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PRINCIPAL INVESTIGATOR: Dr. Deborah Shear

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Dr. Deborah A Shear (PI)
Email: deborah.a.shear.civ@mail.mil

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The Geneva Foundation
917 Pacific Ave, Suite 600
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14. ABSTRACT

Closed head concussion is of significant concern to both military and civilian medicine. 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. This 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 Close Out Report, we provide a summary of all results/deliverables from experiments conducted under this project.

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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, 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/mm3 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.

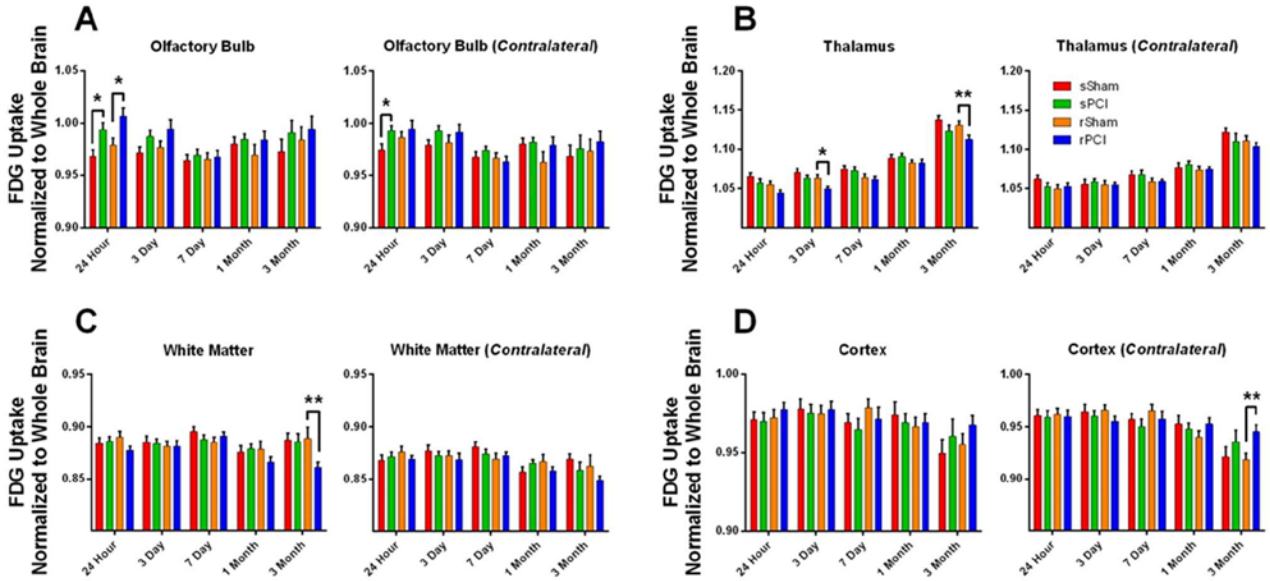


Figure 1: PCI alters FDG uptake. Longitudinal changes in FDG uptake were evaluated following PCI (A-D). Regions are presented here if at least one significant alteration was observed in either PCI group at any time point (* $p < 0.05$, ** $p < 0.01$ against respective sham control; Two-way ANOVA with 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.

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*: Immediately following each PCI impact, rats were returned to their home cage in the supine position and the time to return to an upright position, or righting reflex, was recorded. Rats in the sPCI group had significantly greater righting reflex times than those in the sSham group ($p < 0.01$, Fig. 3A). For repeat injury groups, righting reflex times were assessed after each

sequential 1h impact. While the mean time to right was increased following the first PCI impact over sham control, this did not reach statistical significance ($p = 0.056$, Fig. 3B). Following the 2nd, 3rd, and 4th sequential PCI impacts, the mean time to right was significantly increased over the corresponding sham control anesthesia administration (2nd impact: $p < 0.01$, 3rd and 4th impacts: $p < 0.05$; Fig. 3B).

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

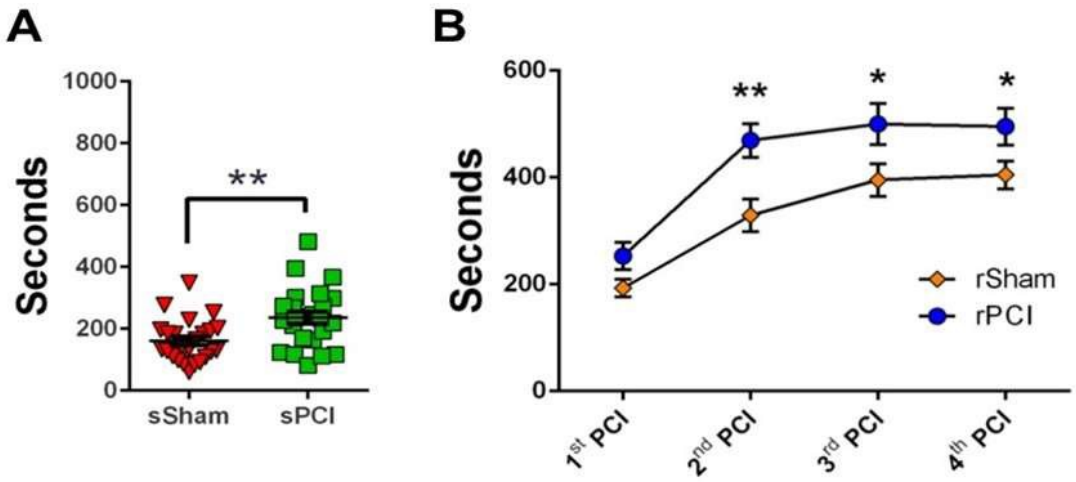


Figure 3: PCI increases righting reflex Time to regain righting reflex was recorded following each PCI injury or sham control manipulation for both the single injury group (A) and repeat injury group (B). Values for each individual rPCI impact were compared against the matched rSham impact (* $p < 0.05$, ** $p < 0.01$). Statistical significance evaluated against the respective sham control by an unpaired t-test (A) or two-way ANOVA with Fisher's LSD post test (B). N = 24,24,22,22 for sSham,sPCI,rSham,rPCI, respectively.

