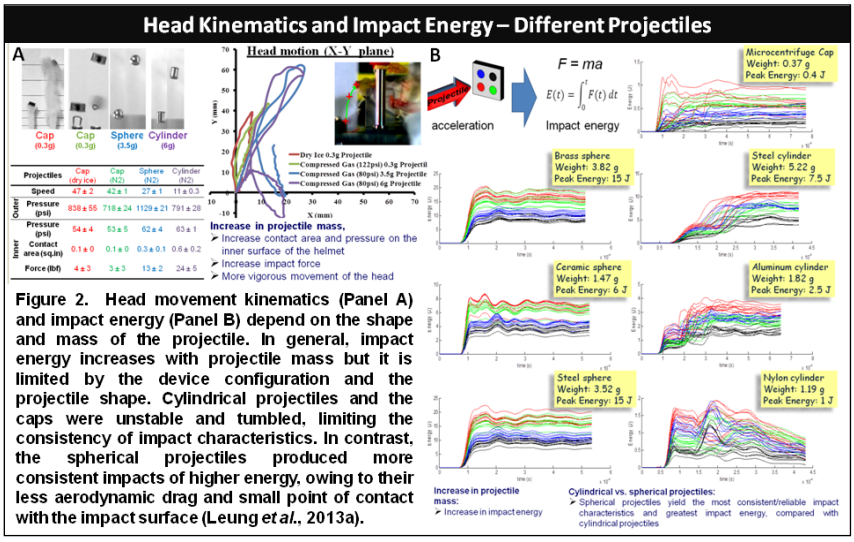


Figure 2. Head movement kinematics (Panel A) and impact energy (Panel B) depend on the shape and mass of the projectile. In general, impact energy increases with projectile mass but it is limited by the device configuration and the projectile shape. Cylindrical projectiles and the caps were unstable 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.***

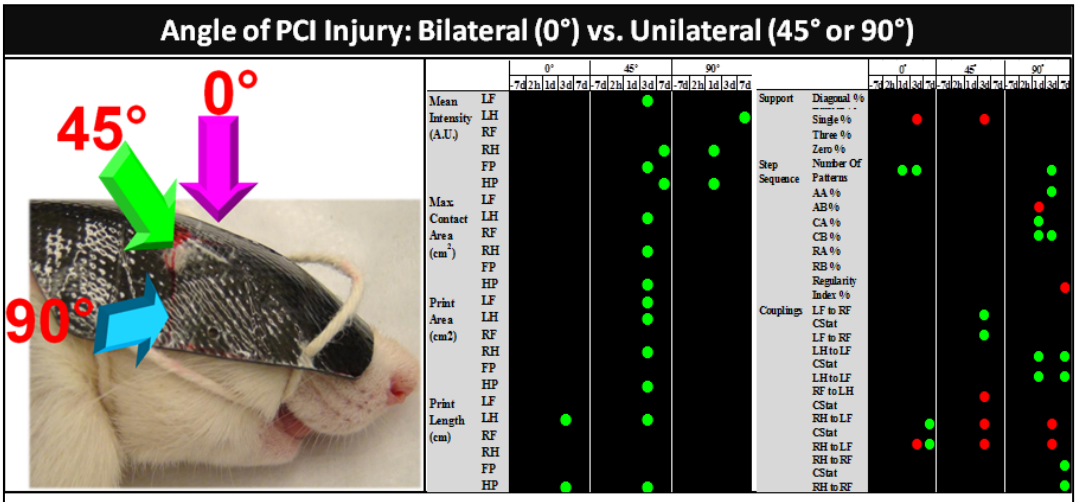


Figure 3. Adult rats were anesthetized with isoflurane, fitted with custom-designed helmets, and subjected to a single PCI angled at 0° (bilateral), 45° or 90° (unilateral) from sagittal plane. Sham controls received the same procedures without PCI. Gait performance was assessed using CatWalk automated 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. (2013b; manuscript in preparation). ***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 advance 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.

OVERALL PROJECT SUMMARY

PHASE 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. Control groups consist of animals that received anesthesia (sham) and animals exposed to repeated PCI (positive PCI control) only.

Task 1.1 (Months 1-12): Evaluate the regional and temporal profile of histopathological changes following a single PCI injury. The following experiments aim at elucidating diffuse axonal injury (DAI), glial response, blood brain barrier (BBB) permeability and brain edema using immunohistochemistry (IHC) at different time points and anatomical regions of the brain following PCI.

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. This study is focused on the expression of beta-amyloid precursor protein (βAPP) and amino cupric silver (CuAg) expression as markers for acute axonal injury. The effects of PCI on axonal injury using APP and CuAg staining were assessed at 6h, 24h, 72h, 7d, 14d and 28d post-PCI.

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 this study, the glial response to PCI injury was being 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.

Regional and temporal histopathological changes following mild concussive brain injury: Following PCI injury, animals (n=8 per time point; 6h, 24h, 72h, 7d) were sacrificed with an overdose of ketamine/xylazine (70 and 6 mg/kg, i.m., respectively) and were transcardially perfused. Brains were harvested and post-fixed in 4% paraformaldehyde for 6 hours and then in 20% sucrose solution. The samples were processed at FD Neurotechnologies Inc. (Ellicott City, MD, USA). A series of coronal free floating brain sections (40 µm; 960 µm interval from +4.0 mm to -7.0 mm from Bregma) were immunostained for β-APP with Vector ABC system. Accumulation of β-APP in axons and axonal bulb formation indicate axonal damage. Another series of coronal sections was stained using FD NeuroSilverTM Kit II (FD Neurotechnologies Inc., Ellicott City, MD, USA) for neurodegeneration. For glial responses, a series of coronal brain sections was immunostained for glial fibrillary acidic protein (GFAP; astrocyte marker) and another series was immunostained for ionized calcium binding adaptor molecule (Iba1; microglia/macrophage marker). Images of the slides were digitized and examined using a BX61 microscope (Olympus, PA). Sham control groups (n=8 per time point; 24h and 7d) were included to control any possible effects of isoflurane anesthesia. A repeated PCI group (n=3) in which animals were subjected to 4 PCIs at 1h interval, was included at one acute post-injury time point

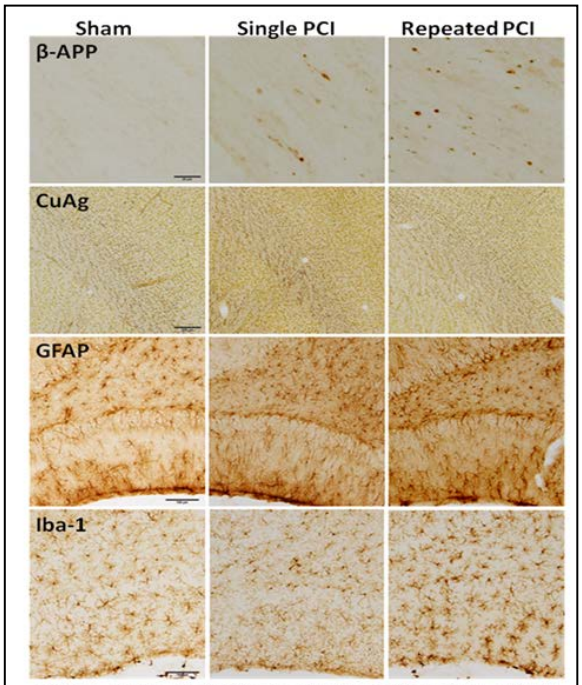


Figure 4. Representative images of brain sections stained with β-APP (first row; corpus callosum; 400X; scale bar: 20 µm); silver staining (2nd row; corpus callosum; 40x; scale bar: 200 µm); GFAP (3rd row; hippocampus; 100x; scale bar: 100 µm); and Iba-1 (4th row; hippocampus; 100x; scale bar: 100 µm).

only (1h following the final PCI injury) to serve as an initial positive control to assess the sensitivity of the outcome measures for detecting injury.

Axonal damage was revealed by the β-APP accumulation and axonal bulb formation at corpus callosum in both single PCI (6h) and repeated control (1h) groups (Figure 5). Single PCI produced axonal injury detected in the corpus callosum. βAPP peaked at 6 hours post-injury in the ipsilateral (injured) hemisphere and remained elevated at 24h, it decreased gradually afterwards on both sides. Following repeated PCI, dramatic increases in axonal injury were detected compared with single PCI. Following repeated PCI, peak increases in βAPP were detected at 24h on both ipsilateral and contralateral sides, with significantly more injury on the ipsilateral side ($p<.05$). While βAPP levels resolved significantly by 14d post-injury, the βAPP immunoreactivity in the brains of animals exposed to repeated PCI group still remained significantly elevated vs sham (Figure 5; $p<.05$). Silver staining (CuAg; Figure 4) was negative in corpus callosum in all groups.

PCI injury produced more hypertrophied and reactive astrocytes (observed with GFAP) predominantly in the right hippocampus compared to the sham control. Threshold quantification revealed significant increases in hippocampal astrocyte activation at 24h post-injury after a single PCI that was evident in both hemispheres (Figure 6; $p<.05$ vs. sham control). After repeated PCI, GFAP activation peaked at 6h post-injury and remained significantly higher than sham out to 72h. Moreover, GFAP immunoreactivity following repeated PCI was significantly higher than that in single PCI group at 6h post-injury. No differences were detected between the two groups at the other time points.

Hippocampal microglia exhibited a rounded amoeboid-like appearance and upregulated Iba-1 protein, suggesting an activation response following PCI (both single and repeated). Threshold quantification revealed an injury-induced biphasic response in hippocampal Iba-1 expression (Figure 7) that peaked at 6h and then again at 72h post-injury. Similar to the trend observed on GFAP-labeled astrocyte activation, significant differences in microglia activation between single and repeated PCI were only detected at 6h post-injury ($p<.05$).

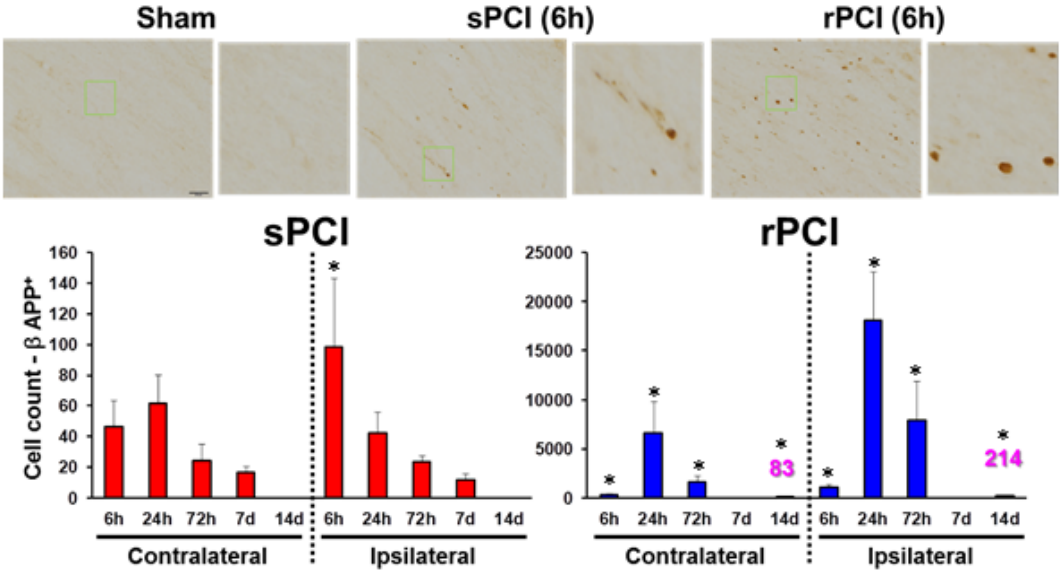


Figure 5. Pictures on top show the βAPP expression in representative brain sections from animals exposed to single and repeated PCI at 24h post-injury. Graph shows threshold quantification of βAPP in single PCI (sPCI) and repeated PCI (rPCI) groups (sham not shown). Significant increases in βAPP expression were detected in the injured (ipsilateral) hemisphere at 6h after sPCI that were dramatically increased and more sustained after rPCI. Note the difference in the scale shown on the Y axis for sPCI and rPCI (* $p < .05$ vs. sham).

