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14. ABSTRACT

Closed head concussions, also known as mild traumatic brain injuries (mTBIs), are of great concern to both military and civilian populations alike. While acute concussion symptoms resolve for most patients, a subset will experience effects that persist chronically. Emphasis has been placed upon identifying prognostic indicators to distinguish these vulnerable patient populations for the purpose of providing enhanced care. Two potential clinically-relevant prognostic indicators include altered brain glucose metabolism as detected by FDG-PET imaging and changes in serum microRNA levels. The aim of this work is to comprehensively characterize longitudinal profiles of these two potential prognostic indicators following single and repeated injuries in a rodent model of closed head concussion. These studies utilize the WRAIR Projectile Concussive Impact (PCI) model, which is a military relevant model of closed head concussion developed under the directive of the Combat Care Casualty Research Program (CCCRP). In this Year 2 Report, we provide finalized results to characterize longitudinal alterations in brain glucose uptake and associated neurobehavioral changes through 6 months following single or repeated closed head concussions. In addition, altered microRNAs following a single concussion are identified through 14 days following injury.

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1. INTRODUCTION:

The WRAIR Projectile Concussive Impact (PCI) model of closed-head mTBI was previously established under the directive of the Combat Casualty Care Research Program (CCCRP). The histopathological, molecular, and acute neurobehavioral profiles of this military-relevant mTBI model, which includes a custom designed helmet and sensor film system provided by the Army Research Laboratory, have been well characterized by previous studies. The primary goals of the current proposal are to a) characterize clinically relevant acute metrics of brain trauma following PCI and b) determine their prognostic value for chronic neurological and cognitive deficits and/or neurodegeneration. The two clinically relevant mTBI metrics assessed here will be brain glucose metabolic dysfunction and alterations in serum microRNA levels. **Following either single or repeated PCI injuries, studies in SOW Major Task 1 will assess brain glucose uptake by [18F] FDG-PET/CT imaging while studies in SOW Major Task 2 will evaluate serum microRNA profiles.** This proposal expands upon our ongoing collaboration with the Uniformed Services University Health Science (USUHS) Translational Imaging Facility, which is highly experienced with the study of brain glucose metabolism in brain trauma models. The long-term objective of this proposal is to determine a clinically relevant mechanism for discerning mTBI patients whose symptoms will persist chronically, thereby identifying which patients may need increased care and treatment to mitigate chronic deficits and neuropathology. The findings from this study will be the basis for future preclinical studies following single or repeat PCI and will inform future clinical studies of mTBI.

2. KEYWORDS:

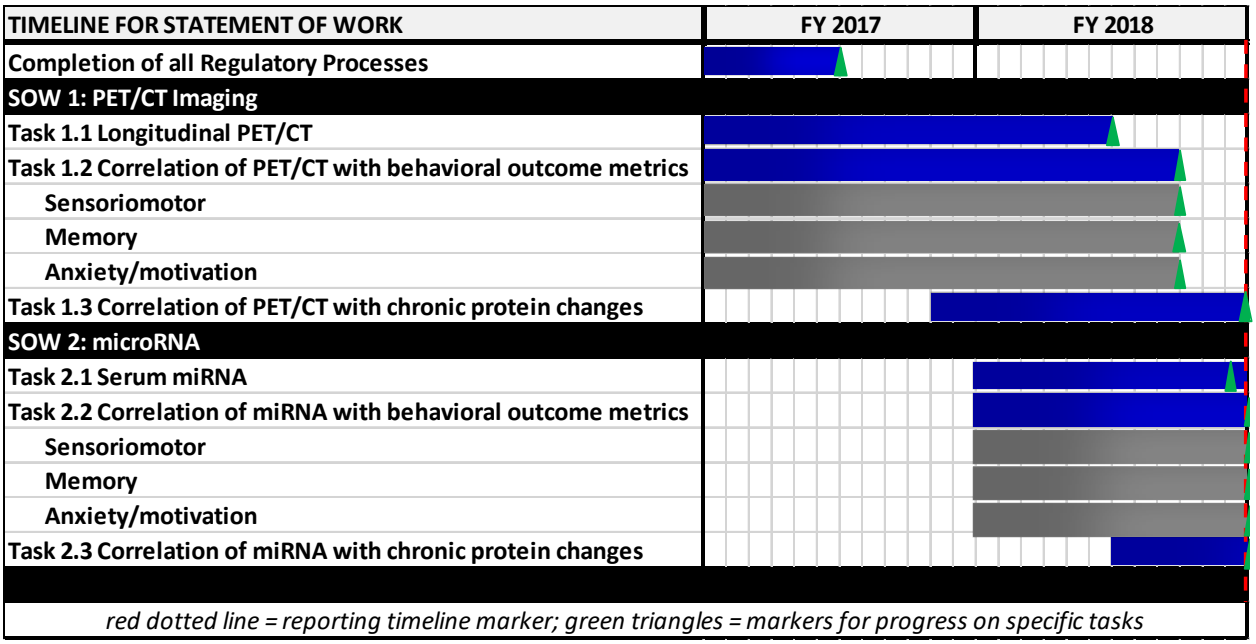
Concussion; Projectile Concussive Impact (PCI); mild TBI (mTBI); repeated mTBI; brain glucose metabolism; FDG-PET/CT imaging; microRNA; neurodegeneration; neurological deficits; behavioral impairment

3. ACCOMPLISHMENTS:

a. What were the major goals of the project?

SOW Major Task 1: Determine if acute brain glucose metabolism dysfunction following single or repeat PCI correlates with longitudinal behavioral outcome measures and chronic protein changes relating to CTE or neurodegenerative pathology.

SOW Major Task 2: Determine if acute changes in serum miRNA biomarkers have prognostic value for deficits in longitudinal behavioral outcome measures and CTE related neuropathology following single or repeat PCI.



b. What was accomplished under these goals?

<u>SOW Major Task 1 (Months 1-24):</u> Determine if acute brain glucose metabolism dysfunction following single or repeat PCI correlates with longitudinal behavioral outcome measures and chronic protein changes relating to CTE or neurodegenerative pathology.

Work for SOW Major Task 1 has been completed. Four different study groups were initiated for this task: single Sham (sSham), single PCI (sPCI), repeated Sham (rSham), and repeated PCI (rPCI). Injuries were induced using the modified PCI device, which has previously been described in great detail (Leung, Larimore et al. 2014). In the repeated sham and injury groups, a total of 4 hits or sham control manipulations were performed for each rat with a one hour interval between procedures. All experimental tasks for SOW Major Task 1 occurred at Site 1 (WRAIR; PI: Dr. Deborah Shear), with the exception of the PET/CT imaging experiments described in Subtask 1.1, which occurred at Site 2 (USUHS; PI: Dr. Bernard Dardzinski).

Subtask 1.1: Determine the acute alterations in brain glucose metabolism in specific regions of interest (ROI) following single and repeated PCI by combined [18F] FDG-PET and CT.

In these experiments, brain region specific uptake of [18F]FDG was measured by PET with corresponding CT as a surrogate for assessing brain glucose metabolism. FDG-PET/CT imaging experiments were conducted at 24h, 3d, 7d, 1m, 3m, and 6m after injury. All imaging was performed at the USUHS Center for Neuroscience and

Regenerative Medicine (CNRM) Translational Imaging Facility (TIF). The morning of the scan, animals were transferred from WRAIR to USUHS/CNRM TIF. All transportation of animals to and from WRAIR and USUHS/TIF was performed by the WRAIR Veterinary Services Program (VSP). PET imagining was performed on the Siemens Inveon PET System. CT imagining was performed on the Siemens Multimodality System during the same acquisition session as the PET Imaging. For analysis, FDG uptake in µCi was determined in both the right (ipsilateral) and left (contralateral) hemispheres in the following broad area regions of interest (ROIs) using the invicroRatAtlas54 on the VivoQuant software: basal ganglia, thalamus, amygdala, cerebellum, cortex, hypothalamus, midbrain, corpus callosum, olfactory bulb, hippocampus, septal area, ventricles, and white matter. FDG concentrations in each right and left ROI were calculated in µCi/mm^3 and were normalized to the concentration of FDG in the whole brain. These normalized values were used for subsequent data analysis.

Altered FDG uptake between PCI injured rats and their corresponding shams (ie, sSham vs sPCI; rSham vs rPCI) were analyzed in both ipsilateral and contralateral ROIs listed above. No comparisons were made between sPCI and rPCI rats due to the effects of multiple anesthesia administrations, which results in significant alterations in the absence of injury. Statistically significant injury effects are described below. Figures for these brain regions were included if a brain region (either ipsilateral or contralateral) demonstrated altered FDG uptake as a consequence of injury at any time point between 24h – 6m.