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. 3).

Gait Analysis: Rats were subjected to the automated gait analysis task at baseline, 2h, 2d, and 1m after injury using The CatWalk Automated Gait Analysis System (Noldus Information Technology, Leesburg, VA) as previously described (Mountney et al., 2013). Briefly, following acclimation to a darkened goal box (5 min), rats completed trial runs across a glass walkway towards the goal box. A camera positioned underneath the walkway recorded illuminated pawprints resulting from direct contact between the paws and glass surface, which were digitized for processing and analysed using the CatWalk XT 9. 55 different gait parameters were assessed. Limited gait alterations

were observed at baseline, 2d, and 1m after injury while robust injury effects were seen at 2h. As such, only the 2h data is presented here.

At 2h, gait dysfunction compared to matched sham controls was detected in all four paws (RF, RH, LF, LH) at both injury severities. The data indicate a greater number of significantly altered parameters and larger percent changes compared to sham controls in the rPCI group than the sPCI group. 26/55 analyzed gait parameters were significantly altered following sPCI compared to sSham ($p < 0.05$) while after rPCI, 33/55 parameters were significantly altered from rSham ($p < 0.05$). The significant differences are presented as percent change from the appropriate sham control for both the sPCI and rPCI injury groups (Fig. 4A-C, non-significant parameters not shown).

Overall, dynamic gait parameters revealed that PCI animals moved more slowly than their corresponding sham controls (Fig. 4A). Temporal parameters indicated that injured

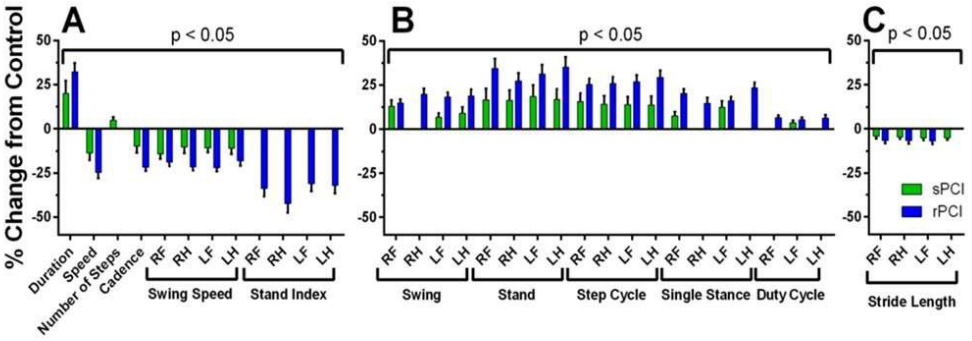


Figure 4: PCI induces acute gait dysfunction Analysis of dynamic (A), temporal (B), and static (C) gait analysis parameters 2 hours following injury reveals significant injury effects in both the single and repeat PCI groups. Values are presented as a percent change in each injury group from the appropriately matched sham control. Statistical significance was determined using raw data values; only parameters which were significant in either PCI group are presented here ($p < 0.05$ from respective sham, unpaired t-test). N = 24,24,20,20 for sSham,sPCI,rSham,rPCI, respectively.

rats spent more time moving through individual gait components compared to matched sham controls (Fig. 4B). Static paw positioning parameters revealed few differences between injured and uninjured rats (Fig. 4C). No injury effects were seen in parameters that examine inter-limb coordination.

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. At 6m, no chronic deficits or sPCI or rPCI were observed as detected by the NSS-R. All NSS-R results were provided in the Year 1 & 2 Annual Reports.

Experiment 1.2.2 Memory Assessments:

Memory assessments were performed at 1 and 3 months after injury using the Morris water maze (MWM) task (Noldus EthoVision XT) with a video-tracking system. Performance on these tasks is also being evaluated at 6 months but is not yet complete in all cohorts. 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.

The acquisition trials of the spatial learning MWM task revealed no significant injury effect in the latency to find the hidden platform compared to matched sham controls at any time point (Fig. 5). Thigmotaxic behavior (perimeter swimming) significantly increased in the sPCI compared to sSham group on the first acquisition trial one month following injury ($p < 0.05$, Fig. 6A) but significantly decreased in the rPCI group compared to rSham in the second acquisition trial at 3 months post-injury ($p < 0.05$, Fig. 6B). Both sPCI and rPCI groups had significantly lower mean thigmotaxic scores during the acquisition trials at 3 months after injury ($p < 0.001$, Fig. 6E) compared to their respective sham controls. No other differences in thigmotaxic swimming behavior were observed. Surprisingly, at 1m, sPCI rats spent significantly more time in the probe trial platform quadrant than sSham animals ($p < 0.001$, Fig. 7A). No other differences in memory retention were observed between injury groups.