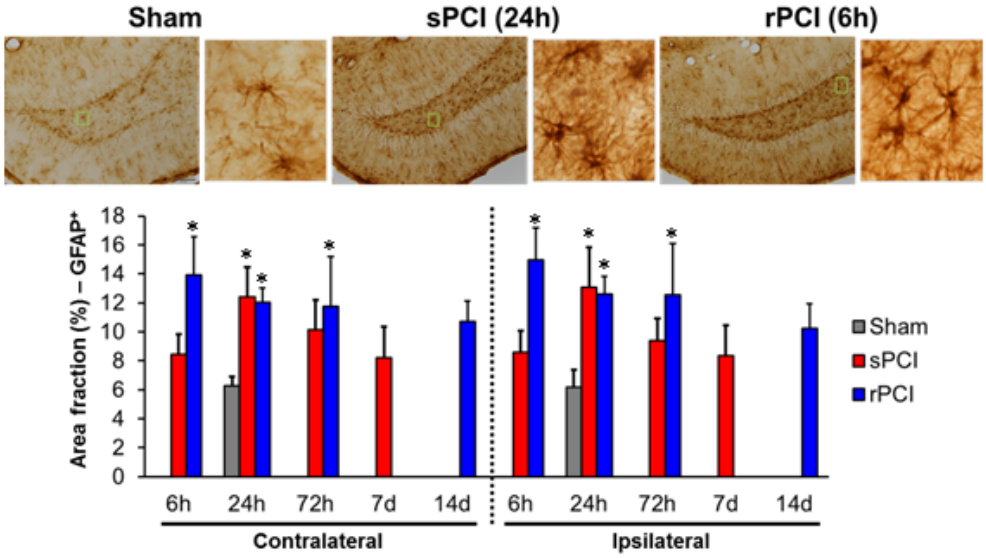


Figure 6. Pictures on top show GFAP expression in representative brain sections from animals exposed to single and repeated PCI at 24h post-injury. Graph shows threshold quantification of GFAP in Sham, single PCI (sPCI) and repeated PCI (rPCI) groups. Significant increases in GFAP expression were detected in both hemispheres at 24h after sPCI and at 6, 24h and 72h after rPCI (* p < .05 vs. sham).

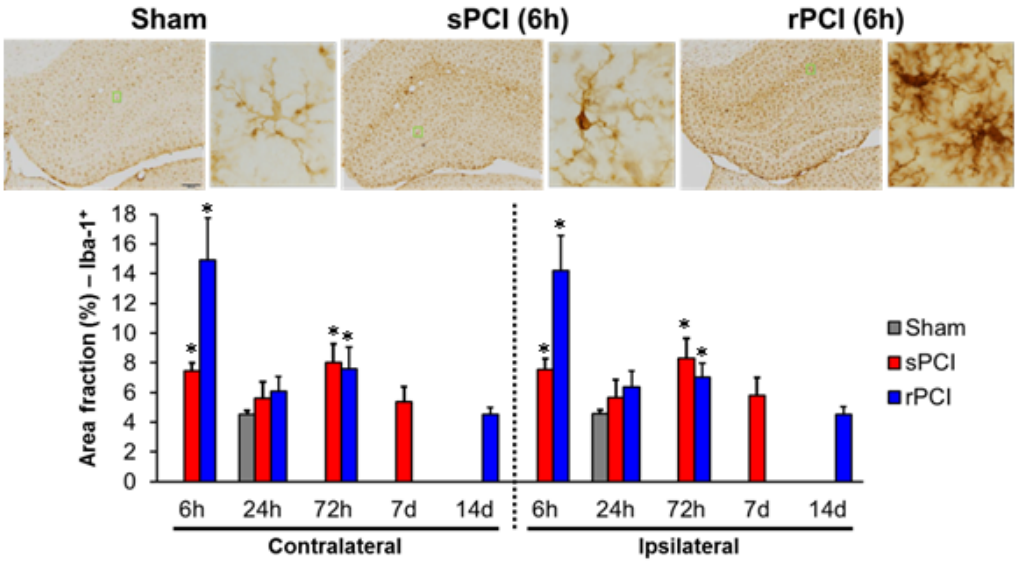


Figure 7. Pictures on top show Iba-1 expression in representative brain sections from animals exposed to single and repeated PCI at 24h post-injury. Graphs show threshold quantification of Iba-1 expression in Sham, single PCI (sPCI) and repeated PCI (rPCI) groups. Significant increases in Iba-1 expression were detected in both hemispheres at 6h and 72h after both sPCI and rPCI (* p < .05 vs. sham).

Experiment 1.1.3. Blood-Brain Barrier (BBB) Permeability: The effects of PCI on BBB permeability were examined at discrete post-injury time points (i.e. 6h, 24h and 72h) using biotin dextran amine (BDA; 3 kDA) to detect BBB disruption. In addition, the involvement of astrocytes and/or tight junctions in the BBB breakdown process were 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).

Effects of single and repeated PCI-induced concussion on blood brain barrier permeability: At the specified post-injury time points, animals were overdosed with ketamine/xylazine and monitored for distinguishing reflexes at 6, 24 or 72h post-PCI. The animals were then decapitated and the brain was rapidly removed and submerged in isopentane that had been super cooled with dry ice. The brains were wrapped in foil and stored at -80oC freezer then shipped to FD Neurotechnologies Inc for processing and staining. Tissue processing and staining protocols have been optimized for fluorescent staining of AQ4 co-labeled with GFAP, zonula occluden-1 (ZO-1) and claudin-5 (Cld5), as shown in Figure 8.

Analysis of BDA staining failed to reveal any evidence of BBB disruption following either single or repeated PCI (not shown). However, results of tight junction protein analysis revealed PCI-induced reductions in Cl-5 expression (Figure 9) in the injured cortex that that were evident at 6 and 72h following a single PCI and at 6h, 24h and 72h after repeated PCI ($p<.05$ vs. sham control). No between group differences were detected on the contralateral cerebral cortex at any time point.

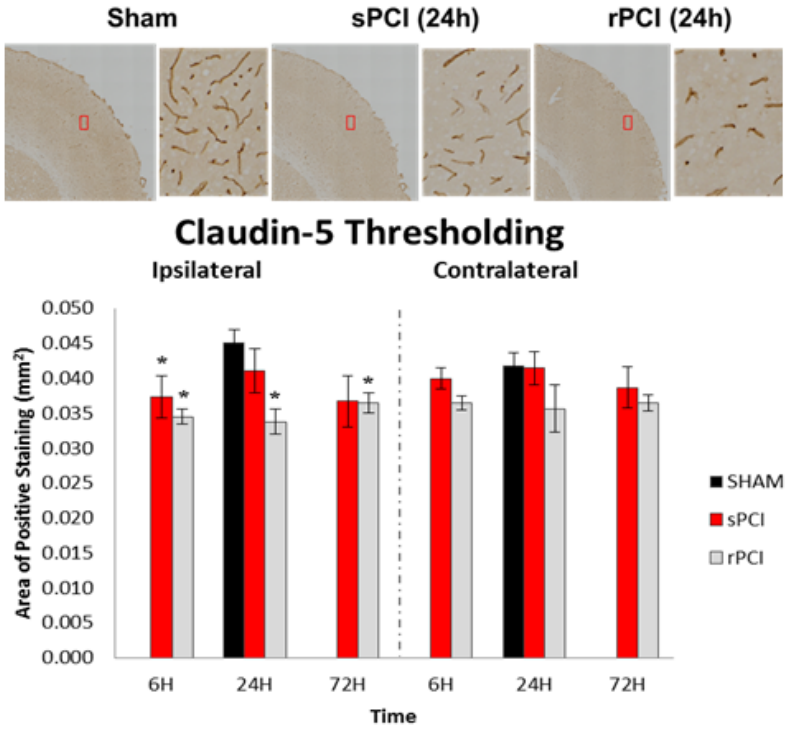


Figure 9. Pictures on top show the regional profile of reduced claudin-5 expression in representative brain sections from animals exposed to single and repeated PCI at 24h post-injury. Graph shows threshold quantification of claudin-5. Significant reductions in claudin-5 expression, indicative of blood brain barrier damage, were evident in the injured hemisphere at 6 hours post-injury (both single and repeated PCI) and at 24h and 72h post-injury (repeated PCI only (*p < .05 compared to sham controls at 24h).

Collectively, the histopathological findings demonstrated that a single PCI is capable of producing quantifiable axonal injury, glial activation and BBB disruption in the absence of any gross pathology. Further, the profiles of these neuropathological changes are time-dependent and region-specific and are sensitive to the cumulative effects of repeated concussive impact.

Task 1.2 Regional and temporal profile of molecular/bionergetic changes following single PCI injury. 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).

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.

Regional and temporal profile of molecular changes following single and repeated PCI injury: Anesthetized rats received zero (sham), one (1xPCI) or repeated (2, 3 or 4xPCI) impacts separated by 1h intervals. Righting reflex (RR) and sensory-motor deficits (revised neurobehavioral severity scale, NSS-R) were recorded immediately following the last impact. RR and NSS-R Methods: Immediately after each PCI, rats were placed into their cages and observed for RR or latency to regain consciousness and ability to turn-over into the supine position using separate cohorts. Forty-five, minutes after each PCI, rats were evaluated using the NSS-R, which consists of 10 tests designed to assay motor, sensory, and reflex skills. Normal responses are assigned “0”, immediate impairment was assigned “1”, and failure to perform the task were assigned “2”. A maximum possible score was 20 and indicative of maximal sensory-motor deficit on all tests.

Changes in protein abundance in GFAP and GFAP breakdown products were evaluated in ipsilateral cortex and hippocampus at 4, 24, and 3 days post-single PCI by western blot. Briefly, tissue was homogenized in radioimmunoprecipitation buffer (RIPA) containing protease and phosphotase inhibitors. Total protein was normalized based on bicinchoninic acid (BCA) assay. Equal amount of total protein were run on polyacrylamide gels and transferred to PVDF membranes using the trans-blot turbo transfer system. Blots were blocked in 5% milk, probed with rabbit anti-GFAP primary antibodies overnight, washed, probed with donkey anti-rabbit secondary antibodies for 2 hours and detected using electrochemiluminescence. Blots were reprobed with anti-beta-actin antibody to control for protein loading. Analysis of band intensity was done using an LAS4000 and ImageQuantTL software (GE Healthcare). As a reference, moderate TBI models have shown increased GFAP expression in tissue starting at 1 and increasing at 3 days post injury.

Serum and CSF GFAP were analyzed using in-house customized Mesoscale ELISAs 1h after the last concussion. Mesoscale ELISA Assays: CSF or serum samples (N = 9-10) were incubated in plates manually coated with GFAP capture antibody (Banyan Biomarkers, Alachua, FL). Plates were then incubated with GFAP detection antibody and sulfo-tagged MSD anti-mouse secondary. GFAP quantitation was determined by electrochemilluminescent (ECL) signal. Protein content was determined from standard curves and accuracy was confirmed with controls. Values shown are shown as mean +/- SEM (ng/mL).

Prior to statistical analysis, outliers were removed with the ROUT method (false discovery rate Q = 0.1%). For RR, NSS-R and CSF GFAP, analysis between groups were compared using 2-way ANOVA with Tukey post-hoc multiple comparisons and analysis within groups was conducted with either 1-way ANOVA (2-4 X PCI) or Student's t-Test (1 X PCI) with Tukey post-hoc. Analysis of serum GFAP was conducted with the 1-tailed Student's t-Test. Correlation analysis was conducted with the Spearman correlation coefficient (r). Statistical significance ($p < 0.05$) is indicated by an asterisk (*t-Test or Spearman correlation) or a phi (Φ, 1- or 2-way ANOVA). All analyses were conducted with Prism (GraphPad v.6). N = 9-10 in each study.