Results regarding FDG uptake following single and repeat PCI from 24h – 3m after injury were reported in the Year 1 Annual Report. At 6m following sPCI, uptake decreased in the ipsilateral ventricles by 2.05% ($p < 0.05$, Fig. 1C) and the ipsilateral and contralateral thalamus by 1.65% and 1.83%, respectively ($p < 0.05$, Fig. 1B). After rPCI, FDG uptake decreased in both the ipsilateral and contralateral thalamus by 1.56% and 1.51%, respectively, at 6m after injury ($p < 0.05$, Fig. 1B). In addition, uptake decreased in the ipsilateral hemisphere by 0.59% but increased in the contralateral hemisphere by 0.64% at 6m after rPCI ($p < 0.01$, Fig. 1F).

No changes in FDG uptake were observed in the ipsilateral or contralateral basal ganglia, amygdala, cerebellum, hypothalamus, midbrain, corpus callosum, hippocampus, and septal area at any time point assessed.

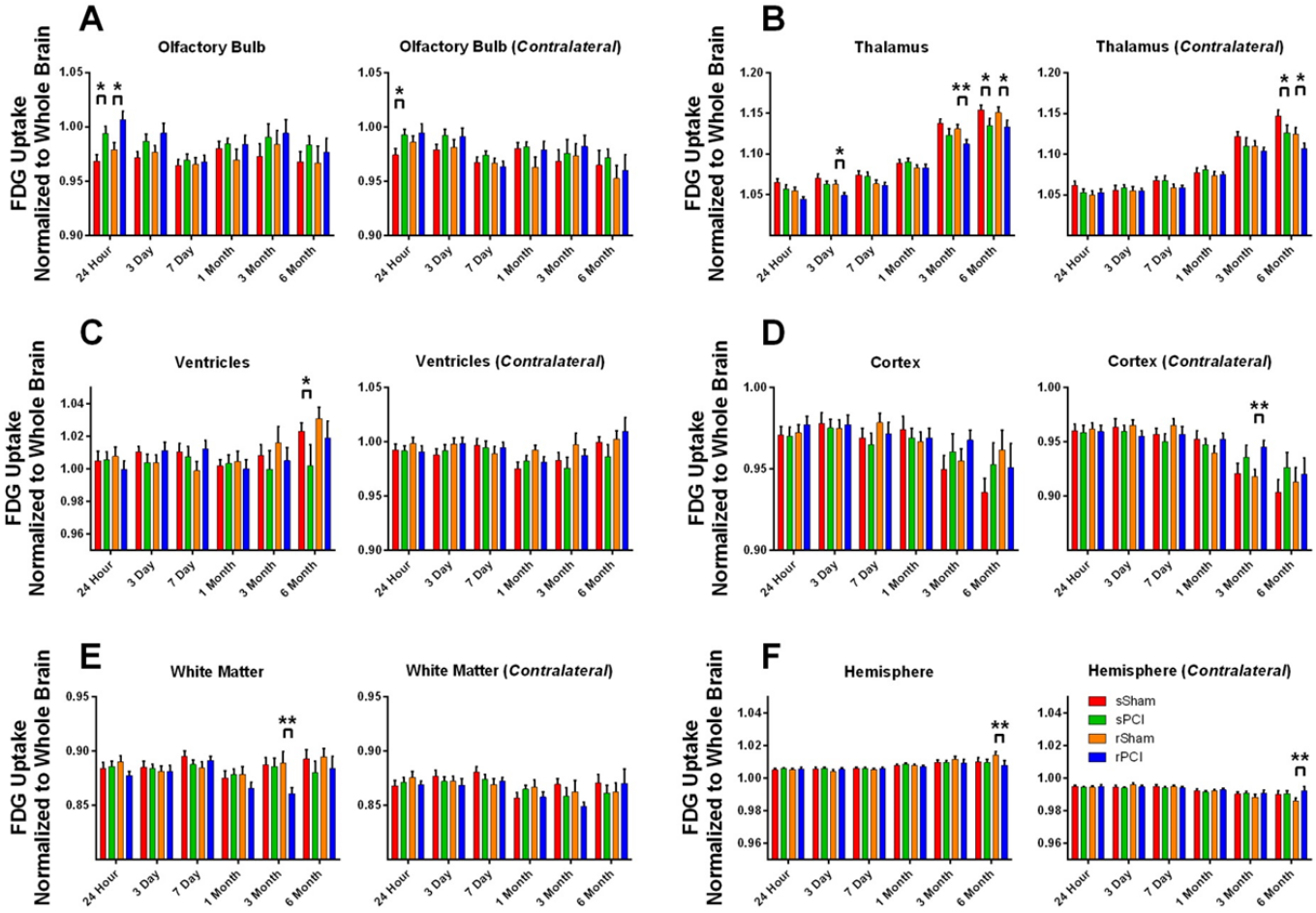


Figure 1: PCI alters FDG uptake. Longitudinal changes in FDG uptake were evaluated following PCI (A-F). Regions are presented here if at least one significant alteration was observed in either PCI group at any time point (* $p < 0.05$, ** $p < 0.01$ against respective sham control; Two-way ANOVA with Fisher's LSD post test). Ns for sSham,sPCI,rSham,rPCI at each time point are as follows: 24h - 22,22,22,22; 3d - 22,22,22,22; 7d - 21,22,22,22; 1m - 24,24,22,22; 3m – 18,16,18,17; 6m – 13,12,15,12.

Subtask 1.2: Determine if brain glucose metabolism correlates with changes in established acute, subacute, and chronic behavioral outcomes following single or repeat PCI.

Experiment 1.2.1 Sensorimotor Assessments:

Righting reflex and Catwalk results were presented in the Year 1 Annual Report.

NSS-R: The Revised Neurological Severity Scale (NSS-R) includes 10 separate neurological tests to evaluate motor, sensory, and reflex skills. These individual tests include a balance beam test, a landing test, a tail raise test, a drag test,

righting reflex, ear reflex, eye blink response, sound reflex, tail reflex, and paw flexion reflex. Performance on each test is scored using the following system: 0 for no impairment, 1 for partial impairment, or 2 for severe impairment. Composite scores for each animal were tabulated at baseline, 4h, 2d, 1m, 3m, and 6m post injury. At baseline, the composite NSS-R scores from all groups were comparable. NSS-R results for 24h – 3m following rPCI were presented in the Year 1 Annual Report. At 6m, no chronic deficits or sPCI or rPCI were observed as detected by the NSS-R (Fig. 2).

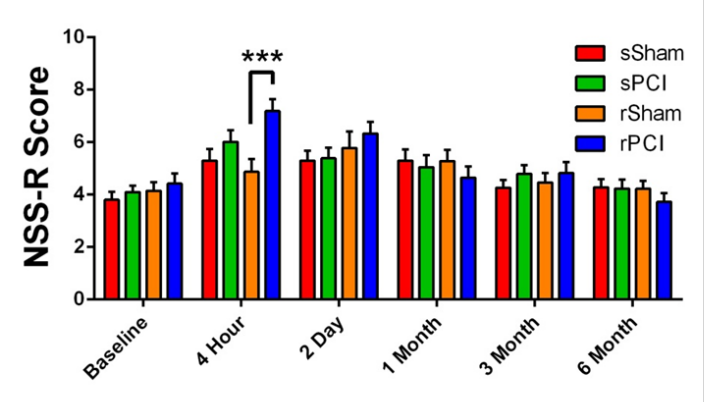


Figure 2: PCI results in acute neurological deficits. Composite NSS-R scores were elevated in the rPCI group at 4h after injury. Statistical significance of the injury groups was evaluated against their respective sham controls at each time point (*** $p < 0.001$, two-way ANOVA with Fisher's LSD post test). Ns for sSham,sPCI,rSham,rPCI at each time point are as follows: baseline - 24,24,22,22; 4h – 24,24,22,22; 2d – 24,24,22,22; 1m - 24,24,22,22; 3m – 22,23,19,21; 6m – 22,23,19,21.

Experiment 1.2.2 Memory Assessments:

Memory assessments were performed at 1, 3, and 6 months after injury using the Morris water maze (MWM) task (Noldus EthoVision XT) with a video-tracking system. The water maze apparatus consisted of a circular pool (75 cm deep; 175 cm diameter) filled with clear water (22 C, room temperature) to a depth of 60 cm. A clear, Plexiglas platform was submerged to a depth of 1 cm from the water surface and placed approximately 35 cm from the wall of the pool. Trials were performed in a darkened room with visual light cues.