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.

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. Correlational analyses between regions of altered brain glucose metabolism and chronic behavioral deficits will be performed following completion of the 6 month behavioral experiments.

No significant correlational relationships were obtained for single injury groups with any parameter assessed. ***For repeat injury groups, however, numerous 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 but did not correlate with chronic alterations in glucose uptake. 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 *

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*	Average Speed	-0.4762	0.0022 **
	Duration	0.4216	0.0067 **
	Stand (RF)	0.421	0.0068 **
	Stand (RH)	0.4053	0.0095 **
	Stand (LH)	0.3805	0.0154 *
	Step Cycle (RF)	0.42	0.007 **
	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)	0.411	0.0084 **
	Stand Index (LH)	0.4076	0.009 **
	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)	-0.402	0.0101 *
	Step Cycle (RH)	-0.3807	0.0154 *
	Step Cycle (LH)	-0.3785	0.016 *
	Step Cycle (RF)	-0.3724	0.0179 *
	Cadence	0.3911	0.0126 *
	Stand (RF)	-0.3752	0.017 *
	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
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 **

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
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 *

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.

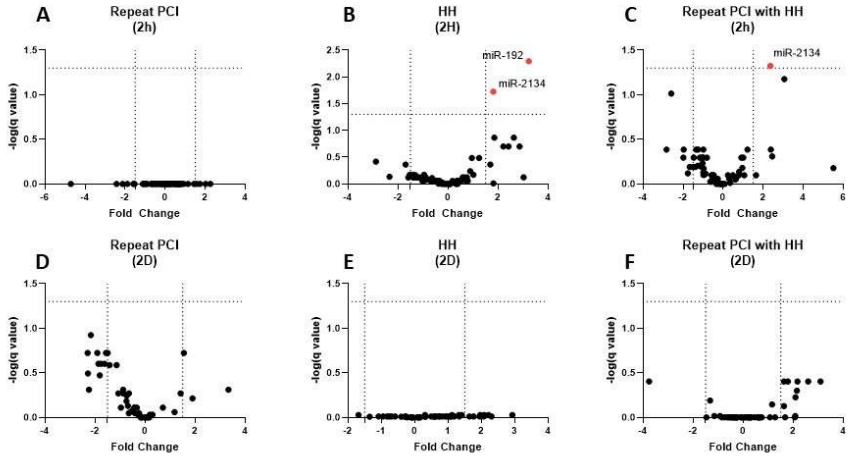
At 6 months following PCI, preliminary analysis of the rSham and rPCI groups demonstrated no change in chronic neurodegeneration as detected by staining for amyloid beta and phosphorylated tau (Fig. 8). As a result, this experiment was not explored further with additional animals or in the sPCI group.

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.

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.

All live animal work for SOW Major Task 2 is 100% completed. In the original experimental design, four different study groups were included in this Task: sSham, sPCI, rSham, and rPCI. Injuries were 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 occurred at Site 1 (WRAIR; PI: Dr. Deborah Shear). miRNA characterization of sSham and sPCI, and of rSham and rPCI has been completed. In addition, because initial results showed only modest trends, additional animals (per group) and additional groups (polytrauma) and (PCI+polytrauma) were added as part of a Year 3 no-cost-extension to determine whether exposure to polytrauma would exacerbate the effects of concussion on FDG-PET imaging data (Site 2) and serum miRNA profiles. Results on serum miRNA profiles were provided in Annual Report 3 (see Figure 5 below). Overall, results showed that miRNAs were more significantly altered following rPCI vs. sPCI (Fig. 5 A, D). Conversely, following polytrauma alone, miRNA signaling was altered at the 2h post-injury time point but not at 2 days post-injury. However, in animals that received both PCI and polytrauma, miRNA changes were detected at both post-injury time points. Notably, miR-2134 is a pre-synaptic/astrocytic microRNA that is involved in neural plasticity. The finding that we see an elevation of this microRNA level at 2h post injury is an injury specific observation, rather than an actual gene expression modulation. It may be that the synaptic shear forces exerted by concussion is causing the release of this microRNA to the blood. miR-192 on the other hand is in involved in blood coagulation and venous thrombosis (PMID: 32160777).



Target	Fold Change	P value	t ratio	df	q value
mmu-miR-2134	2.359	0.000587	4.337	15.00	0.047451

Figure 5. Volcano plots showing alterations in miRNA at 2 hours (top row) and 48 hours post-injury. Inserts shows fold changes for combined PCI+HH (polytrauma) injury effects.

Subtask 2.1: Determine the acute serum miRNA biomarker change profiles following single or repeat PCI occurring alone or in conjunction with induced hypoxemia and hemorrhagic shock (HH = polytrauma).

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.

Experiment 2.2.1 Sensorimotor Assessments: This experiment assessed sensorimotor function following PCI with the following tasks: Righting Reflex immediately after injury; CatWalk gait analysis at 2h post injury, and the Neurological Severity Scale – Revised (NSS-R) at 48 hours, 3 months, and 6 months post injury.

Experiment 2.2.2 Memory Assessments: The Morris water maze task data collected in Exp. 1.2.2 was used to evaluate potential correlations between memory dysfunction and miRNA profiles at 1 and 3 months following injury. No significant correlations were detected.

Experiment 2.2.3 Anxiety and Motivation: The elevated plus maze task results from Exp 1.2.3 were used to determine potential correlations between anxiety and motivation at 1, 3, and 6 months post injury. No significant correlations were detected.