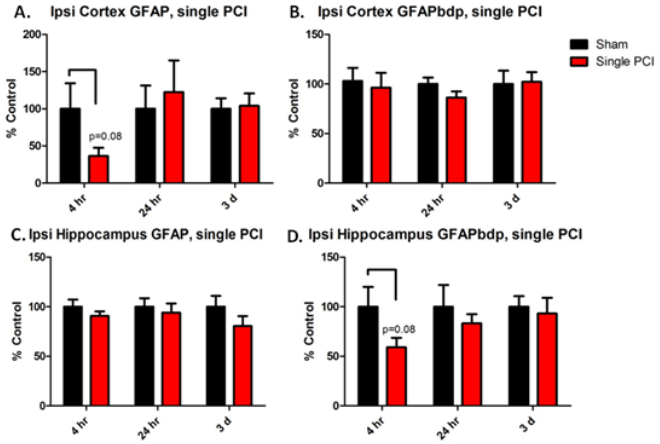


Figure 10. GFAP and GFAPbdp levels in (A,B) ipsilateral cortical tissue and in the (C,D) ipsilateral hippocampal tissue following single PCI. Protein levels from single PCI animals versus sham animals was evaluated at time points 4, 24 hr, 3 days post injury. The 50 kDa band corresponding to GFAP was measured. No significant alterations in GFAP or GFAPbdp expression were detected (error bars: standard error of the mean, n=9-10 per group).

<u>Delayed consciousness, sensory-motor deficits, and GFAP levels in repeated concussive impact</u>: Results showed significant PCI-induced alterations in RR (Figure 11) and NSS-R scores (Figure 12) following a single PCI ($p<0.05$ vs. Sham) both of which also showed significant (1.6–1.7 fold) increases in magnitude following the 2nd PCI ($p<0.05$ vs. 1xPCI). Although both RR and NSS-R remained significantly elevated following a 3rd or 4th PCI ($p<0.05$ vs. 1xPCI or Sham), no additional "stepwise" increases were detected relative to the 2nd impact ($p<0.05$ vs. 2xPCI). Levels of GFAP in CSF increased to 1.9 ng/mL after a single PCI ($p<0.05$ vs. sham) and were further increased following repeated concussions with mean levels of 3.9 ng/mL (2xPCI), 2.7 ng/mL (3xPCI) and 7.5 ng/mL (4xPCI) (Figure 13; $p<0.05$ vs. Sham). Notably, a significant correlation was detected between RR scores and CSF GFAP following 4xPCI (Figure 14; $p<0.05$). Consistent with this observation, Figure 13 shows that serum GFAP levels analyzed in the 4xPCI group were significantly increased vs. sham (4xPCI=0.06 ng/mL; Sham=0.02 ng/mL; $p<0.05$). ***These results suggest that single and repeated PCI lead to neurological impairments that are associated with increased abundance of GFAP in brain, CSF and serum.***

Table 1. The Revised Neurological Severity Scale	
General balance	Ability to balance and walk on a balance beam
Landing test	Display of landing reflex
Tail raise test	Observation of limb flexion and extension when lifted by the tail
Drag test	Resistance to being gently pulled backward
Righting reflex	Ability to right its posture after being placed on its back
Eye blink response	Normal blinking response to eye stimulation
Ear Reflex	Response after gentle ear touch
Sound reflex	Display of startle and fear posture
Tail reflex	Vocalization following gentle pinch of the tail
Paw flexion reflex	Hindlimb withdrawal reflex following gentle foot pinch

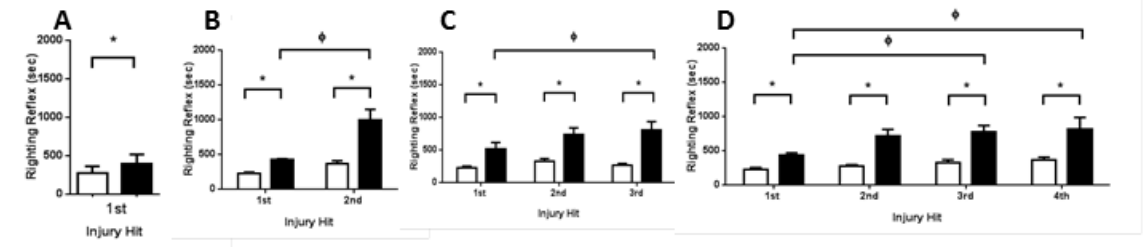


Figure 11. Righting reflex increases after single and repeat PCI. The number of hits (x-axis) and righting reflex in seconds (y-axis) are displayed for each injury type. (A) 1X, (B) 2X, (C) 3X, and (D) 4X. Sham (white bars) and PCI (black bars) groups are displayed as the mean +/- SEM.

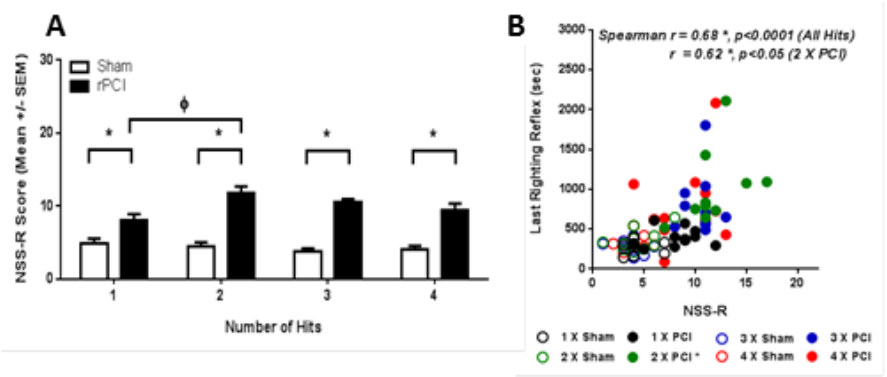


Figure 12. Repeated PCI Leads to Sensory-Motor Deficit that Correlates to Impaired Righting Reflex. (A)The number of hits (x-axis) and NSS-R score (y-axis) are displayed for each injury single (1X) and repeated (2X- 4X) PCI. Sham (white bars) and PCI (black bars) groups are displayed as the mean +/- SEM. (B) Spearman correlation between the NSS-R score and the last RR (sec). Single (black), 2X (green), 3X (blue) and 4X (red) PCI groups are indicated. Sham (open circle) and PCI (closed circle) groups are displayed. Values indicate the mean +/- SEM for each group and injury type.

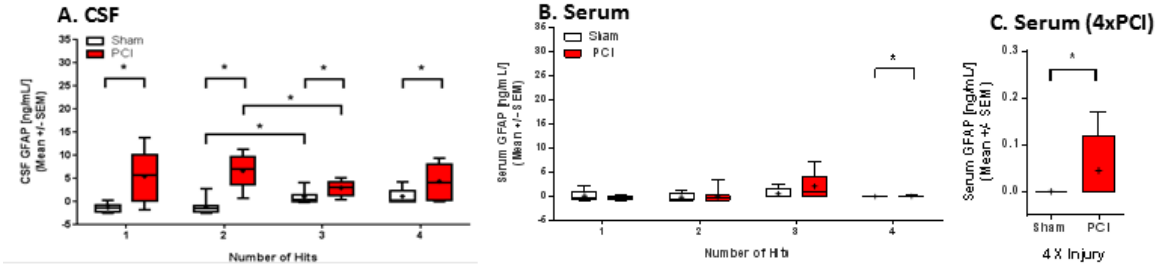


Figure 13. GFAP Increases in Biofluids One Hour after Single and Repeated PCI: (A) Quantitation of GFAP in (A) CSF or (B) in serum after single (1X) or repeated (2-4X) PCI. Values indicate the mean fold change +/- SEM of GFAP in the serum or CSF of sham (white bars) compared to PCI (red bars).

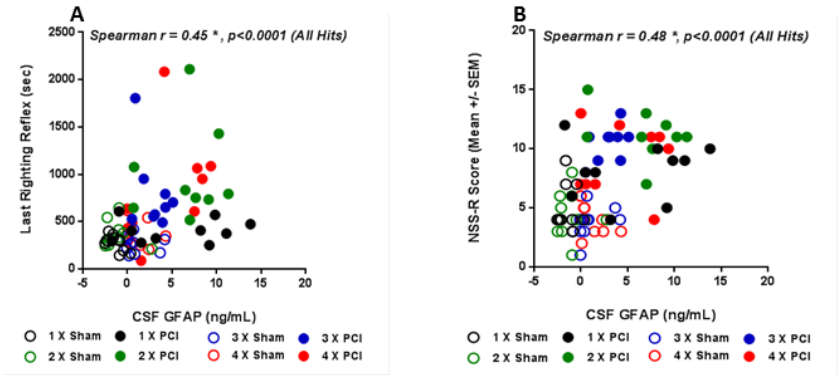
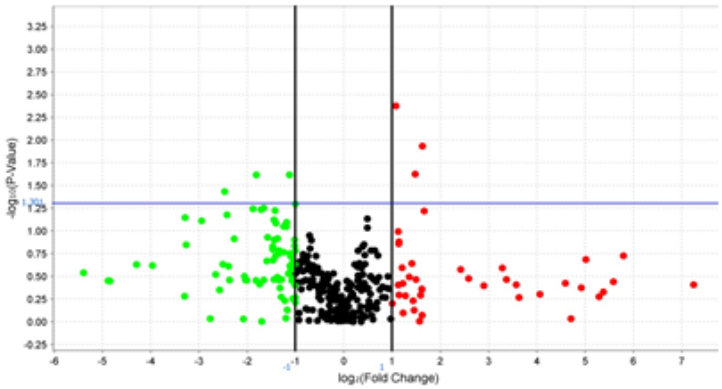


Figure 14. CSF GFAP Quantity in CSF Correlates to RR and NSS-R Deficits: (A) Spearman correlation between CSF GFAP (x-axis) and either (A) the last RR (seconds) or (B) the NSS-R score. Single (black), 2X (green), 3X (blue) and 4X (red) PCI groups are indicated. Sham (open circle) and PCI (closed circle) groups are displayed. Values indicate the mean +/- SEM for each group and injury type.

Micro-RNAs as a Novel Biomarker for mTBI: Both human and rodent TBI studies have identified acute phase modulation of microRNAs (miRNAs) that regulate inflammatory and neurodegenerative proteins. As stable molecules, miRNAs thus represent a class of potential brain biomarker candidates to evaluate TBI. To date, miRNAs have been studied in several models of TBI, and there is also evidence that long-term miRNA dysregulation occurs in neurodegenerative disorders and in spinal cord injury (Mitchell et al. 2008; Liu et al. 2009; Risdall et al. 2011; Balakathiresan et al. 2012; Junn et al. 2012). Accordingly, we have conducted miRNA array analysis of serum following a single mTBI. Our preliminary results are shown in a "volcano" plot of microRNAs assessed in microarray analysis of total RNA isolated from rodent serum in PCI or SHAM animals. ***At 24h post-injury, we detected significant alterations in a number of miRNAs in serum following a single PCI (Figure 15).***

A



B

DOWNREGULATED			UPREGULATED		
microRNA	FOLD CHANGE	p value	microRNA	FOLD CHANGE	p value
miR 193b	1.13	0.024	miR 1897-3p	1.47	0.024
miR 195	1	0.051	miR 183#	1.63	0.012
miR 351	1.81	0.024	miR 335#	1.08	0.004
miR 350	2.47	0.037			

Figure 15. MicroRNA Modulation 4h after One Hit PCI. A. Volcano plot of microRNAs assessed in microarray analysis of total RNA isolated from rodent serum in PCI or SHAM animals. The plot displays the relationship between fold-change and significance between the two groups, using a scatter plot view. The blue horizontal line denotes level of significance (p=0.05). The y-axis is the negative $\log_{10}$ of p values (a higher value indicates greater significance) and the x-axis is the difference in expression between two experimental groups as measured in $\log_2$. The red dots represent specific microRNAs that are increased, while the green dots represent microRNAs that are decreased. B. Represents microRNAs that are decreased, increased and the respective p values.

Acute Changes in CTE-related Markers Tau and Phosphorylated Tau Follow Repeat PCI: Additional experiments were conducted to determine whether there are acute changes in levels of full length Tau protein and phosphorylated Tau (AT8 epitope) following repeat mTBI. For this purpose, Animals were injured 4 times with an interval of 1 hr between injury (n=10). Repeat sham (rSham) animals underwent sham procedure (no projectile) 4 times with an interval of 1 hr between proceedures (n=10). At 3 days post-injury brains were dissected into regions of interest and snap frozen. Tissue was homogenized in 1xRIPA plus protease and phosphatase inhibitors. Total protein levels were determined using the BCA method. Tau and phosphorylated Tau (AT8) levels were evaluated by western blot. Blots were re-probed for beta actin and target band intensity was normalized to beta actin levels for each sample.