Spatial Learning: In the spatial learning task, the rat was placed in the pool (snout facing the pool-wall) at one of four equally spaced starting positions: north (N), south (S), east (E), and west (W). Each rat was allowed to swim freely in the pool until finding the submerged platform or until 60 sec had elapsed. If the rat did not find the platform in 60 sec, it was manually guided there. Once on the platform,

rats were allowed to rest for 10 sec prior to removal and return to their home cage. Rats were given 2 trials per day (5 min. ITI) for 4 consecutive days followed by a missing platform (probe) trial on the 5th day to assess memory retention. The platform location varied for each time point tested. The primary outcome measures were: (1) latency (sec) to find the hidden platform; (2) percent time spent swimming in outer annulus (thigmotaxic behavior); and (3) percent time searching in the target (missing platform) zone during the probe trial.

Results from 24h – 3m after injury were reported in the Year 1 Annual Report. No significant effects of sPCI or rPCI were observed at 6m following injury (Fig. 3-5).

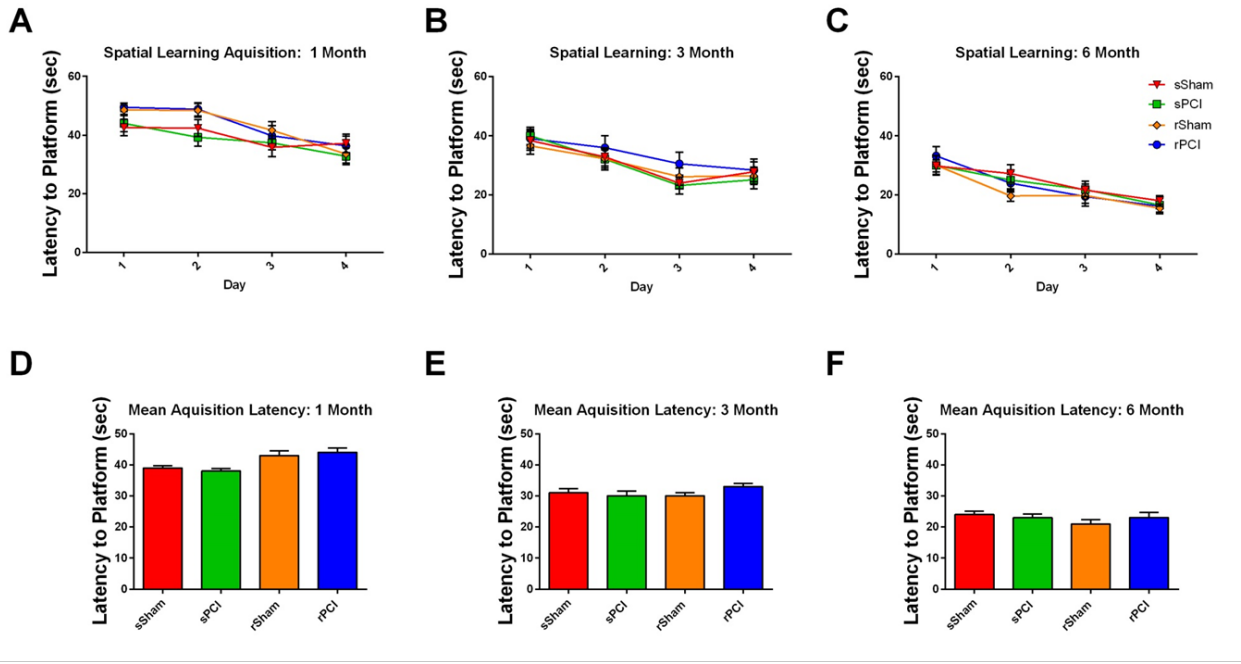


Figure 3: PCI does not alter spatial learning acquisition. Spatial learning was assessed after PCI in the Morris water maze task. No injury effects were observed compared to matched sham controls in individual daily trials (A - C, Two-way repeated measures ANOVA with Fisher's LSD post test) or mean acquisition latencies (D - F, unpaired t-test). Ns for sSham,sPCI,rSham,rPCI at each time point are as follows: 1m - 24,24,22,22; 3m – 24,23,22,20; 6m – 24,23,19,20.

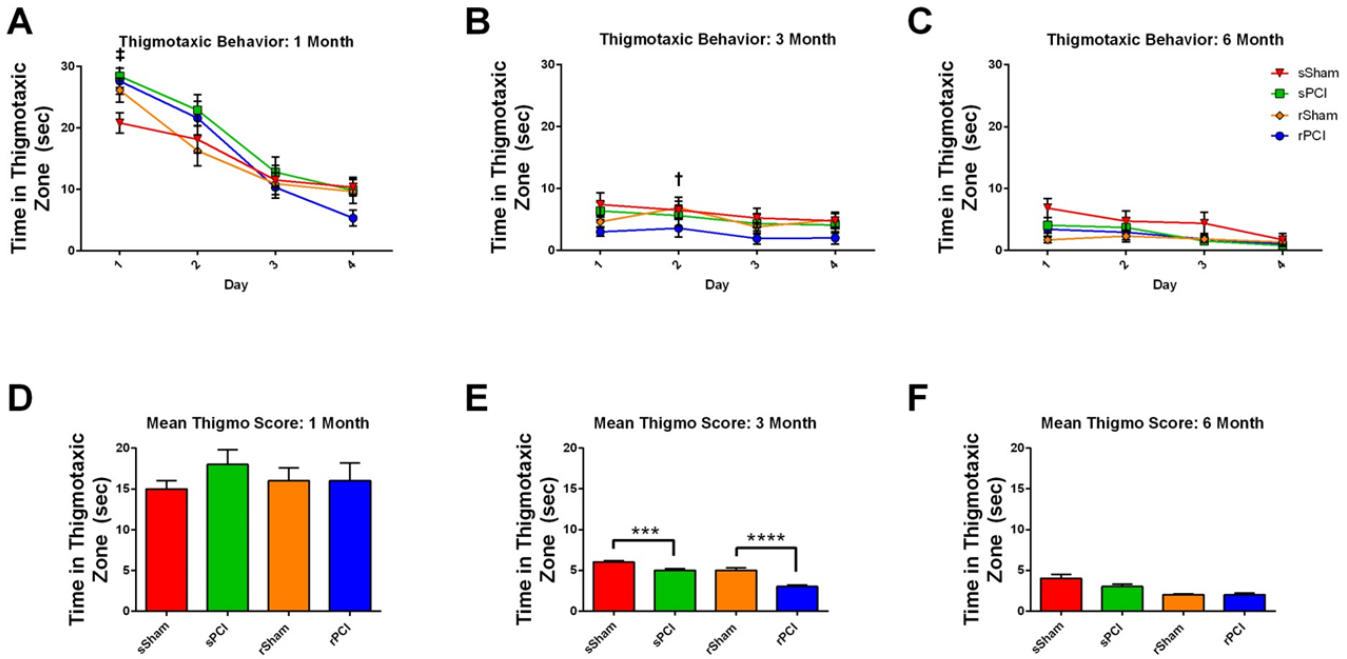


Figure 4: PCI affects thigmotaxic swimming behavior. Differences in thigmotaxic swimming in the spatial learning acquisition trials of the Morris water maze task were assessed by individual acquisition days (A - C, ‡ $p < 0.05$ sSham vs sPCI, † $p < 0.05$ rSham vs rPCI, Two-way repeated measures ANOVA with Fisher's LSD post test) and mean duration in zone (D - F, ***$p = 0.001$, **** $p < 0.0001$ against respective sham control, unpaired t-test). Ns for sSham,sPCI,rSham,rPCI at each time point are as follows: 1m - 24,24,22,22; 3m – 24,23,22,20; 6m – 24,23,19,20.