Experiment 2.2.4 Correlation Analysis: Correlation analyses between acute serum miRNA and behavioral outcome metrics following PCI were evaluated. No significant correlations were detected between these outcome metrics.

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.

Experiment 2.3.1 Neurodegenerative Pathology: Chronic alterations in proteins related to neurodegenerative disease pathology, such as tau, phosphorylated tau, and amyloid precursor protein, will be evaluated following PCI. Histopathological processing was delayed due to shutdowns resulting from the pandemic. Brain specimens are currently being processed by FuDu technologies and we anticipate the analysis of those sections will be completed by the end of the current NCE

Experiment 2.3.2 Correlation Analysis: Correlation analyses between acute serum miRNA profiles and protein hallmarks of neurodegeneration following PCI will be evaluated. Animals were divided into 6 groups (n=6/group): Sham, Hemorrhage and Hypoxia (HH60), single projectile concussive injury (sPCI), sPCI+HH60, repeated PCI (rPCI), and rPCI+HH60. Serum was collected from each animal at 2 hours and 24 hours after injury. A biomarker panel (GFAP, UCHL1, NF-L, tau) was conducted on serum samples using the SIMOA HD-1 Neurology 4-plex assay.

Our results (Figure 6 below) suggest that hemorrhage and hypoxia play an important role in the biomarker levels. In the absence of TBI, hemorrhage and hypoxia increase GFAP and UCHL1 at 24 hours after injury. sPCI and rPCI increased NF-L at 2 hours but we did not observe any changes to GFAP, UCHL1, or tau. PCI also caused no changes

at the 24 hour time point. When hemorrhage and hypoxia occur as a comorbidity to TBI, the biomarker levels increased significantly. GFAP, NF-L, and UCHL-1 increased significantly at 2 hours with GFAP and UCHL1 levels remaining increased at 24 hours. These results demonstrate that extracranial injuries exacerbate biomarker levels and may augment neuropathology.

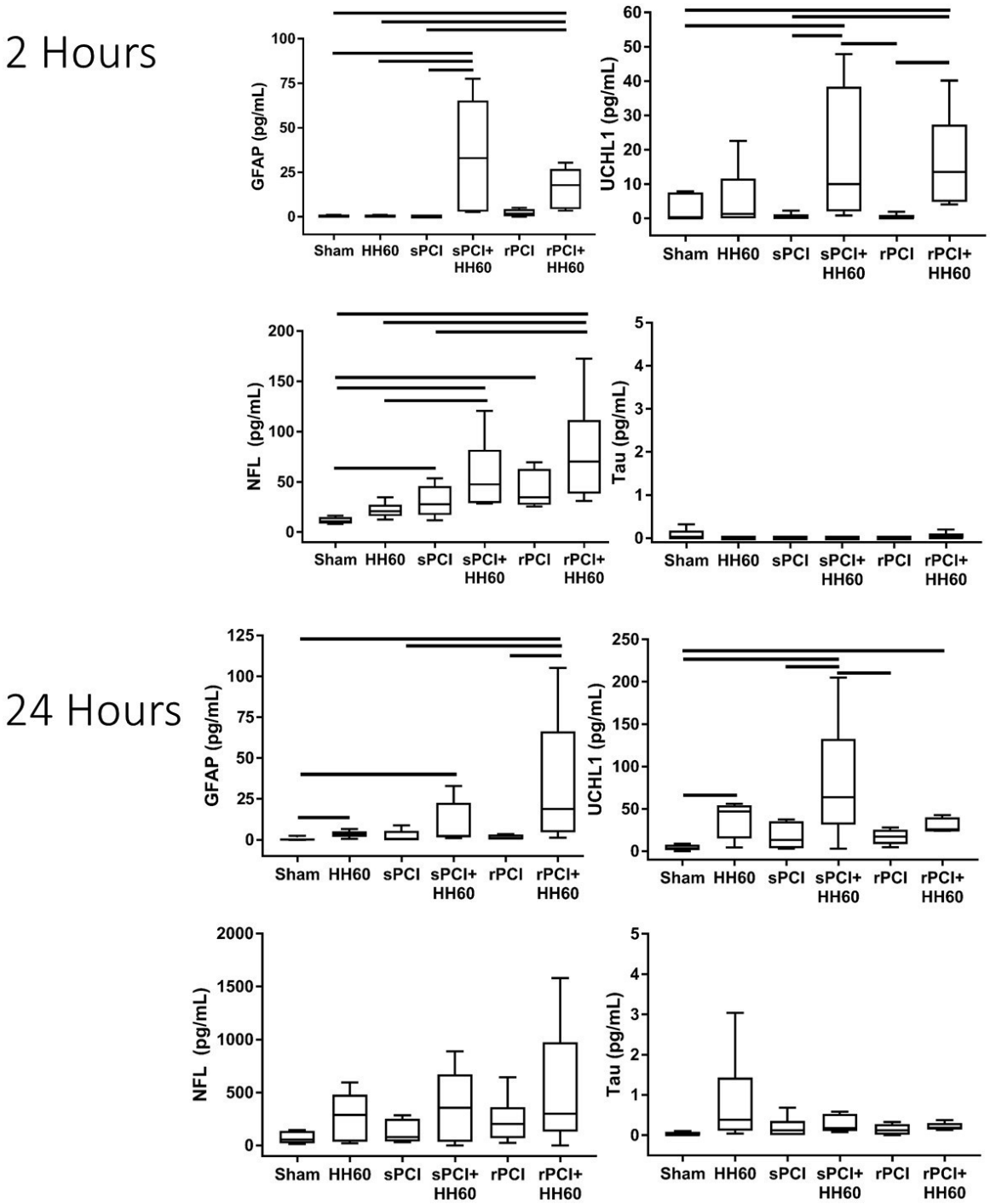


Figure 6. Box plots showing serum levels for GFAP, UCHL1, NFL, and Tau at 2 hours and 24 hours post-injury.