Phosphorylated Tau (Serine 202/ epitope AT8) and total Tau (epitope Tau 5) levels were significantly increased in ipsilateral hippocampus following repeated PCI (Figure . Molecular analysis of the PCI model is ongoing, including the analysis these CTE related markers at subacute and chronic time points. **Increased levels of phosphorylated Tau and total Tau in the injured hippocampus following PCI suggest the existence of increased cellular stress in this brain region.**

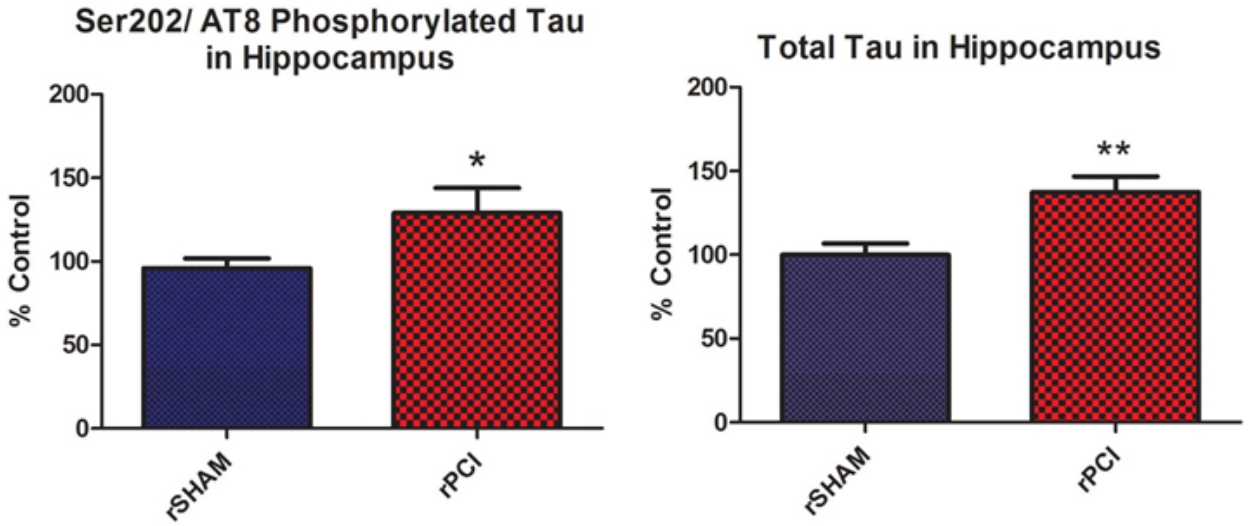
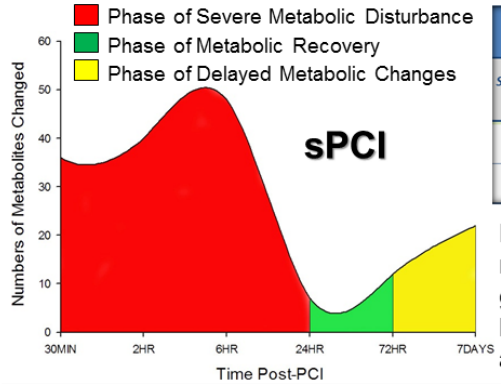


Figure 15: rPCI Increases Phosphorylated Tau and Full length Tau Levels in Ipsilateral Hippocampus: Protein levels from rPCI versus rSham animals were determined in ipsilateral hippocampus at 3 days post-injury. Analysis using Student's t-test indicated a significant increase in both Tau phosphorylations (*p<0.05) and full length Total Tau (p<0.01) mean levels. Error bars: standard error of the mean**

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.

Following single PCI (sPCI) injury, animals were euthanized 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. Cerebral metabolic tissues were collected at 30min, 2h, 6h, 24h, 72h, and 7 days following a sPCI (sPCI). Samples from the repeated PCI (rPCI), serving as positive control, were collected at 2h only (4 hits spaced 1h apart) (n=6 per time point). All samples were processed by Metabolon Inc. to generate global metabolic profiles based on a library of over 4,000 known brain related metabolites.

METABOLOMIC PROFILE



rPCI	Statistical Comparisons One-Way ANOVA Contrasts						
Significantly Altered Biochemicals	PosCtrl Sham	sPCI 0.5h Sham	sPCI 2h Sham	sPCI 6h Sham	sPCI 24h Sham	sPCI 72h Sham	sPCI 168h Sham
Total biochemicals $p \le 0.05$	55	36	40	48	7	12	22
Biochemicals (↑|↓)	23|32	8|28	4|36	8|40	2|5	6|6	10|12

PCI was accompanied by differences in neurotransmitter related metabolites. Alterations in glutathione metabolism suggest differences in redox homeostasis following injury. Glycogen metabolism and glucose utilization were altered following PCI.

Metabolic Pathways Changed:

- ✓Glucose Metabolism
- ✓TCA Cycle Metabolism
- ✓Lipid Oxidation
- ✓Neuropeptide Synthesis
- ✓Glutathione Metabolism
- ✓Arginine Metabolism
- ✓Nucleotide Metabolism
- ✓Polyunsaturated Fatty Acids

Metabolomics Platform Technology:

Sample processing: Samples were prepared using the automated MicroLab STAR® system from Hamilton Company. A recovery standard was added prior to the first step in the extraction process for QC purposes. Sample preparation was conducted using aqueous methanol extraction process to remove the protein fraction while allowing maximum recovery of small molecules.

Ultrahigh performance liquid chromatography/Mass Spectroscopy (UPLC/MS/MS): The LC/MS portion of the platform was based on a Waters ACQUITY ultra-performance liquid chromatography (UPLC) and a Thermo-Finnigan linear trap quadrupole (LTQ) mass spectrometer, which consisted of an electrospray ionization (ESI) source and linear ion-trap (LIT) mass analyzer. The MS analysis alternated between MS and data-dependent MS2 scans using dynamic exclusion.

Gas chromatography/Mass Spectroscopy (GC/MS): The samples destined for GC/MS analysis were re-dried under vacuum desiccation for a minimum of 24 hours prior to being derivatized under dried nitrogen using bistrimethyl-silyl-triflouroacetamide (BSTFA). Samples were analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron impact ionization.

Glucose metabolism: Glucose is critical for the generation of cellular energy, nucleic acids, and biomass. Both positive control and sPCI tissues possessed elevated glucose levels compared to sham controls (**Figure 16**). Higher levels of sorbitol (generated by the reduction of excess glucose) and fructose in these tissues suggest altered glucose availability that may arise from a change in uptake and/or utilization. In support, further analysis revealed that the glycogen metabolites, such as maltotetraose, were elevated in positive control and sPCI tissues and may reflect catabolism to support glycolytic and anabolic pathways.

Evidence in the literature demonstrates that glucose is rapidly converted to lactate following TBI and may signify a shift to a non-oxidative metabolism (Clausen, Hillered, & Gustafsson, 2011; O'Connell et al., 2005; Scafidi et al., 2009). In agreement, higher levels of glucose 6-phosphate (G6P), 3-phosphoglycerate, pyruvate, and lactate were observed in positive control and sPCI tissues beginning at 6 hours and lasting for 7 days. Aside from energy metabolism, differences in G6P may also be indicative of alterations in pentose phosphate pathway (PPP) metabolism. At 2 and 6 hours post sPCI, brain tissue exhibited elevated levels of the PPP metabolites sedoheptulose 7-phosphate and the isobar for pentulose 5-phosphates. The PPP plays a critical role in promoting anabolic growth, nucleotide biogenesis, and replenishing NADPH necessary for glutathione reductase function. Thus, it is proposed that the shunting of glycolytic intermediates into the PPP may be critical for preventing secondary injury and initiating recovery.

TCA cycle: Differences in glucose metabolism suggested that the TCA cycle may also be altered in response to PCI. Positive control and sPCI samples exhibited initially elevated levels of the TCA cycle intermediates citrate and alpha-ketoglutarate, but lower levels of succinate compared to sham controls (**Figure 17**). These observations may reflect a change in enzyme function considering published studies demonstrate that alpha-ketoglutarate dehydrogenase (KGI
activity of TCA cycle enzymes in the brain and is exquisitely sensitive to oxidative inhibition (Tretter & Adam-Vizi, 2005). Alternatively, lower levels of succinate may be indicative of enhanced succinate dehydrogenase activity as evidence in the literature demonstrates TBI can induce an increase of mitochondrial and peroxisomal relative mass with higher succinate dehydrogenase activity (Borges,

Cerejo, Santos, Sarmento, & Azevedo, 2004). Furthermore, NAD+ levels were elevated post sPCI and in positive controls, while NADH levels were reduced (until later time points) potentially suggesting a decline in oxidative metabolism. Although ATP levels were higher in these tissues, this may partially represent hyper succinate dehydrogenase activity as discussed earlier and/or elevated glycolytic metabolism. Together, these findings suggest that mitochondrial metabolism may be disrupted or altered following sPCI (Verweij et al., 2000).

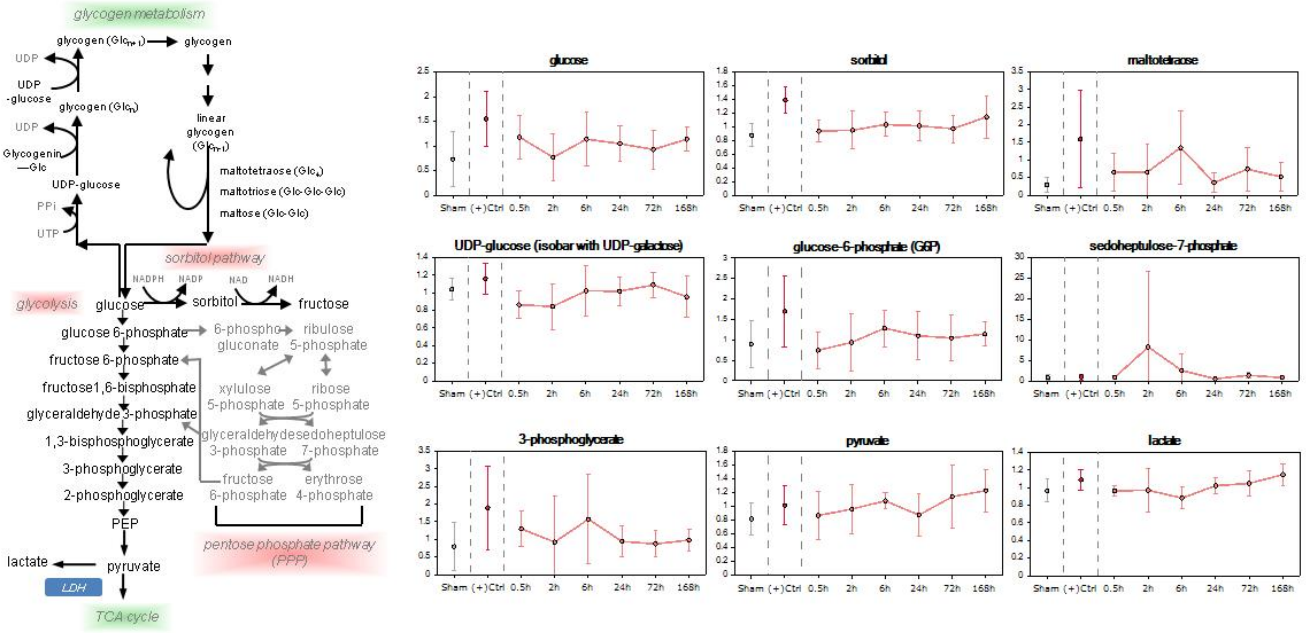


Figure 16. High creatine phosphate levels may reflect a difference in ATP/ADP buffering capacity, while elevated levels of the polyamine putrescine may contribute to