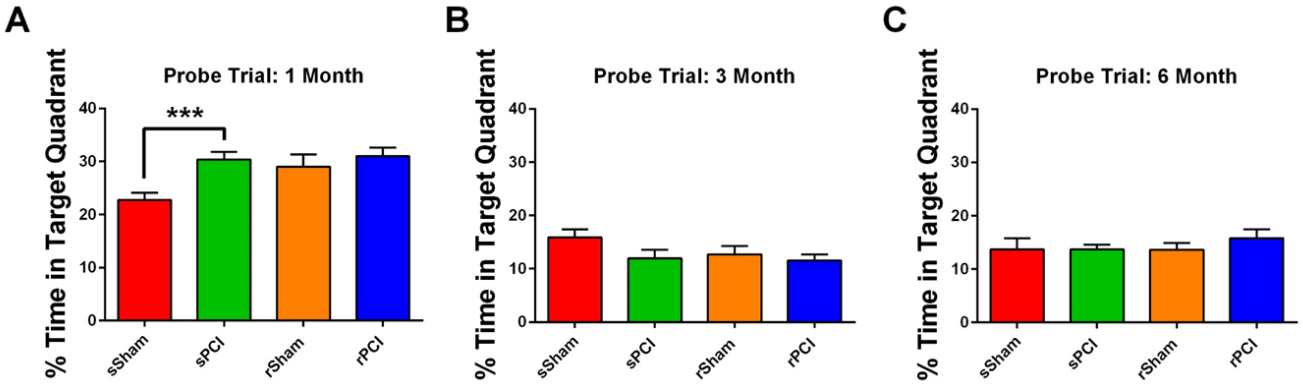


Figure 5: PCI has mild effects on memory retention. Percent of time spent searching the target quadrant for the missing platform was evaluated during the probe trial. Significant injury effects were assessed against the corresponding sham control group (A - C, ***$p < 0.001$, unpaired t-test). Ns for sSham,sPCI,rSham,rPCI at each time point are as follows: 1m - 24,24,22,22; 3m – 24,23,22,20; 6m – 24,23,19,20.

Working Memory: The working memory testing was a delayed matching-to-place task that consisted of two sets of two trials each with a 5 minute inter-set interval. Within a single set, the second trial occurred immediately following the first. The starting position and platform location remained consistent for both trials within a set but was moved to a new starting position and platform location between trial sets. Results from 1m and 3m after injury were previously reported in the Year 1 Annual Report. At 6 months after injury, both sPCI and rPCI groups had a higher latency to locate the hidden platform compared to their respective shams in individual trials ($p < 0.01$ and $p < 0.05$, respectively). However, impairments in working memory as determined by the difference in latency to locate the platform between trials in each trial set were not observed at any time following PCI (Fig. 6).

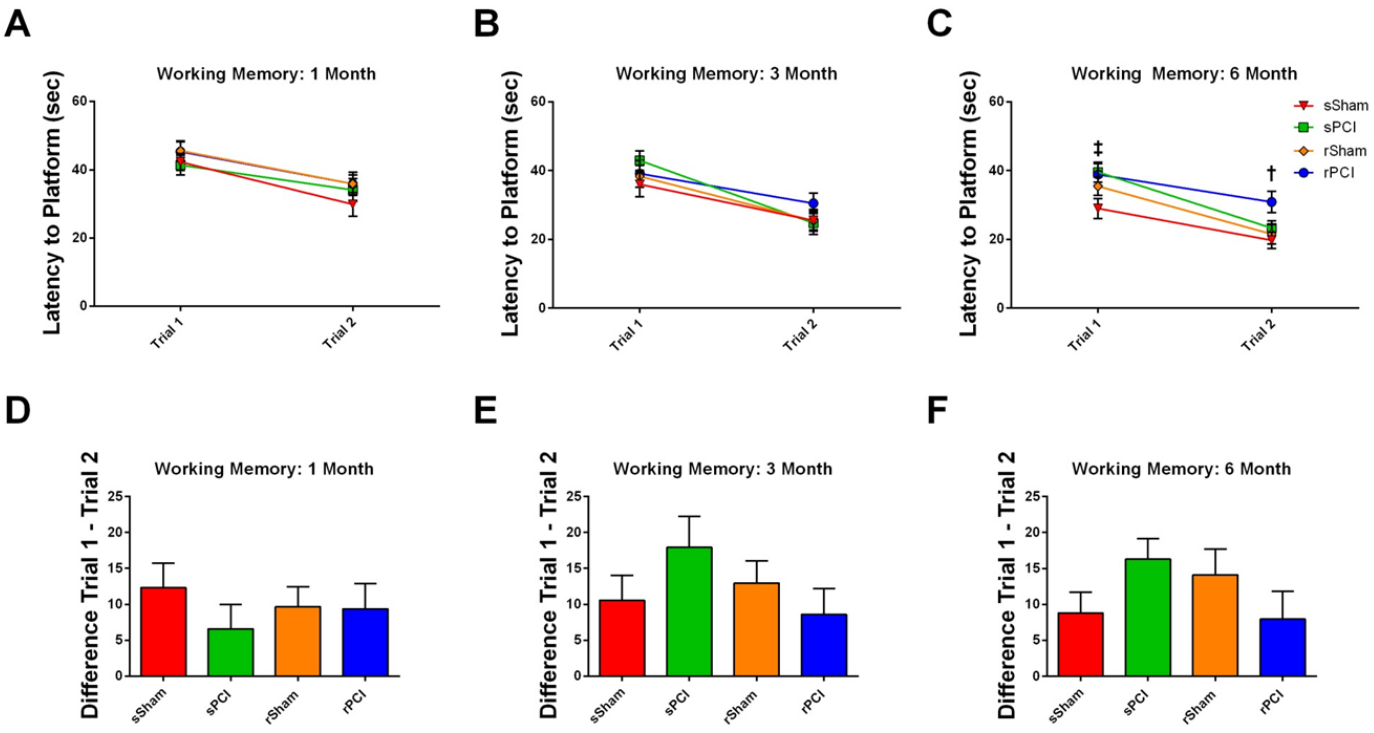


Figure 6: PCI does not result in working memory deficits. PCI groups showed mild increases in latency to locate the hidden platform in individual trials at 6 months post injury (C, ‡ $p < 0.01$ sSham vs sPCI, † $p < 0.05$ rSham vs rPCI, Two-way repeated measures ANOVA with Fisher's LSD post test). However, no effects on working memory as determined by the latency difference between trials 1 and 2 (D - F, unpaired t-test) were observed. Ns for sSham,sPCI,rSham,rPCI at each time point are as follows: 1m - 24,24,22,22; 3m – 20,19,22,20; 6m – 24,23,19,20.

Experiment 1.2.3 Anxiety and Motivation:

Anxiety behavior was assessed prior to injury and at 1, 3, and 6 months after injury with the elevated plus maze (EPM). The EPM (Noldus Technologies) consisted of two perpendicular intersecting walkways elevated 1 meter above the floor. One walkway (2 arms) had no wall while the other walkway (2 arms) had high walls. Rats were placed in an open arm facing the center of the maze and were allowed to explore for 5 minutes. Animal movements were recorded and analyzed using Ethovision software (Noldus Technologies). All trials were performed in a darkened room without the experimenter present. The primary outcome measures were duration in open or closed arms, frequency of entering open or closed arms, distance travelled, and velocity.

Results from 1m and 3m following injury were previously reported in the Year 1 Annual Report. No significant alterations were observed between injured rats and matched sham controls at 6m for arm durations, arm entries, distance travelled, or velocity.