SOW Major Task 2 Summary and Conclusions: There are no results or conclusions to present at this time for SOW Major Task

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 and 2 were presented in poster format at the 2017 – 2019 National Neurotrauma Symposiums. Details of this presentation may be found in Section 6 (Products) of this report. Additionally, a manuscript to disseminate the results of SOW Major Task 1 has been submitted for publication in a peer reviewed journal.

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

N/A

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 following concussive injury will inform future pre-clinical and clinical studies that examine metabolic disturbances following mTBI. The data generated from this study thus far also highlight the potential importance of acquiring a baseline PET imaging scan to assess changes after injury on an individual basis. This is an important consideration in the design of future preclinical imaging studies.

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 future preclinical and clinical studies to

better define these changes, may impact the usage and duration of use of FDG-PET imaging clinically following concussion.

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

Nothing to report.

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

Nothing to report.

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.**

  See Appendix A.

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

Nothing to report.

Other publications, conference papers, and presentations.

See Appendix B

- **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.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

a. What individuals have worked on the project?

Name:	Dr. 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:	Dr. Bernard Wilfred
Project Role:	Associate
Investigator Research Identifier:	
Nearest person month worked:	2
Contribution to Project:	PCI Injuries; Behavioral Assessments; Data Analysis; Reporting
Funding Support:	CCCRP
Name:	Dr. Angela Boutte
Project Role:	Associate
Investigator Research Identifier:	
Nearest person month worked:	2
Contribution to Project:	Data Analysis;
Reporting Funding Support:	The Geneva

Foundation

Name:	Dr. Zachary Bailey
Project Role:	Research
Associate Research Identifier:	
Nearest person month worked:	4
Contribution to Project:	PCI – Polytrauma Injuries; Serum biomarkers; Behavioral Assessments
Funding Support:	CCCRP

Name:	Katherine Cardiff
Project Role:	Research
Associate Research Identifier:	
Nearest person month worked:	2
Contribution to Project:	PCI – Polytrauma Injuries; Serum biomarkers; Behavioral Assessments
Funding Support:	The Geneva Foundation

Name:	Dr. 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?

Nothing to report.

c. What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

Not applicable.

9. APPENDICES

Appendix A: Manuscript Draft
Appendix B: Poster Presentations

APPENDIX A

Cerebral glucose uptake is altered following repeated exposure to repeated concussion

Bernard J. Dardzinski[2, 3], Shalini Jaiswal[2], Andrew Knutsen[2], Kristen DeDominicis[1], Hongna Pan[2], Lai Yee Leung[2], Nathanael Allison[2,3], Mark D. Whiting[2], and Deborah Shear[1]

1. Brain Trauma Neuroprotection Program, Center for Military Psychiatry and Neuroscience, WRAIR, Silver Spring, MD
2. Translational Imaging Core, CNRM, USUHS, Bethesda, MD
3. Radiology and Radiological Sciences, USUHS, Bethesda, MD

Corresponding Author
Deborah A. Shear, PhD
Director, Brain Trauma Neuroprotection
Walter Reed Army Institute of Research
503 Robert Grant Ave, Silver Spring MD 20910

Key Words: traumatic brain injury, concussion, extracranial trauma, polytrauma, hypoxemia, hemorrhagic shock

Abstract

INTRODUCTION: Extracranial trauma, including hypoxemia and hemorrhagic shock (HS), play a role in the management of traumatic brain injury (TBI), especially on the battlefield. A number of studies indicate that TBI combined with extracranial injury may produce outcomes worse than those seen from TBI alone, yet the precise mechanisms by which extracranial insults influence outcome are unclear. Extracranial trauma may produce abnormal metabolic demands on the brain above and beyond that seen by TBI alone, thereby exacerbating the effects of injury. While many studies have examined these factors in moderate to severe TBI, the role of extracranial insult combined with repetitive, mild TBI (rmTBI) has not been well studied. The current study was designed to characterize the brains metabolic response to rmTBI, with or without combined hypoxemia and HS, using [^{18}F]FDG-PET imaging.

METHODS: Adult, male rats underwent rmTBI, with or without combined hypoxemia and HS, or repeat sham procedure as controls. Three rmTBIs were administered at 5 min intervals using the WRAIR projectile concussive impact device, a non-invasive method of studying blunt impact injury to the brain. Sixty minutes of transient hypoxemia was induced 15 min following the last rmTBI, followed by 5 min of normoxia, and then a 60 min period of hemorrhagic shock induced by tail vein bleeding. Physiological monitoring of arterial

pressure, heart rate, and breathing rate were recorded throughout all procedures. Serial [^{18}F]FDG-PET/CT scans were obtained at baseline prior to injury and at day 1 and 7 after injury using a Siemens Inveon preclinical scanner.