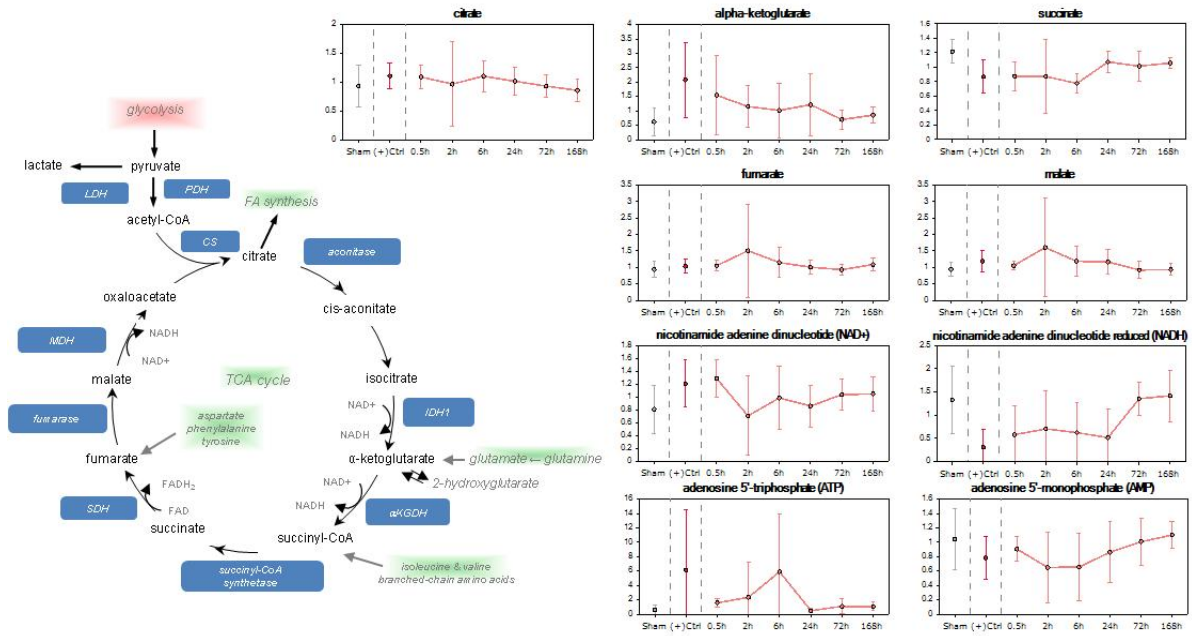


Figure 17. Mitochondrial metabolism may be disrupted post projectile

Amino Acid Related Metabolites

Neuropeptides: Compared to sham controls, no significant differences were observed in N-acetylaspartate (NAA) and N-acetyl-aspartyl-glutamate (NAAG) levels in positive control and sPCI samples regardless of time point (**Figure 18**) which may suggest limited tissues damage. In contrast, gamma-aminobutyric acid (GABA) levels trended higher in positive control and sPCI tissues immediately following injury. GABA predominantly exerts inhibitory effects on the central nervous system, but has also been reported to depolarize neurons and increase intracellular Ca++ in response to trauma for up to a week (van den Pol, Obrietan, & Chen, 1996). Differences in GABA levels may partially result from alterations in GABA receptor expression as documented following traumatic brain injury (Raible, Frey, Cruz Del Angel, Russek, & Brooks-Kayal, 2012) and may play a role in directing and enhancing the outgrowth of regenerating neurites.

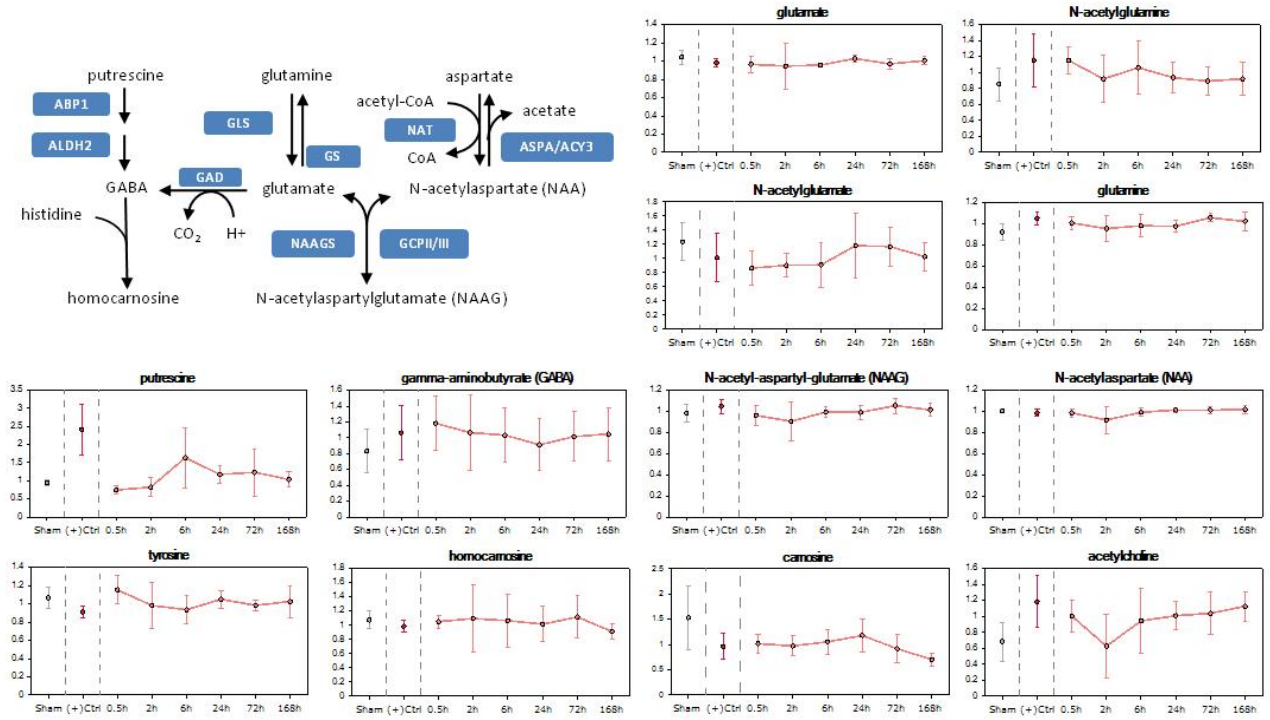


Figure 18. Neuropeptide related metabolites are altered following PCI.

Aside from differences in uptake and release, changes in neuropeptide levels may be a consequence of altered synthesis. Specifically, higher levels of the polyamine putrescine may support GABA synthesis, while modestly lower levels of glutamate may suggest depletion to support GABA biogenesis. Considering homocarnosine levels did not significantly vary; differences in GABA were likely a result of altered synthesis or uptake as opposed to degradation. Furthermore, reduced levels of related carnosine in both positive control and sPCI tissues may reflect the depletion of this neuroprotective antioxidant (Boldyrev et al., 1997), while higher levels of histidine in positive control tissues may reflect protein turnover and provide protection against oxidative stress (Bae & Majid, 2013). Notably, lower levels of glutamate were also accompanied by a reduction in N-acetylglutamate, while both glutamine and N-acetylglutamine accumulated in sPCI and positive control rats. This inverse relationship in glutamine/glutamate availability may be notable considering

published studies demonstrate the ratio between these two amino acids can reflect the severity of neurological injury (Shutter, Tong, & Holshouser, 2004).

In addition to neuropeptides, sPCI and positive control tissues possessed elevated levels of acetylcholine. Acetylcholine is an excitatory neurotransmitter that can create a wide variety of cellular responses including opening cation channels, releasing Ca2+ from intracellular storage sites, and modulating activities of K+ and Ca2+ channels. Higher acetylcholine levels may be notable considering evidence in the literature demonstrates that a magnitude of TBI sufficient to produce spatial memory deficits can result in a reduction in the release of acetylcholine within the hippocampus (Dixon, Bao, Long, & Hayes, 1996).

Glutathione metabolism

TBI can be accompanied by an accumulation of high energy oxidants and free radicals that can contribute to secondary tissue damage (Cornelius et al., 2013). Although oxidized glutathione (GSSG) levels were similar between all conditions, both sPCI and positive control samples exhibited an initial decline in reduced glutathione (GSH) levels prior to 24 hours (**Figure 19**). The exposure of GSH to reactivate oxygen species (ROS) induces cysteine oxidation, generating a disulfide bond with another oxidized molecule of glutathione forming oxidized glutathione (GSSG). Thus, an imbalance between GSH and GSSG levels may reflect a change in oxidative stress following TBI. Notably, GSH levels were elevated in sPCI samples by 72 hours and 7 days and may be indicative of a restoration of redox homeostasis.

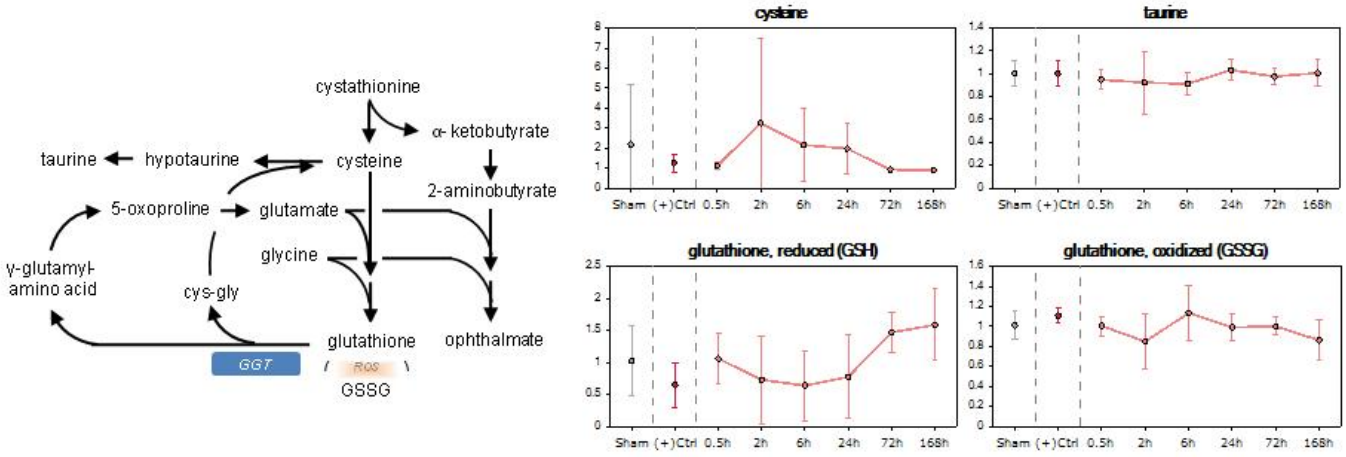


Figure 19. Glutathione metabolism is disrupted following PCI.

Conclusions: Collectively, these findings suggest that PCI induced mild TBI can significantly alter the metabolic profile of rat brain tissue. This disruption was greatest following repetitive PCI and generated a biochemical signature that may warrant further investigation for diagnostic potential. Notably, metabolic alterations at 2 hours in positive controls were delayed until 6 hours in sPCI tissues and may suggest increased sensitivity of repetitively exposed animals to metabolic dysfunction. Specifically, PCI was accompanied by differences in neurotransmitter related metabolites such as GABA and acetylcholine that may influence behavior and recovery, and changes in polyamine and glutathione metabolism that may influence tissue repair and redox homeostasis following injury. Additionally, glycogen metabolism and glucose utilization were enhanced following PCI and may compensate for limited TCA cycle that may be indicative of restricted mitochondrial

function. Finally, differences in nucleic acid metabolism may impact availability for tissue repair and contribute to oxidative stress.

Task 1.3 (Months 1-18). Evaluate the neurobehavioral (motor, cognitive, and affective) profile following PCI injury.

Righting Reflex: Immediately following PCI injury, all rats displayed significantly increased Righting Reflex (RR) compared to uninjured anesthesia-alone controls. In animals receiving subsequent impacts, RR remained elevated following repetitive PCI compared to sham control (rSHAM). Repeated measure Two-Way ANOVA showed a significant main effect of group, a main effect of impact number, and a significant interaction effect of group and impact number, indicating increased RR with multiple hits (Figure 20). Notably, multiple comparisons of RR at sequential impacts showed significant increases between the first impact and all three subsequent impacts but no cumulative effect (eg. no difference between second and third, and third and fourth).