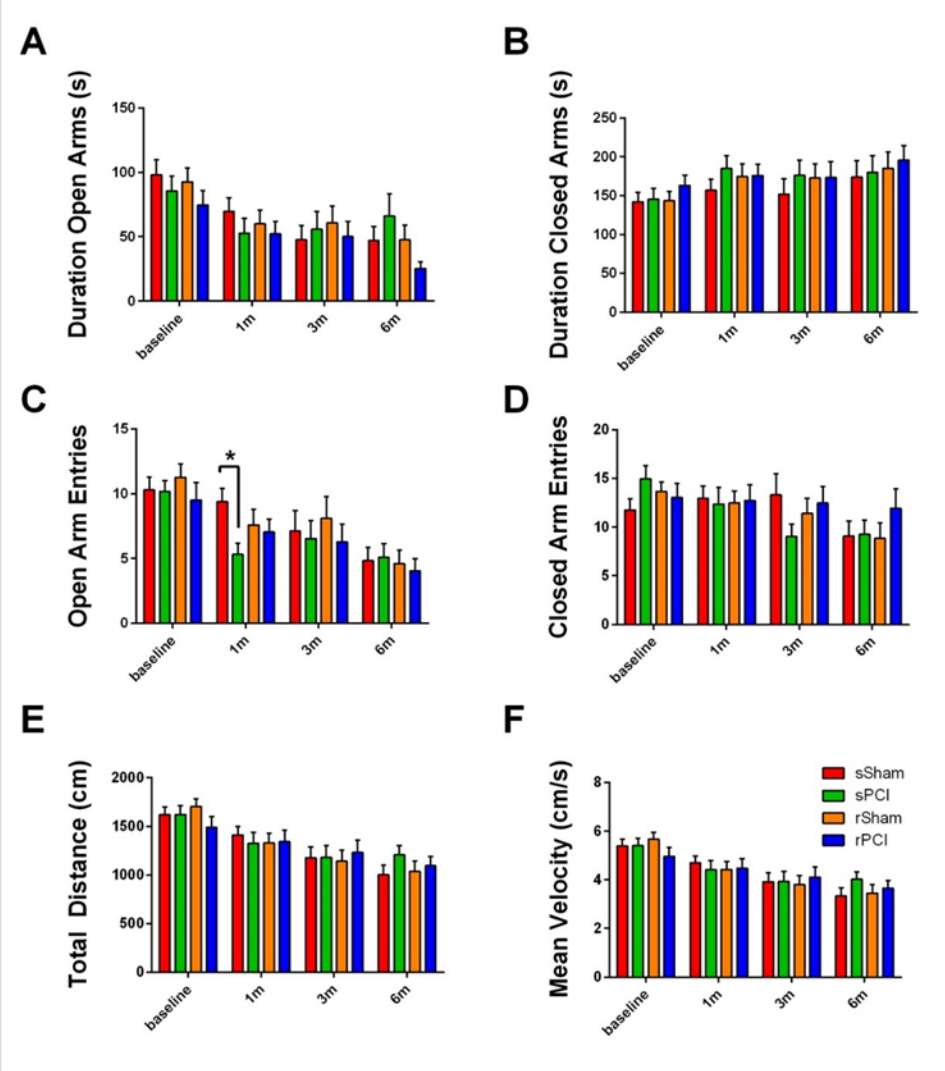


Figure 7: PCI results in limited anxiety-like behavior. The longitudinal effects of PCI on anxiety-like behavior were assessed using the elevated plus maze task. Significant alterations in open arm parameters (A&C), closed arm parameters (B&D), distance (E), and velocity (F) were evaluated for each PCI group against the respective sham (*$p < 0.05$, Two-way ANOVA with Fisher's LSD post test). Ns for sSham,sPCI,rSham,rPCI at each time point are as follows: baseline - 24,24,22,22; 1m – 24,24,22,22; 3m – 24,23,22,21; 6m – 23,23,19,20.

Experiment 1.2.4 Correlation Analysis:

To assess if clinically relevant metrics of concussion may have prognostic value for acute - chronic alterations in brain glucose metabolism, correlational analyses between significantly altered brain regions of FDG uptake and injury impact

factors, righting reflex times, and significantly altered gait parameters were performed.

No significant correlational relationships were obtained for single injury groups with any parameter assessed. For repeat injury groups, however, many significant correlations between acute concussion metrics and longitudinal FDG uptake alterations were obtained. For clarity and ease of interpretation, weak correlations ($-0.35 < r < 0.35$) have been omitted. Metrics which quantify the strength of the injury impact (Table 1) correlated significantly with acutely altered FDG-PET ROIs and with each hemisphere at 6 months post injury. Conversely, righting reflex (Table 2), which acts as a measure of loss of consciousness in the rat, correlated with acute through chronic changes in FDG uptake. Numerous significantly altered gait parameters detected at 2h post injury correlated with acutely (Table 3) and chronically (Table 4) altered FDG-PET ROIs.

FDG-PET ROI	Outcome Measure	Pearson r	p value
Olfactory Bulb (Ipsilateral) *24 Hour*	*Pressure (PSI)*		
	4th Hit	0.5598	0.0067 **
	3rd Hit	0.5515	0.0078 **
	SUM	0.4702	0.0315 *
Thalamus (Ipsilateral) *3 Day*	*Pressure (PSI)*		
	2nd Hit	-0.5499	0.008 **
	SUM	-0.5291	0.0137 *
	Force (lbs)		
	2nd Hit	-0.5385	0.0143 *
	3rd Hit	-0.4793	0.0325 *
Hemisphere (Contralateral) *6 Month*	*Force (lbs)*		
	4th Hit	0.7031	0.0158 *
	SUM	0.6480	0.0428 *
	Pressure (PSI)		
	2nd Hit	0.6179	0.0428 *
	SUM	0.6480	0.0428 *
Hemisphere (Ipsilateral) *6 Month*	*Force (lbs)*		
	4th Hit	-0.7029	0.0159 *
	SUM	-0.6477	0.0429 *
	Pressure (PSI)		
	2nd Hit	-0.6182	0.0426 *
	SUM	-0.6482	0.0426 *

Table 1: Significant results from two-tailed Pearson correlation analyses of injury impact factors with significantly altered FDG-PET ROIs. All data is from the repeat injury groups.

FDG-PET ROI	Outcome Measure	Pearson r	p value
Olfactory Bulb (Ipsilateral) *24 Hour*	*Righting Reflex (s)*		
	SUM	0.5822	< 0.0001 ****
	3rd Hit	0.5146	0.0004 ***
	4th Hit	0.5017	0.0005 ***
	2nd Hit	0.3719	0.0129 *
Cortex (Contralateral) *3 Month*	*Righting Reflex (s)*		
	SUM	0.4514	0.0065 **
Thalamus (Ipsilateral) *3 Month*	*Righting Reflex (s)*		
	4th Hit	-0.4742	0.004 **
	SUM	-0.4552	0.006 **
Thalamus (Ipsilateral) *6 Month*	*Righting Reflex (s)*		
	4th Hit	-0.5148	0.0101 *

Table 2: Significant results from two-tailed Pearson correlation analyses of righting reflex with significantly altered FDG-PET ROIs. All data is from the repeat injury groups.

FDG-PET ROI	Outcome Measure	Pearson r	p value
Olfactory Bulb (Ipsilateral) *24 Hour*	Average Speed	-0.4762	0.0022 **
	Duration	0.4216	0.0067 **
	Stand (RF)	0.4210	0.0068 **
	Stand (RH)	0.4053	0.0095 **
	Stand (LH)	0.3805	0.0154 *
	Step Cycle (RF)	0.4200	0.0070 **
	Step Cycle (LF)	0.3799	0.0156 *
	Step Cycle (RH)	0.3781	0.0162 *
	Step Cycle (LH)	0.3513	0.0262 *
	Swing Speed (LF)	-0.4138	0.0079 **
	Duty Cycle (RF)	0.4110	0.0084 **
	Stand Index (LH)	0.4076	0.0090 **
	Stand Index (RH)	0.3668	0.0216 *
	Single Stance (RF)	0.3692	0.0191 *
	Stride Length (RH)	-0.3679	0.0195 *
	Stride Length (LH)	-0.3544	0.0249 *
	Cadence	-0.3538	0.0251 *
Thalamus (Ipsilateral) *3 Day*	Swing Speed (RH)	0.4586	0.0029 **
	Swing Speed (RF)	0.3837	0.0145 *
	Swing Speed (LF)	0.3829	0.0148 *
	Step Cycle (LF)	-0.4020	0.0101 *
	Step Cycle (RH)	-0.3807	0.0154 *
	Step Cycle (LH)	-0.3785	0.0160 *
	Step Cycle (RF)	-0.3724	0.0179 *
	Cadence	0.3911	0.0126 *
	Stand (RF)	-0.3752	0.0170 *
	Stand (LH)	-0.3673	0.0197 *
	Stand (LF)	-0.3635	0.0211 *
	Swing (RF)	-0.3609	0.0221 *

Table 3: Significant results from two-tailed Pearson correlation analyses of gait parameters with acute FDG-PET ROIs. Only gait parameters and ROIs which were significantly different from sham controls were assessed for a correlational relationship. All data is from the repeat injury groups.

FDG-PET ROI	Outcome Measure	Pearson r	p value
Cortex (Contralateral) *3 Month*	Swing Speed (LH)	-0.4058	0.0156 *
	Swing (LH)	0.3878	0.0213 *
	Single Stance (RH)	0.3625	0.0324 *
Thalamus (Ipsilateral) *3 Month*	Swing Speed (LH)	0.4927	0.0026 **
	Swing Speed (RH)	0.3658	0.0307 *
	Stand Index (RF)	-0.4404	0.0081 **
	Swing (LH)	-0.4302	0.0099 **
	Stand (RF)	-0.3558	0.0359 *
White Matter (Ipsilateral) *3 Month*	Swing (RF)	-0.3596	0.0398 *
	Swing Speed (LH)	0.3509	0.0452 *
Thalamus (Contralateral) *6 Month*	Stride Length (LF)	-0.4347	0.0382 *

Table 4: Significant results from two-tailed Pearson correlation analyses of gait parameters with chronic FDG-PET ROIs. Only gait parameters and ROIs which were significantly different from sham controls were assessed for a correlational relationship. All data is from the repeat injury groups.

Subtask 1.3: Determine if acute brain glucose metabolism dysfunction following a single or repeat PCI correlates with chronic protein changes relating to CTE or neurodegenerative pathology (tau, tau phosphorylation, and amyloid precursor protein) using end-term protein analysis.