RESULTS: rmTBI altered acute physiology, yet this effect was not significantly changed with combined hypoxemia and HS. Significant changes in mean arterial pressure were noted with the hypoxemia and HS group without rmTBI. rmTBI alone produced significant acute (day 1) changes in brain glucose metabolism in the caudate putamen, while hypoxemia and HS alone produced significant metabolic changes in the mamillary nucleus and primary somatosensory cortex. rmTBI combined with hypoxemia and HS produced the most widespread metabolic changes, with numerous brain regions showing altered glucose metabolism at day 1 post-injury. By day 7 post-injury, none of the groups showed sustained alterations in brain glucose metabolism as measured by FDG-PET.

CONCLUSION: These findings suggest a complex metabolic response to rmTBI combined with hypoxemia and HS that can be detected early after injury using FDG-PET imaging. The widespread and dynamic changes in brain metabolism suggest a possible synergistic effect of rmTBI and extracranial injury compared to those injuries alone. Future studies are needed to determine the eventual effects on neuropathological and functional outcome. Overall, these results provide promise for understanding and eventually treating the metabolic dysfunction seen in response to combined rmTBI and extracranial trauma.

Introduction

Extracranial trauma plays an important role in the pathophysiology of traumatic brain injury (TBI) and has been associated with increased morbidity and mortality in human TBI patients.[1] A number of clinical and experimental studies indicate that extracranial trauma may exacerbate the effects of TBI and increase the vulnerability of the brain to insult. The precise mechanisms by which extracranial injuries may modify TBI pathophysiology have been reviewed previously[2] and remain under intense investigation, yet it is becoming increasingly clear that injuries leading to hypoxemia and hemorrhagic shock may exacerbate the effects of TBI.[3–6] While the effects of concomitant extracranial injury have been well documented in moderate to severe TBI, it is unclear how hypoxemia and hemorrhagic shock (HS) interact with the pathophysiology of repetitive,

mild TBIs (rmTBI).

On the battlefield, the majority of head injuries are classified as mTBI, they are frequently repetitive, and typically occur with concomitant extracranial injury[7]; thus, the synergistic effects between rmTBI and other injuries is of special concern to the military population. The relationship between mTBI and extracranial injury is not limited to the military, as many forms of high-impact trauma, including falls, heavy object accidents, and motor vehicle accidents, involve concomitant damage to the body or major organ systems.[8] Experimental models of polytrauma have revealed a number of deleterious effects of hypoxemia and hemorrhagic shock when combined with mTBI, including exacerbation of neuronal death,[9] enhanced cytotoxic edema,[10] increased metabolic disturbances,[11] increased astrocytosis, apoptosis, and vascular damage,[12] and impaired motor and cognitive functioning.[13] However, the complex interactions between rmTBI and secondary extracranial injury are only beginning to be understood.

Previous studies indicate that mTBI alters brain glucose metabolism, and the evaluation of glucose utilization by ^{18}F-fluorodeoxyglucose ([^{18}F]FDG)-PET imaging has been used as a biomarker to assess the effects of TBI in both human[14–16] and experimental models of trauma.[17–19] These studies have uncovered complex regional and time-dependent alterations in blood glucose utilization during the early period following trauma that cannot be observed without advanced imaging modalities. However, to date the effects of combined rmTBI and extracranial injury on regional brain glucose metabolism have not been investigated. Building on previous studies, we sought to evaluate changes in brain metabolism as determined by [^{18}F]FDG imaging experiments in rats up to 7 days following trauma. We characterized the effects of rmTBI, with or without combined hypoxemia and hemorrhagic shock, in a clinically and military-relevant model of closed-head injury. Changes in physiological parameters (heart rate, breathing rate, mean arterial pressure) were examined at baseline and during the acute post-injury period. Regional blood glucose metabolism in brain was measured by [^{18}F]FDG uptake at baseline, day 1, and day 7 post-injury. Given the susceptibility of the brain to rmTBI, the known effects of mTBI on brain metabolism, and the possible synergistic effects of extracranial injury, we hypothesized greater variability in the metabolic response to rmTBI when combined with hypoxemia and HS.

Materials and Methods

Animals

Adult, male Sprague-Dawley rats weighing 270-350 g were divided into 4 groups (n=48, 12 animals/group): repeat Sham (rSham), repeat mTBI (rmTBI), rSham combined with hypoxemia and hemorrhagic shock (rSham + HH), and rmTBI combined with HH (rmTBI + HH). The overall experimental timeline for PET/CT, rmTBI, and HH are shown in Figure 1. All experiments complied fully with the principles set forth in the "Guide for the Care and Use of Laboratory Animals" prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (DHEW pub. No. (NIH) 85-23, 2985) and were approved by the Walter Reed Army Institute of Research and Uniformed Services University Institutional Animal Care and Use Committee.