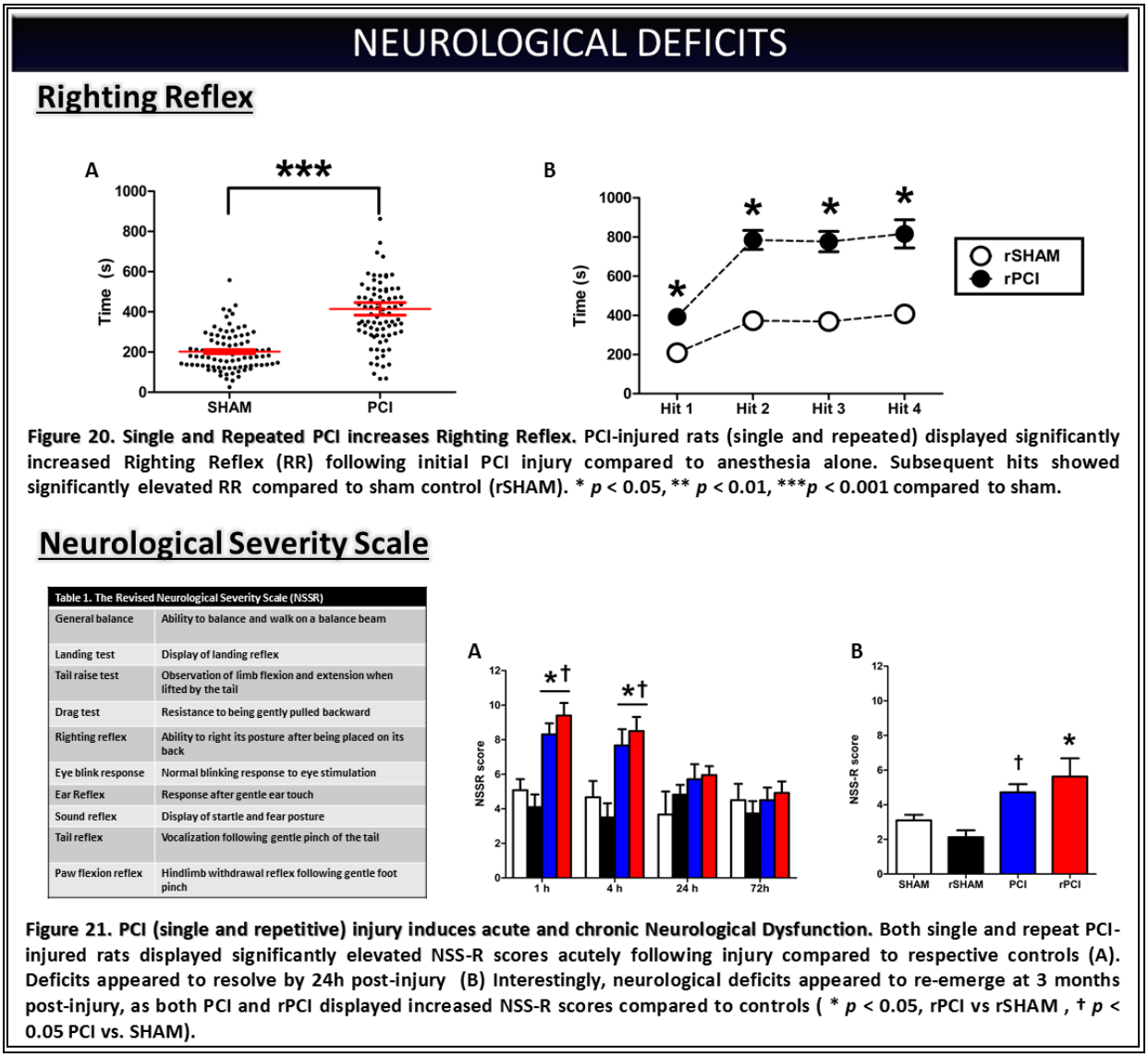


Figure 20. Single and Repeated PCI increases Righting Reflex. PCI-injured rats (single and repeated) displayed significantly increased Righting Reflex (RR) following initial PCI injury compared to anesthesia alone. Subsequent hits showed significantly elevated RR compared to sham control (rSHAM). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to sham.

Figure 21. PCI (single and repetitive) injury induces acute and chronic Neurological Dysfunction. Both single and repeat PCI-injured rats displayed significantly elevated NSS-R scores acutely following injury compared to respective controls (A). Deficits appeared to resolve by 24h post-injury (B) Interestingly, neurological deficits appeared to re-emerge at 3 months post-injury, as both PCI and rPCI displayed increased NSS-R scores compared to controls (* $p < 0.05$, rPCI vs rSHAM , † $p < 0.05$ PCI vs. SHAM).

Neurological Severity Scale Revised (NSSR): Neurological deficits were scored using the Neurological Severity Scale-Revised at 1, 4, 24, and 72h post-injury. The revised NSS-R consists of 10 tests designed to assay motor, sensory, and reflex skills. For a completely normal response on each test, zero points are assigned. For an intermediate response demonstrating moderate

impairment, one point was assigned. For a complete lack of ability to perform the test, two points were assigned. On the 10-task panel higher score indicated greater the impairment, with 20 points representing the maximum (i.e., worst) outcome. All assessments were conducted by experimenters blind to groups.

NSSR Results: Neurological deficits manifested in both single and repeat PCI within one hour, remained elevated, and returned to sham control levels by 24h post-injury (Figure 21). Two-Way ANOVA showed a significant main effect of group, a main effect of time, and a significant interaction effect of group and time, indicating transient yet significant injury-induced neurological changes which resolved acutely following injury. Neurological deficits manifested in both single and repeat PCI within one hour, remained elevated, and returned to sham control levels by 24h post-injury (Figure 21). Two-Way ANOVA showed a significant main effect of group, a main effect of time, and a significant interaction effect of group and time, indicating transient yet significant injury-induced neurological changes that resolved acutely following injury.

Sensorimotor Dysfunction:

Gait Analysis. Dynamic balance requires maintenance while in motion (gait). The presence of normal gait and postural stability are highly dependent on the somatosensory sensory system which is comprised both proprioception and sensation. Since disruption in these pathways, as a result of brain injury, can manifest in abnormal walking patterns, we assessed sensorimotor alterations following single and repeated PCI using the CatWalk gait analysis system.

Rats were subjected to gait assessment 3 days prior to PCI (baseline measures) and at 2h, 24h, 72h, 9d, 28d, 3 months post-injury using the CatWalk automated gait analysis system (Noldus Information Technology Inc., Leesburg, VA) as previously described (Mountney et al 2013). The CatWalk is a highly sensitive device consisting of a 1.3 m long glass plate illuminated on the side by dim fluorescent lighting. In a dark (unlit) room, animals are placed on the walkway and allowed to traverse from one end to the other. Direct contact between the paw and glass surface results in light reflection in the form of illuminated footprints. Footprint images are video-recorded by a camera positioned under the walkway. Briefly, rats were first acclimated in darkened goal box positioned at one end of the runway (5 min duration). Following acclimation, the animals were removed from the box and completed 3 consecutive runs (averaging approximately 2 s/run) with 2 min inter-trial intervals in the box between each run. No pre-injury training was necessary. Runs with walking velocities >30% variation were excluded from analysis. The images from each trial were converted into digital signature and processed using CatWalk XT 9.1 software with a minimum threshold set at 80 (a.u. ranging from 0 to 225). Following footprint identification and labeling, data pertaining to static and dynamic gait parameters were generated for each trial. The mean scores from 3 consecutive trials (per animal/time points) were analyzed for statistical significance.

Gait Walk Results: At all post-injury time points animals from all groups did not show prominent or overt gait disturbances and a large proportion of gait parameters remained unchanged in injured animals compared to respective controls over the course of the study. However, PCI-injured rats, both single and repeated, displayed a number of significant yet subtle sensorimotor abnormalities. Single PCI resulted in deficits on a limited number of dynamic gait parameters at 2h post-injury that were resolved by 24h post-PCI. In contrast, repeated PCI produced significant deficits across 28 separate gait parameters, the majority of which were sustained out to 72h post-injury, but most of

which resolved by 9d post-PCI (Table 1). In all injured rats, gait deficits were not restricted to one side or body position, but instead appeared bilaterally and equally distributed on both front and hind paws. Rats displayed abnormal gait patterns with increased stance phase, swing phase, and step cycle as well as decreases in swing speed and cadence (Figure 22). The stance phase was further subdivided into 3 segments, including (1) initial dual limb stance, (2) single limb stance, and (3) terminal dual limb stance. Notably, these three parameters were elevated for 72h following repeated PCI, indicating impairments in proprioception and pain sensitivity. Significant increases of three-point support and corresponding decreases in dual limb support were noted in rPCI rats at 3, 14, and 28d post-injury. Despite significant gait abnormalities, step patterns and regularity index (indices of coordination) showed no differences between groups.

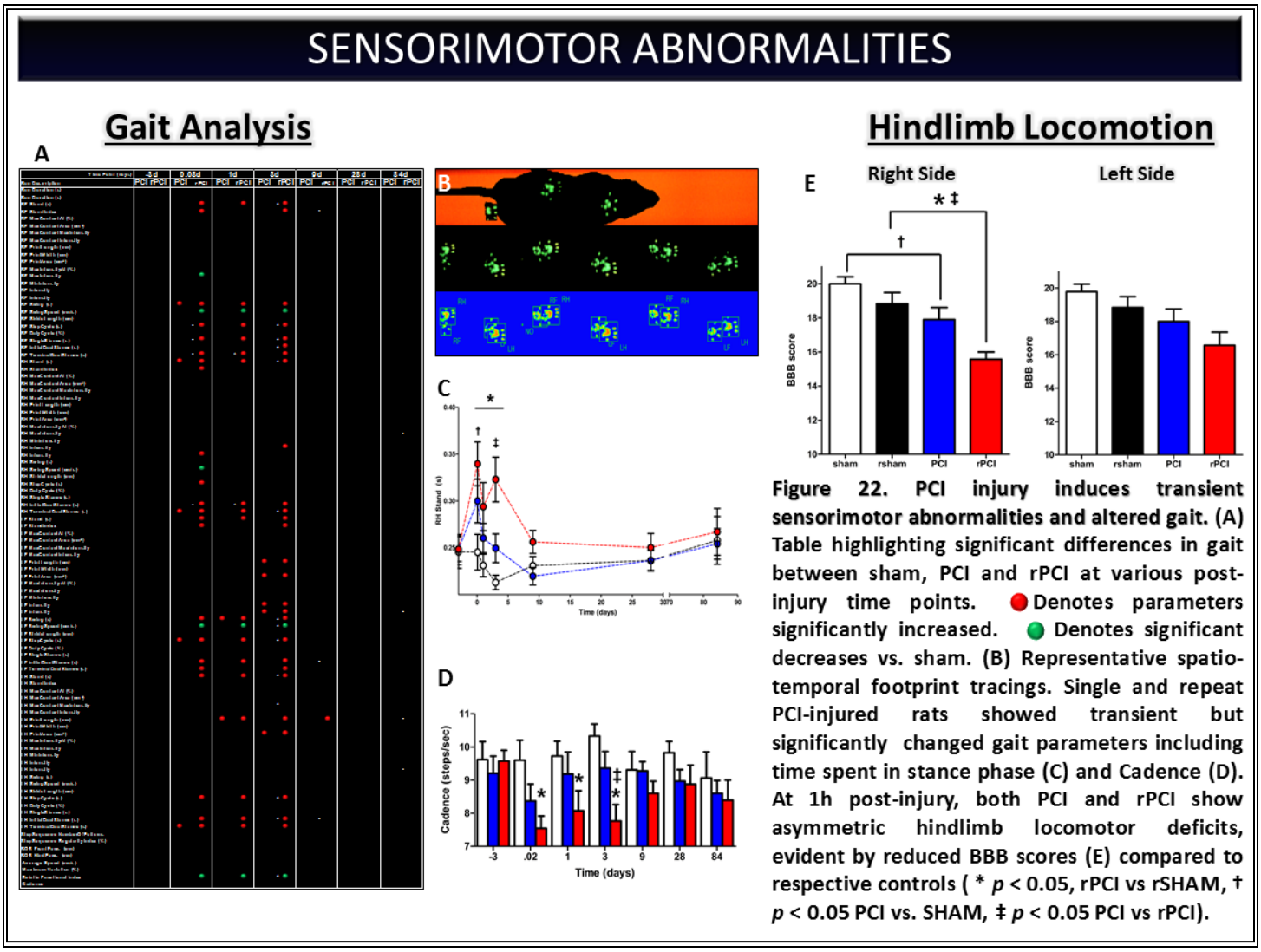


Figure 22. PCI injury induces transient sensorimotor abnormalities and altered gait. (A) Table highlighting significant differences in gait between sham, PCI and rPCI at various post-injury time points. Denotes parameters significantly increased. Denotes significant decreases vs. sham. (B) Representative spatio-temporal footprint tracings. Single and repeat PCI-injured rats showed transient but significantly changed gait parameters including time spent in stance phase (C) and Cadence (D). At 1h post-injury, both PCI and rPCI show asymmetric hindlimb locomotor deficits, evident by reduced BBB scores (E) compared to respective controls (* $p < 0.05$, rPCI vs rSHAM, † $p < 0.05$ PCI vs. SHAM, ‡ $p < 0.05$ PCI vs rPCI).