Experiment 1.3.1 Neurodegenerative Pathology:

The effect of PCI on the neurodegenerative markers amyloid beta and phosphorylated tau was evaluated. At 6 months following PCI, rats were perfused with 4% paraformaldehyde and brains were removed for evaluation by immunohistochemistry. Paraffin embedded coronal brain sections were stained with 6E10 (amyloid beta) and AT8 (phosphorylated tau) antibodies. Positive staining was quantified in both the ipsilateral and contralateral hemispheres. Results were summed across six slices per rat and analyzed as a percent of positive staining relative to total slice area.

At 6 months following PCI, preliminary analysis of the rSham and rPCI groups demonstrated no change in chronic neurodegeneration as detected by staining for amyloid beta and phosphorylated tau (Fig. 8). As a result, this experiment was not explored further with additional animals or in the sPCI group.

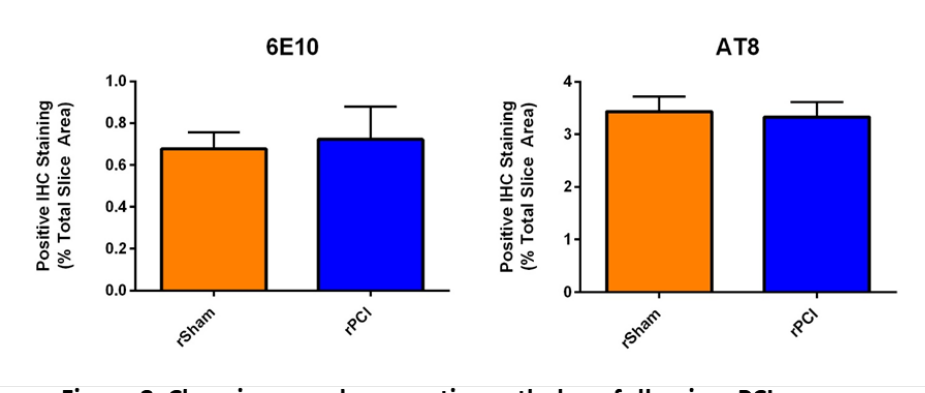


Figure 8: Chronic neurodegenerative pathology following rPCI. Immunohistochemistry staining for amyloid beta (6E10) and phosphorylated tau (AT8) were performed at 6 months following rPCI. Data is quantified as the percent of positive staining related to total slice area. No alterations between rSham and rPCI groups were observed (unpaired t-test). N = 3/group.

Experiment 1.3.2 Correlation Analysis:

Correlation analysis between neurodegenerative pathology markers and brain glucose metabolic dysfunction was not performed since no significant neurodegenerative pathology was observed following injury.

SOW Major Task 1 Summary and Conclusions:
Work for SOW Major Task 1 has been completed. The primary goal of SOW Major Task 1 was to characterize longitudinal alterations in brain glucose metabolism with FDG-PET imaging following single or repeated concussions induced with the WRAIR PCI model. Work through 3 month endpoints was previously reported in the Year 1 Annual Report. Assessment at 6 months has now been completed and demonstrated chronic alterations in FDG uptake which occurred in both ipsilateral and contralateral hemispheres. The data at 6 months post injury provide additional support to the previously reported conclusion that brain glucose uptake following PCI follows a pattern of acute hypermetabolism with hypometabolism prevailing chronically. This was especially apparent in the thalamus, where decreased uptake was observed in both the sPCI and rPCI groups both ipsilateral and contralateral to the injury impact location. Given the importance of this brain region in relaying sensory and motor signals to the cortex, future research into thalamic dysfunction may provide insight into the etiology of chronic concussion symptomology.

Secondary goals of SOW Major Task 1 included the characterization of neurobehavioral deficits and chronic neurodegenerative pathology after single and repeat PCI. Behavioral deficits through 3 months post injury were previously reported in the Year 1 Annual Report. At 6 months post injury, no alterations were observed in the spatial learning and probe trials of the MWM or in behavior in the EPM. In the working memory MWM, while both PCI groups took longer to find the hidden platform in individual trials, no changes were seen in working memory when assessing the difference between trial 1 and trial 2. In considering all behavioral deficits from 24h – 6m, PCI resulted in acute neurobehavioral deficits but minimal changes in anxiety and cognitive performance at 1m and 3m following injury. By 6m, all behavioral deficits had resolved. Additionally, at 6m post injury, no evidence for chronic neurodegenerative pathology was observed in rPCI rats compared to rSham rats.

<u>SOW Major Task 2:</u> Determine if acute changes in serum miRNA biomarkers have prognostic value for deficits in longitudinal behavioral outcome measures and CTE related neuropathology following single or repeat PCI.

Work for SOW Major Task 2 is underway. Four different study groups will be included in this Task: sSham, sPCI, rSham, and rPCI. Injuries will be induced using the modified PCI device, which has previously been described in great detail (Leung, Larimore et al. 2014). All experimental tasks for SOW Major Task 2 will occur at Site 1 (WRAIR; PI: Dr. Deborah Shear). miRNA characterization of sSham and sPCI is complete; characterization of rSham and rPCI has been delayed due to issues with commercial product availability. These issues are now resolved and work is currently in progress.

Subtask 2.1: Determine the acute serum miRNA biomarker change profiles following single or repeat PCI.

Acute – subacute serum miRNA biomarkers have been profiled in sSham and sPCI groups at 4h, 24h, 3d, 7d, and 14d following injury. The mirVana miRNA isolation kit (ThermoFisher) was utilized to isolate miRNA from serum samples. Resulting miRNA was reverse transcribed into cDNA and preamplified using Megaplex Rodent Primer Pools A&B (ThermoFisher). Preamplified products were loaded onto TaqMan OpenArray Rodent microRNA Panels for subsequent real-time PCR using the QuantStudio 12K Flex system. Data was analyzed with Expression Suite software and Thewrmofisher cloud based RQ application.

The OpenArray Rodent miRNA panel assesses 750 rodent miRNA sequences for each study sample, providing a discovery based platform to identify altered miRNAs following injury. Volcano plots for each time point demonstrated that PCI significantly upregulated and downregulated miRNAs at 4h, 24h, and 3d compared to sSham (**Fig. 9**). By 7d, no significantly altered miRNAs were identified. Data at 14d post injury also indicated no significant changes as a consequence of sPCI (data not shown). Details regarding the altered miRNAs and their corresponding fold changes are presented in **Table 5**.

Serum microRNA data from repeat PCI samples (2h and 2 days post Injury) are shown in Volcano plots (**Fig. 10** A and B) All the data is normalized to the corresponding sham groups. Detailed list of altered microRNAs are described in **Table 6**

Serum microRNAs profiling from a combined insult with repeat PCI and hypoxemia and hemorrhagic shock (Polytrauma) was also pursued as an additional undertaking.The results are shown in (**Fig. 11** A and B) and the details of the altered microRNAs are shown in **Table 7**