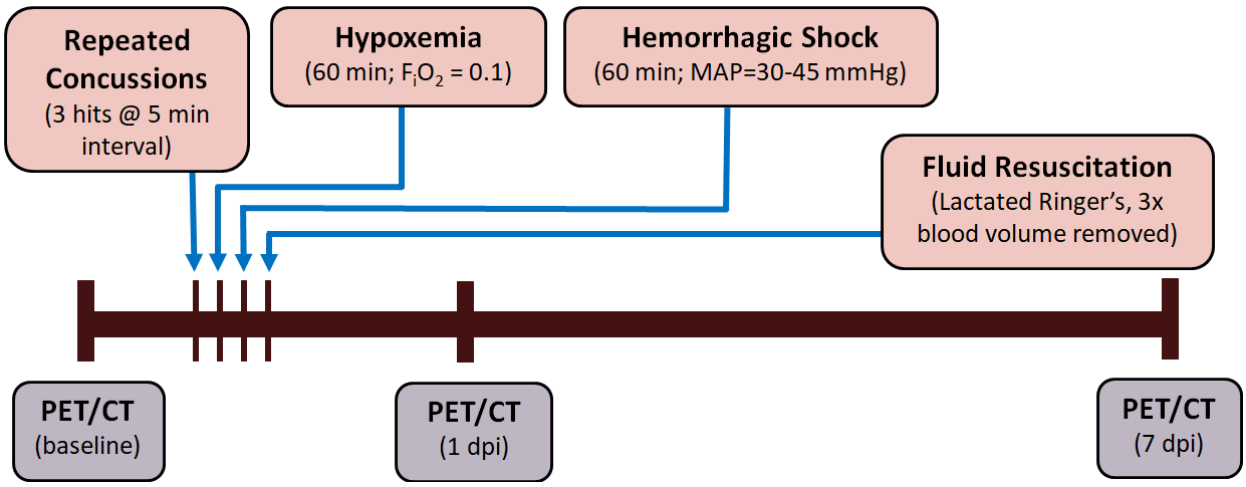


Figure 1. Experimental timeline for positron emission tomography, repeat mild traumatic brain injury, hypoxemia + hemorrhagic shock, and fluid resuscitation procedures.

Projectile Concussive Impact

We used the second generation WRAIR projectile concussive impact (PCI) model which was developed for preclinical study of concussion and uses a non-invasive, closed-head injury caused by blunt impact.[20] Injury procedures were performed as previously described. Animals were anesthetized with isoflurane (5% for induction and 2% for maintenance in air/oxygen mixture (fraction of inspired oxygen (FiO2) = 0.26, until after the three impacts). A custom-designed helmet (Army Research Lab, Aberdeen Providing Ground, MD) was fastened to the head using sections of string. Pressure sensor films (Fujifilm pre-scale pressure sensitive film) were adhered to the inner and outer surfaces of the helmet to record the distribution and magnitude of pressure from the small projectile (3.52 g stainless steel sphere). Animals were then placed in a dorsal lateral position on

the elevated platform with the head positioned over an opening of the platform and lightly restrained with an elastic band placed 50 mm above the head, allowing the head and cervical spine to move freely while maintaining the position of the body on the platform (Figure 2). Rats were subjected to three repetitive injuries at five minute intervals targeted at the right temporoparietal surface of the helmet covering the rat's head. The inner and outer pressure sensor films were removed from the helmets and scanned for analysis with the Topaq Pressure Analysis System using a specially calibrated densitometric scanner (Sensor Products, Inc., NJ). Animals in all groups underwent identical procedures including anesthesia and helmet fixation, but only animals in the rmTBI and rmTBI + HH groups were administered injuries with the PCI device.

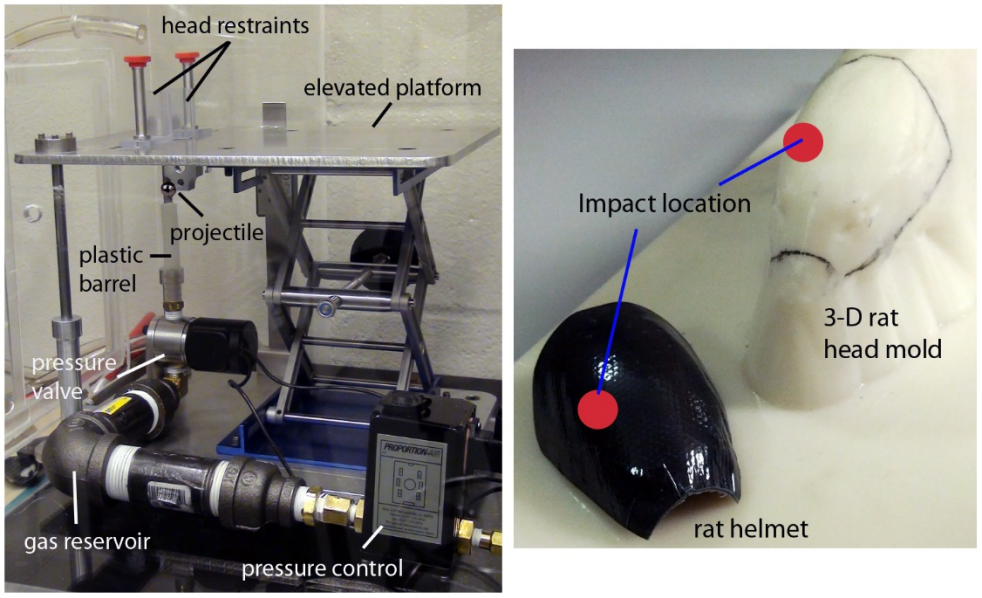


Figure 2. WRAIR Projectile Concussive Impact (PCI) device and rodent helmet. The PCI device causes a non-invasive, closed head injury by blunt impact.