Vestibular/Balance Assessment: The Rotemex-5 rotarod apparatus (Columbus Instruments, Columbus, OH) was used to measure motor coordination and balance. Four different assessment paradigms were tested for their ability to detect differences between sham and injured groups. Separate cohorts were used for each paradigm. Under normal conditions, stable balance is achieved by the integration of sensory information from the visual, vestibular, and somatosensory systems. This input is processed and used to select appropriate motor responses for the maintenance of postural equilibrium; however, following brain injury, balance/vestibular function is highly disrupted

and, in TBI patients, is often one of the most prominent symptoms of both self-reporting and objective measures. As such, we preformed neurofunctional assessments aimed at assessing these metrics.

Vestibular/Balance Results: Following PCI rats were assessed on the rotarod (two trials/time point) starting at 15min post-injury, and then repeatedly tested at 1 h, 24h, 7d and 14d. Two-Way Repeat Measures ANOVA showed a significant group effect, time effect, and a significant group x time interaction. Rats which sustained rPCI displayed significantly reduced latencies on the rotarod at early post-injury time points (15min and 1h) which appeared to resolve and remain stable by 24h. A similar, albeit not significant, trend was seen following a single PCI. Given the apparent "rapid" recovery, we plotted individual data points for latency to fall for each trial during the training (constant speed) and testing (accelerating speed) phases to determine the rate at which vestibular function returned (Figure 24). Although linear regression analysis indicated no significant difference in the calculated slope value over the acute period, regression values for the intercepts for both single and repeat PCI significantly deviated from matched controls and from each other, (p <0.001, for PCI-SHAM, rPCI-rSHAM, and PCI-rPCI). This analysis suggests that PCI does not hinder the animal's ability to learn to balance, but rather decreases the initial starting point, counteracting or reducing any benefit received during the initial training. To determine the effects of repetitive balance testing, separate cohorts of animals were pretrained and tested only at 24h post-injury (Figure 24). A significant deficit was detected at 24h only in the repetitive PCI group when compared to all other groups (* p < 0.05 rPCI vs rSHAM, ‡ p < 0.05 rPCI vs PCI). The apparent temporal incongruence of the recovery time between our two testing paradigms at these early time points (<1h and 24h) highlights the strong influence of the learning component of repetitive testing which may mask functional deficits still present after injury.

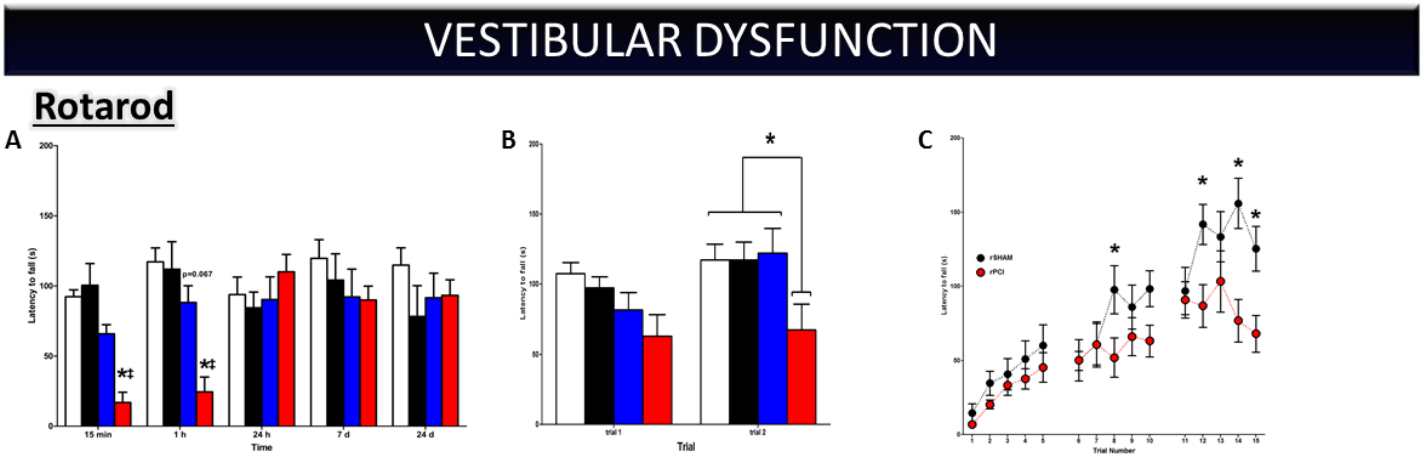


Figure 23. Single and Repeated PCI induced acute and chronic vestibular dysfunction. Balance and vestibular function was assessed on the rotarod starting at 15min post-injury. Rats which sustained rPCI displayed significantly reduced latencies on the rotarod at early post-injury time points (15min and 1h) which appeared to resolve and remain stable by 24h. A similar, albeit not significant, trend was seen following a single PCI. (B) To determine the effects of repetitive balance testing, separate cohorts of animals were tested only at 24h post-injury. A significant deficit was detected only in the repetitive PCI group when compared to all other groups ($p < 0.05$). (C) To examine chronic motor deficits, at three months post-injury, rats were challenged by three sets of 5 trials (15 total) with no pre-training. Although both rPCI and rSHAM groups initially tracked together, rPCI appeared to reach worse performance levels during the exercise stress test compared to rSHAM (* $p < 0.05$, rPCI vs rSHAM , † $p < 0.05$ PCI vs.

Neuroendocrine Response: We investigated post-injury serum levels of the neuroendocrine stress-related hormone, corticosterone (CORT), and observed significant CORT changes in serum levels in both single and repeat PCI groups compared to controls. Two way repeat measure ANOVA showed a significant group effect [$F(3, 14) = 8.98$; $P \leq 0.05$], time effect [$F(2, 28) = 5.38$; $P \leq 0.05$], and a significant group x time interaction [$F(6, 28) = 3.22$; $P \leq 0.05$]. Post-hoc analysis indicated significant differences at the 1h post-injury time point between injured animals and their respective controls but not between single and repeated PCI ($p > 0.05$), suggesting that the rise in cortisol levels was not dependent on number of impacts. To further characterize the acute neuroendocrine response, we screened serum and cerebrospinal fluid (CSF) for 29 distinct rat inflammatory cytokines and chemokines at 1h, 24h and 14d post-injury. At one-hour post-injury, we found significant upregulation of CINC-1 in the serum of rPCI rats compared to all other groups (7.5-fold over rSHAM, $p<0.05$); however, CINC-1 levels returned to control levels by 24h post-injury and no other changes were detected in serum neuroinflammatory markers including IL-6, IL-4, IL-10, and TNF-alpha at any other time point. Conversely, in CSF, levels of two neuroinflammatory cytokines were significantly upregulated in both single and repeat PCI at 24h post-injury. Rodents exposed to multiple impacts significant increases in S-selectin and TIMP-1 and a trend towards increased levels of siCAM showed showing a disproportional increase in the number and time course of cytokines altered compared to levels detected in the serum. Analysis of 1h CSF samples is underway. The differences in the temporal profile suggest a local rather than systemic inflammatory response following PCI (Figure 24).

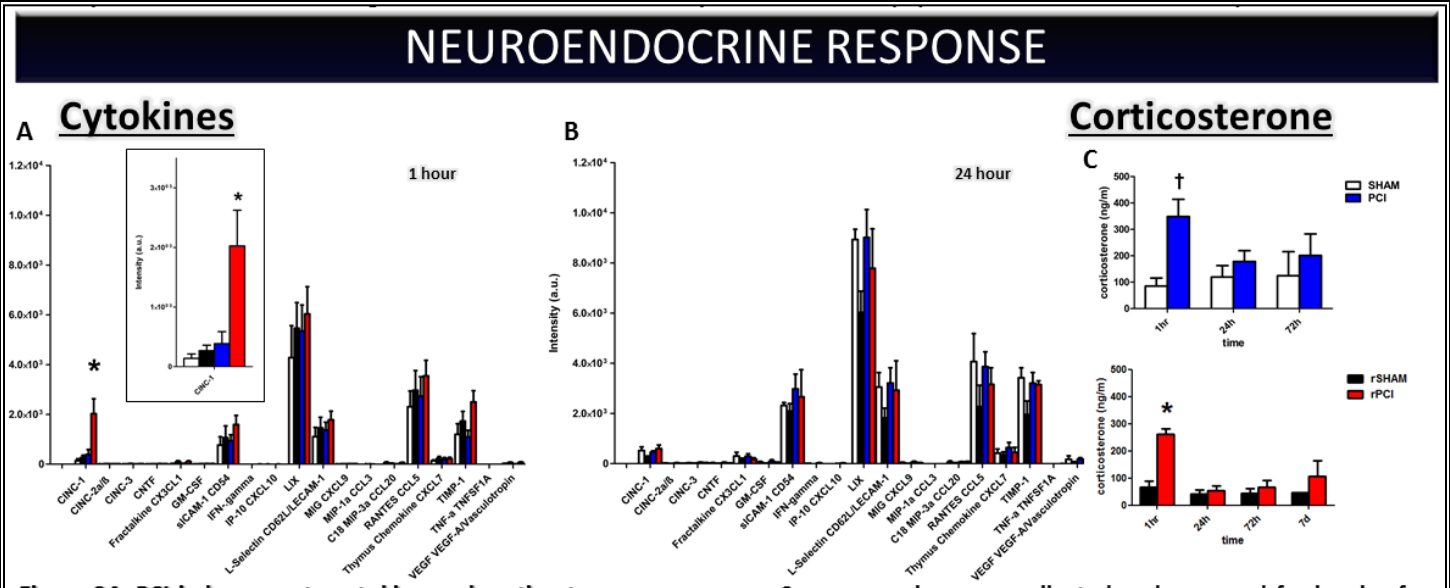


Figure 24. PCI induces acute cytokine and corticosterone responses. Serum samples were collected and screened for levels of inflammatory cytokines. Animals subjected to rPCI showed elevated cytokine levels at 1h which returned to sham levels by 24h post-PCI (A, B). CINC-1 (inset) was dramatically elevated to levels > 7.5 and > 2.5-fold higher in rPCI and PCI compared to respective sham. Both PCI and rPCI rats showed significant increases in corticosterone (CORT) at 1 h post-injury (C). Subsequently, CORT levels were reduced to respective sham levels at later post-injury time points († $p < 0.05$, PCI vs SHAM; * $p < 0.05$, rPCI vs rSHAM,)

Cognitive Assessment: To validate the sensitivity of the cognitive assessment in the PCI model, rats were randomly assigned into two groups: sham and repeated PCI. The sham control group received a single anesthesia (4% isoflurane), whereas the PCI positive control group received four PCIs at 1 hour intervals. At 72 hours post-injury, all rats were subjected to a five-day working memory

test using Morris water maze. Rats were tested in a circular pool (75cm deep and 175cm in diameter) filled with clear water. The position of the platform changed each day rotating within the four quadrants (Northeast, Northwest, Southeast and Southwest). The rats were placed in the pool at the same starting position (i.e. West) throughout the experiment. Each rat received 4 trials per day for 5 consecutive days. Each trial lasted 60 seconds with an inter-trial interval (ITI) for 5 minutes. The latency to find the platform was recorded and used for statistical analysis. Cognitive abilities were assessed in the Morris water maze (MWM) at 1 month and 6 months post-PCI. All rats were exposed to a series of MWM tasks designed to assess:

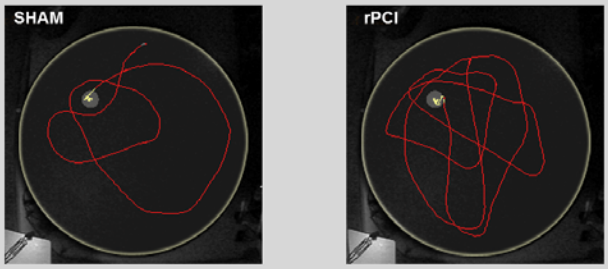
(1) Spatial reference memory: a four-day spatial learning task to find the hidden platform.