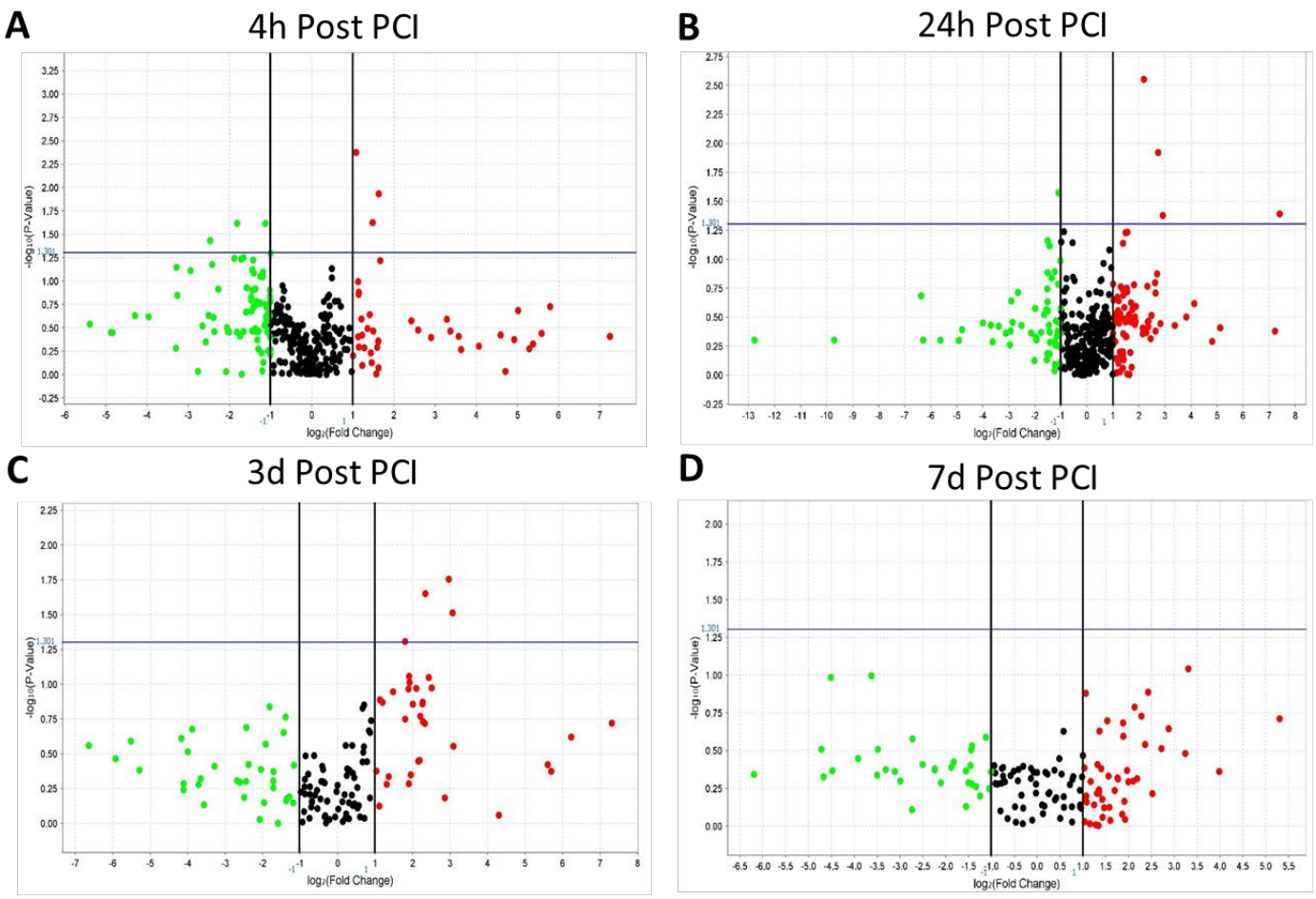
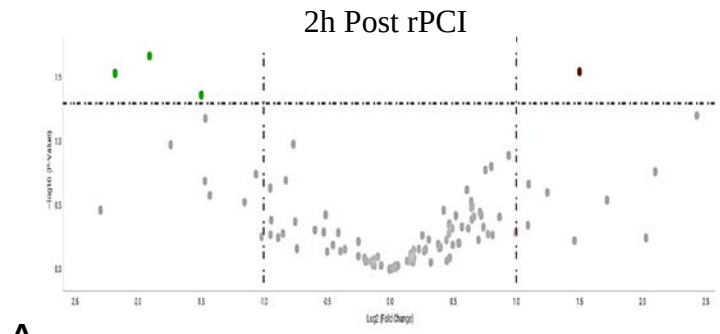


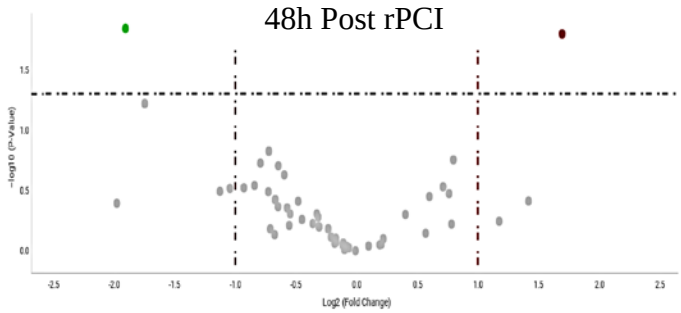
Figure 9: Volcano plots of serum miRNAs. Serum miRNA was assessed at 4h (A), 24h (B), 3d (C), and 7d (D) following sPCI. Vertical lines represent 2 fold increase or decreases. Each dot represents an individual miRNA (green = decreased, black = unchanged, red = increased). The horizontal blue line denotes level of significance, with any dot above this line being statistically significant compared to sham rats. miRNAs which fall in the upper left region are significantly decreased, while miRNAs in the upper right region are significantly increased. N = 10/group and time point.

Time Post Injury	miRNA	log2(Fold Change)	p value
4 hours	miR-350	-2.47	0.037 *
	miR-351	-1.81	0.024 *
	miR-193b	-1.13	0.024 *
	miR-335#	1.08	0.004 **
	miR-1897-3p	1.47	0.024 *
	miR-183#	1.63	0.012 *
24 hours	miR-1897-3p	-1.08	0.027 *
	miR-Let-7f	2.18	0.003 **
	miR-190	2.74	0.012 *
	miR-350	2.93	0.041 *
	miR-199a	7.40	0.042 *
3 days	miR-30a-5p	1.80	0.049 *
	miR-192	2.30	0.022 *
	miR-412	3.00	0.031 *
	miR-150	3.00	0.018 *

Table 5: Detailed list of significantly altered miRNAs following sPCI as presented in Figure 9.



A



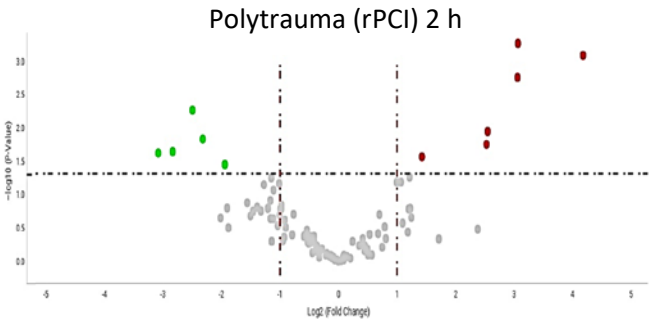
B

Fig. 10: Significant target fold changes for repeat PCI at A) 2 hours and B) 48 hours post injury. Targets with significant fold changes are indicated by a red or green dot, representing up-regulation and down-regulation, respectively. Vertical dotted lines indicate cut off for threshold values for Fold Change (±1.0) and horizontal dotted line indicate $\log_{10}$ of p-value (0.05).

Time Point	Target	Log2(Fold Change)	P-value
rPCI, 2 hours	miR-322	1.5	0.03
	miR-101b	-2.2	0.03
	miR-138	-1.9	0.02
	miR-206	-1.5	0.04
rPCI, 48 hours	miR-872-3p	1.7	0.02
	miR-20b	-1.9	0.01

Table 6: Detailed list of significant microRNAs, as presented in Fig.10

C



D

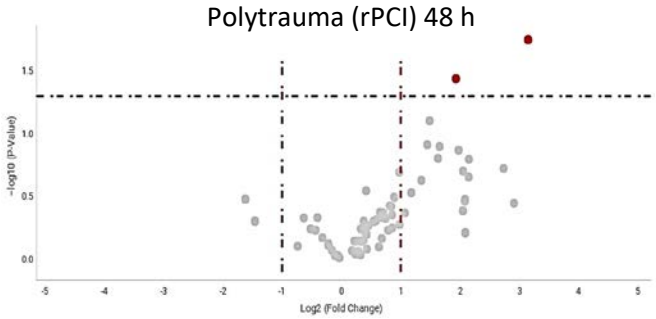


Fig. 11: Significant target fold changes for repeat PCI with hypoxemia and hemorrhagic stroke at C) 2 hours and D) 48 hours post injury. Targets with significant fold changes are indicated by a red or green dot, representing up-regulation and down-regulation, respectively. Vertical dotted lines indicate cut off for threshold values for Fold Change (±1.0) and horizontal dotted line indicate $\log_{10}$ of p-value (0.05).

Time Point	Target	FLog2 (Fold Change)	P-value
rPCI+HH, 2 hours	U6	4.2	0.00
	mmu-miR-685	3.1	0.00
	hsa-miR-192	3.1	0.00
	mmu-miR-429	2.6	0.01
	hsa-miR-122	2.5	0.02
	mmu-miR-2134	1.4	0.03
	mmu-miR-1937C	-3.0	0.02
	mmu-miR-1937B	-2.8	0.02
	hsa-miR-425	-2.5	0.01
	hsa-miR-138	-2.3	0.02
	hsa-miR-20b	-1.9	0.04
rPCI+HH, 48 hours	hsa-miR-133a	3.1	0.02
	mmu-miR-872-3p	2.0	0.04

Table 7: Detailed list of significant microRNAs, as presented in Fig.11

Subtask 2.2: Determine if acute miRNA biomarker profiles correlate with changes in established acute, subacute and chronic behavioral outcomes following single or repeat PCI.