(2) Memory retention: a missing platform (probe) trial.

(3) Working memory: a one-day matching-to-place task (2 pairs of trials).

Primary outcome metrics consisted of the latency to find the hidden platform, % time spent in the platform zone searching for the missing platform during the probe trial, and latency difference (delta) to locate the platform within a trial set in the working memory test (results shown in insert below).

RESULTS



Trace indicates a rat swimming to search for the hidden platform to escape from the water (Yellow trace – platform; Red trace – outside of platform)

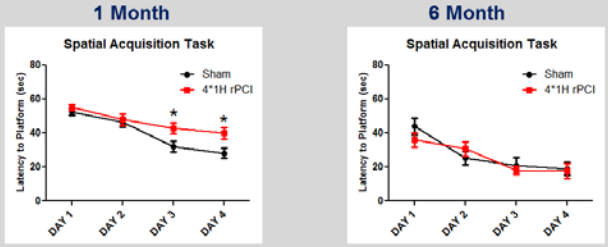
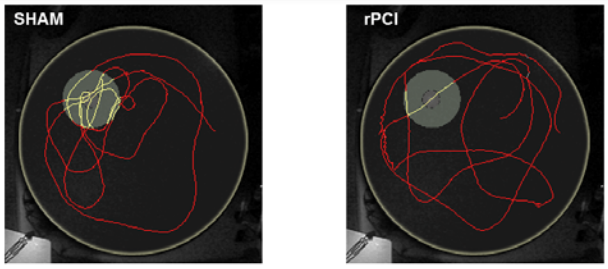


Figure 1. At 1 month post-injury, results of the spatial learning task showed that the average latencies to locate the hidden "escape" platform were significantly longer in rPCI rats over the last two days of the MWM testing compared to sham controls ($p<0.05$). At 6 months post-injury, no differences were detected between rPCI and sham controls on the spatial learning trial.



Trace indicates a rat swimming to search for the missing platform during the probe trial (Yellow trace – platform zone; Red trace – outside of platform zone)

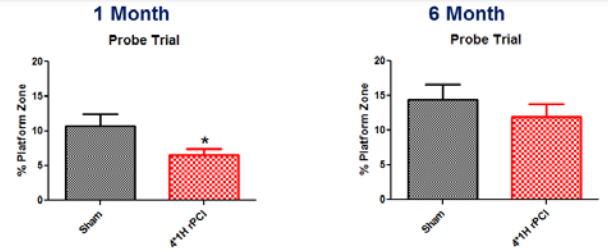
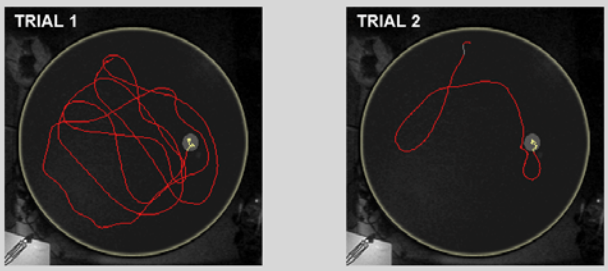


Figure 2. At 1 month post-injury, rPCI rats spent significantly less time in the platform zone searching for the missing platform during the probe trial in the memory retention task ($p<0.05$). At 6 months post-injury, no differences were detected between rPCI and sham controls on the probe trial.



Trace indicates a rat swimming to search for the hidden platform in a set of trials (trail 1 and 2) (Yellow trace – platform; Red trace – outside of platform)

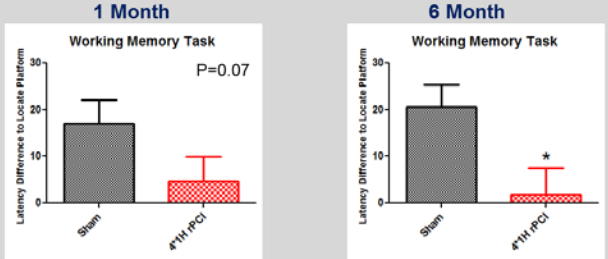


Figure 3. At 1 month post-injury, PCI-injured animals showed a trend toward worse performance, but this failed to reach statistical significance compared to sham controls on the working memory task ($p=0.07$). At 6 months post-injury, rPCI rats exhibited significantly worsened working memory performance compared to sham controls ($p<0.05$).

Overall, the results indicate that the MWM is capable of detecting cognitive deficits following mTBI and thus may be useful in assessing the effects of single vs. repeated injuries induced at varied intervals in the PCI model. Furthermore, the results show that rPCI produced significant cognitive deficits in both spatial learning abilities and in working memory abilities in a time-dependent fashion that may be indicative of progressive pathology and warrant further investigation.

Task 1.4.1 Evaluate quantitative electrophysiological (qEEG) profile of PCI-induced abnormalities in brain wave patterns: For this Task, we plan to apply EEG power spectrum analysis to examine EEG power shift and altered EEG coherence following PCI, through continuous EEG recording out to 72h post-PCI, followed by a 2h recording on post-injury Days 5, 7, and 14. Experimental groups will consist of sham, PW controls, and PCI (n=15/group; N=45).

Rats were randomly divided into two groups: one group received a single PCI injury (N = 10) and another group received the sham procedure (N = 10). Immediately after PCI or sham exposure, rats were surgically prepared with EEG electrode implantation. Both procedures were conducted under isoflurane anesthesia. Upon awakening from anesthesia, rats were placed individually in an EEG recording chamber and connected to a digital EEG recording system. Bipolar EEG recordings were collected continuously from the left and right hemispheres for 14 days post injury (Figure 25)

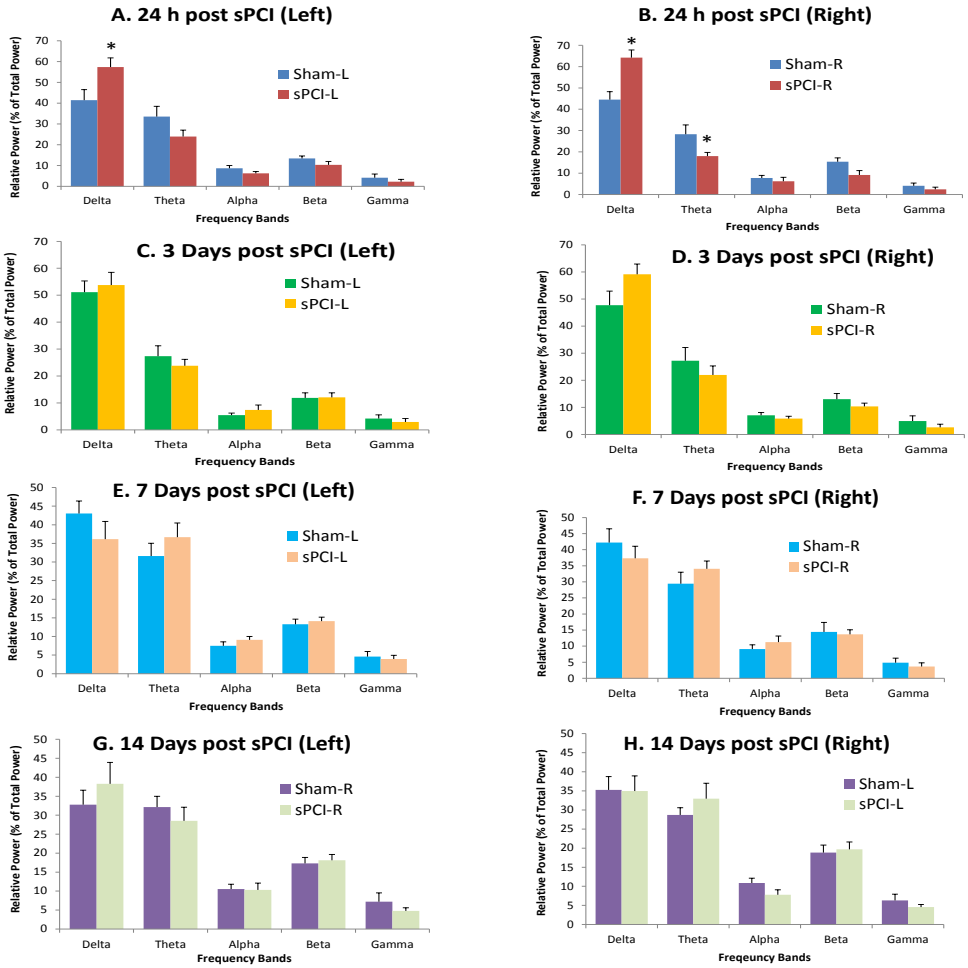


Figure 25. EEG power spectrum analysis shows that a single PCI induced transient EEG slowing in both hemispheres evidenced by the bilateral increase in EEG delta activity within 24 h post injury (A and B) . Normal EEG activities were resumed within 3 days post injury (C-H).

At the end of EEG recording, quantitative EEG (qEEG) power spectral analysis were performed based on a 60-sec awake EEG epoch selected from each rat at 1, 3, 7 and 14 days post single PCI or sham injury. The EEG global frequency band was divided into delta (0.1 - 4 Hz), theta (4 - 8 Hz), alpha (8 - 12 Hz), beta (12 - 30 Hz), and gamma (30 - 100 Hz) bands. The EEG relative power of each band of the left and right hemispheres was calculated at each time point for each rat.

EEG Results: EEG power spectrum analysis indicated that compared to sham condition, a single PCI induced transient EEG slowing in both hemispheres evidenced by bilateral increase in EEG delta activity within 24 h post injury (Figure 25A & B). The increased delta activity was compensated by the decrease in power in all other EEG bands, with the most significant compensation offered in the theta band in the ipsilateral hemisphere (Figure 25). Similar trend of EEG slowing was observed in the ipsilateral hemisphere on Day 3 post PCI. By the 7th day post injury and thereafter, normal EEG activities were restored in both hemispheres (Figure 25E-H).

EEG Summary: EEG slowing is one of the hall marks for brain functional abnormality following brain injury. However, it can be subtle and undetectable from conventional visual EEG signals following a mTBI. Quantitative EEG is a sensitive measure of EEG changes and can be used for discriminate brain injury severities, and therefore has been considered as a useful tool in diagnosis and measurement of mTBI. ***In this study the application of qEEG power spectrum analysis detected bilateral and transient alternations of brain electrical activities following a single PCI.*** The significance of this finding requires further investigation of correlations of EEG changes with other histopathological and/or behavioral changes in order to validate its diagnostic and prognostic values.

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.

REPORTABLE OUTCOMES

(All reportable outcomes since project inception are shown; those from the 2013-2014 funding year are shown in bold font):

1. 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)
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. The 31st National Neurotrauma Symposium. Nashville, Tennessee, USA. August 2013.
3. 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.
4. 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.

5. 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.
6. 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.
7. 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)
8. 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.

CONCLUSION

Phase I studies designed to evaluate the time course effects of a single concussion on clinically relevant outcome measures have been completed. 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 have been initiated. The WRAIR PCI model was refined to provide a consistent and highly reproducible concussion in rats. Experimental results demonstrating clinically relevant signs including neuropathological, molecular, metabolic and neurobehavioral (sensorimotor and cognitive) abnormalities while remaining within the criteria defined for mTBI. The model is fully non-invasive and does not involve any surgical procedures, and creates highly reproducible head impact injuries across subjects. As such, it provides a valuable tool for studying the injury mechanism of closed-head impacts, particularly repeated concussion. By virtue of its reproducibility, high throughput (20 animals can be injured per hr), simple design and relative ease of fabrication, the model will provide an optimal exploratory platform for future diagnostic and therapeutic preclinical studies.

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APPENDICES

1. n/a