Since limited positive findings with the proposed acute, subacute, and chronic behavioral outcome measures were observed following PCI in subtask 1.2, this experiment was not pursued.

Subtask 2.3: Determine if acute miRNA biomarker profiles following a single or repeat PCI correlates with chronic protein changes relating to CTE or neurodegenerative pathology (tau, tau phosphorylation, and amyloid precursor protein) using end-term protein analysis.

Since no positive findings in chronic protein changes following PCI as determined by end-term protein analysis were seen in Subtask 1.3, this experiment was not pursued.

SOW Major Task 2 Summary and Conclusions:

Work for SOW Major Task 2 is in progress. Characterization of serum miRNA profiles following single concussion injuries in the rat have been completed through 14 days following injury. Experiments with the rSham and rPCI groups have been delayed due to issues with product availability, however, these issues have been resolved. Additional time is requested to complete miRNA profiling work following repeat PCI injuries.

Significantly altered serum miRNAs as a consequence of injury were identified at 4 hours, 24 hours, and 3 days following sPCI. By 7 and 14 days post injury, no significantly altered serum miRNAs were identified. This indicates that altered serum miRNA profiles following mild concussion injury are detectable acutely after injury but resolve in the subacute period in rodents. Thus, the use of miRNAs as serum biomarkers to detect injury may be best suited for acute use, although it should be noted that altered serum miRNAs have been identified as late as 15 days post injury in human TBI patients with additional extracranial injuries (Di Pietro, Ragusa et al. 2017).

Upregulated and downregulated miRNAs were identified in this study, which is consistent with previous studies profiling miRNAs following TBI in both rodents and humans (Sharma, Chandran et al. 2014, Di Pietro, Ragusa et al. 2017). Both miR-350 and miR-1897-3p were altered at 4 and 24 hours. Interestingly, these miRNAs were differentially up or downregulated depending upon the time point assessed. Should similar patterns be discernable in human TBI patients, whether these candidate miRNAs are upregulated or downregulated may have utility in determining time post injury. The current data do not inform if miRNA dysregulation following TBI is be dependent upon injury severity or number of

injury impacts sustained. Thus, completion of serum miRNA profiling experiments following rPCI will highlight discrepancies in miRNA expression based upon injury severity. This information will be of importance in moving forward with the validation of target miRNAs.

c. What opportunities for training and professional development has the project provided?

Nothing to report.

d. How were the results disseminated to communities of interest?

Selected results from SOW Major Task 1 were presented in poster format at the 2018 International National Neurotrauma Symposium held in Toronto, Canada. Details of this presentation may be found in Section 6 (Products) of this report.

e. **What do you plan to do during the next reporting period to accomplish the goals?**

In the next reporting period, we will finish the proposed characterization of miRNA profiles following repeat PCI. Collection of these study samples is currently underway.

4. IMPACT:

a. What was the impact on the development of the principal discipline(s) of the project?

The brain regions identified in this project as being sensitive to glucose metabolic dysregulation through six months following concussive injury will inform future pre-clinical and clinical studies that examine metabolic disturbances following mTBI. The general pattern of acute cerebral hypermetabolism and chronic hypometabolism detected following PCI indicates that robust comprehensive clinical studies are needed following concussion injury to clearly define patterns of brain glucose metabolism since the clinical studies (typically with small numbers of participants) demonstrate an overall pattern of hypometabolism. Additionally, the miRNAs identified as sensitive to change following sPCI suggest useful targets for investigation following human concussion injuries.

b. What was the impact on other disciplines?

Nothing to report.

c. What was the impact on technology transfer?

Results from SOW Major Task 1 demonstrating chronic disruptions in brain glucose metabolic activity, in conjunction with findings from future preclinical and clinical studies to better define these changes, may impact the implementation and duration of use of FDG-PET imaging clinically following concussion. Additionally, results from SOW Major Task 2 that identify specific alterations in serum miRNA following PCI may aid in the identification of appropriate targets to include in a blood based biomarker assay to detect concussion in humans.

d. What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS:

a. Changes in approach and reasons for change

Subtasks 2.2 and 2.3 were not pursued. This decision was based upon relevant results from Subtasks 1.2 and 1.3. These data indicated that PCI resulted in limited or no positive findings in the behavioral outcome metrics or in chronic protein changes as proposed, thus the same experiments were not repeated for Task 2.

b. Actual or anticipated problems or delays and actions or plans to resolve them

Delays were encountered in microRNA work for Task 2 due to product backorders. This issue has been resolved with the vendor and work is underway to ensure study completion.

c. Changes that had a significant impact on expenditures

Nothing to report.

d. Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report.

e. Significant changes in use or care of human subjects

Nothing to report.

f. Significant changes in use or care of vertebrate animals.

Nothing to report.

g. Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

  Journal publications.

  Nothing to report.

  Books or other non-periodical, one-time publications.

  Nothing to report.

  Other publications, conference papers, and presentations.

  DeDominicis KE, Jaiswal S, Cardiff K, Leung LY, Pan H, Hoy A, Knutsen A, Dardzinski B, Shear D. (2018) Repeat concussions with or without concomitant polytrauma alter acute physiology and cerebral glucose uptake in rats. Poster presentation. 3rd International Neurotrauma Symposium in Toronto, Canada.

- **Website(s) or other Internet site(s)**

  Nothing to report.

- **Technologies or techniques**

  Nothing to report.

- **Inventions, patent applications, and/or licenses**

  Nothing to report.

- **Other Products**

  This project has demonstrated that the previously established WRAIR PCI model of mild head trauma captures the chronic metabolic depression which has previously been described in clinical patients following brain injury, thus supporting its use as an effective animal model in which to study this phenomenon. This work has also identified target microRNAs for validation as blood based biomarkers of single concussion injuries. These target microRNAs may be useful in the determining the diagnosis, prognosis, and treatment of concussive injuries.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

a. What individuals have worked on the project?

Name:	Deborah Shear
Project Role:	Principal Investigator (Site 1)
Research Identifier:	
Nearest person month worked:	2
Contribution to Project:	Data Analysis; Reporting
Funding Support:	CCCRP

Name:	Kristen DeDominicis
Project Role:	Associate Investigator
Research Identifier:	
Nearest person month worked:	6
Contribution to Project:	PCI Injuries; Behavioral Assessments; Data Analysis; Reporting
Funding Support:	CCCRP

Name:	Bernard Srambical Wilfred
Project Role:	Associate Investigator
Research Identifier:	
Nearest person month worked:	6
Contribution to Project:	microRNA data generation, data analysis; Reporting
Funding Support:	CCCRP

Name:	Savannah Barannikov
Project Role:	Research Associate
Research Identifier:	
Nearest person month worked:	6
Contribution to Project:	Data Analysis; Reporting
Funding Support:	CCCRP

Name:	Katherine Cardiff
Project Role:	Research Associate
Research Identifier:	
Nearest person month worked:	2
Contribution to Project:	PCI Injuries; Behavioral Assessments
Funding Support:	CCCRP

Name:	Shalini Jaiswal
Project Role:	Research Associate
Research Identifier:	
Nearest person month worked:	2
Contribution to Project:	PET/CT Imaging; Data Analysis

Funding Support:

Name:	Bernard Dardzinski
Project Role:	Principal Investigator (Site 2)
Research Identifier:	
Nearest person month worked:	
Contribution to Project:	PET/CT Imaging; Data Analysis; Reporting
Funding Support:	

b. Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Dr. Kristen DeDominicis (Associate Investigator) has left WRAIR/BTNN.

c. What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

Not applicable.

9. APPENDICES

References

Di Pietro, V., M. Ragusa, D. Davies, Z. Su, J. Hazeldine, G. Lazzarino, L. J. Hill, N. Crombie, M. Foster, M. Purrello, A. Logan and A. Belli (2017). "MicroRNAs as Novel Biomarkers for the Diagnosis and Prognosis of Mild and Severe Traumatic Brain Injury." J Neurotrauma **34**(11): 1948-1956.

Leung, L. Y., Z. Larimore, L. Holmes, C. Cartagena, A. Mountney, Y. Deng-Bryant, K. Schmid, D. Shear and F. Tortella (2014). "The WRAIR projectile concussive impact model of mild traumatic brain injury: re-design, testing and preclinical validation." Ann Biomed Eng **42**(8): 1618-1630.

Sharma, A., R. Chandran, E. S. Barry, M. Bhomia, M. A. Hutchison, N. S. Balakathiresan, N. E. Grunberg and R. K. Maheshwari (2014). "Identification of serum microRNA signatures for diagnosis of mild traumatic brain injury in a closed head injury model." PLoS One **9**(11): e112019.