Hypoxemia + Hemorrhagic Shock

Anesthesia was induced with 3.5% isoflurane delivered in air/oxygen mixture (fraction of inspired oxygen (FiO2) = 0.26) and maintained at 1.5% throughout the surgery. Prior to the PCI procedures, the right femoral artery and vein were cannulated for mean arterial pressure (MAP) monitoring and fluid resuscitation, respectively, and the tail artery was cannulated for inducing hemorrhagic shock by blood withdrawal. Fifteen minutes following the third rmTBI, transient hypoxemia was induced by reducing FiO2 to 0.1 (10% oxygen balanced with 90% nitrogen), resulting in a PaO2 of < 40 mmHg. Normoxia (FiO2 = 0.26) was restored after 60 min of hypoxemia. Five minutes following restoration of normoxia, transient hemorrhagic shock was induced

by withdrawing blood via the tail arterial catheter using a withdrawal pump (Harvard Apparatus, Holliston, MA) at a constant rate of 0.25 ml/100 g/min to reduce MAP to 30–45 mmHg (monitored via femoral artery catheter connecting to a blood pressure transducer). Hemorrhagic shock was maintained for 60 min before receiving fluid resuscitation with lactated Ringer's solution (Hospira, Lake Forest, IL) via the femoral vein catheter. The infusion volume was three times the blood volume withdrawn. Sham-only and rmTBI-only groups underwent identical surgical procedures and anesthesia maintenance but did not receive HS, hypoxemia, or fluid resuscitation. Throughout the procedure, physiological recordings were taken at five-minute intervals and averaged over two minutes.

PET/CT Imaging, Reconstruction, and Analysis

Serial [^{18}F]FDG-PET/CT scans were obtained at baseline 2 days prior to injury, as well as at day 1 and 7 after injury using a Siemens Inveon preclinical scanner. Animals were anesthetized with isoflurane, (4.5% for induction and 1-2% for maintenance in 100% oxygen at 2 L/min) and [^{18}F]FDG (1.79 ± 0.13 mCi) was injected via lateral tail vein. For conscious uptake, animals were kept in a separate cage (without food and water) on a heating pad maintained at 37°C. The total uptake time before PET imaging was 45 min (30 min awake, and 15 min anesthetized) Physiologic monitoring during imaging included measurements of temperature, respiration rate, heart rate, and oxygen saturation. A 30-minute static PET scan was acquired in list mode (350-650 keV energy window, 3.483 ns coincidence timing window) with an axial field of view of 12.7 cm. A brief CT scan (3 bed in 'rat mode', 500 μA, 80 kVp) was acquired following the PET scans for anatomical localization, attenuation, and scatter corrections. The PET data were reconstructed with Inveon Acquisition Workplace software, version 2.0 (Siemens Medical Solutions, Erlangen, Germany). Corrections were applied for dead-time, decay, attenuation and scatter and a requested resolution smoothing setting of 0.8 mm was used. A three-dimensional ordered-subset expectation maximization/maximum a posteriori (OSEM 3D/SP-MAP) iterative protocol (2 OSEM3D iterations and 18 MAP iterations) was used for reconstruction. The image matrix was 256 x 256 with final voxel dimensions of 0.39 x 0.39 x 0.80 mm. The intrinsic resolution of the system was 1.4 mm full width half maximum (FWHM) at the center of the field of view. The CT images were reconstructed using Feldkamp algorithm with beam hardening and HU corrections applied.

The voxel-based analysis requires that all images be in the same space. This was accomplished by creating a template from the PET data and then registering each data set to the template. All registrations were performed using ANTs v2.1. The PET data were resampled to the CT resolution, cropped using both the PET and CT, and rigidly registered to the CT. The CT data were rigidly registered to the VivoQuant CT template, and the PET data were transformed using the computed transformations. A mask was generated from the VivoQuant atlas and applied to the baseline PET data. A template was created using the script antsMultivariateTemplateConstruction2.sh from a random sampling of the data at baseline.[21] Each masked PET image was registered to the PET template using the nonlinear registration algorithm in ANTs.[22] For SPM analysis, SUVw images were computed and loaded into SPM12 for analysis. The images were masked and smoothed using a 1.2 mm isotropic Gaussian kernel.

Statistical Analysis

Physiological data were analyzed with two-way, repeated measures ANOVA with Tukey's post-hoc tests for between group comparisons using GraphPad Prism, v7.01 (GraphPad Software, San Diego, CA). Physiological data are reported as mean ± standard error of the mean. For the voxel-based analysis of ^{18}[F]FDG uptake (SPMv12), the SUVw response was modeled using a factorial design with the factors group and time. P values for the voxel-based analysis are uncorrected and reported at cluster level unless otherwise noted. Effect size was calculated with d as $d = 2T/\sqrt{df}$.

Results

Physiological Data

Figure 3 illustrates the results of physiological recordings of MAP, heart rate, and respiration rate at 5 min intervals following rmTBI, hypoxemia, hemorrhagic shock, and fluid resuscitation. Two-way, repeated-measures ANOVA revealed significant changes in all three physiological measures. For MAP, there was a significant effect of Group ($F_{3,39} = 85.63$, $p < .0001$), a significant effect of Time ($F_{32,1248} = 69.25$, $p < .0001$), and a significant interaction ($F_{96,1248} = 19.56$, $p < .0001$). For heart rate, the effect of group was not significant ($F_{3,39} = 2.65$, $p = .06$), while the effect of Time ($F_{32,1248} = 2.63$, $p < .0001$) and the Group × Time interaction ($F_{96,1248} = 3.08$, $p < .0001$) were both significant. Respiration rate followed a similar pattern to heart rate, with

no significant overall effect of Group ($F_{3,39} = .89$, $p = .45$) and a significant effect of Time ($F_{32,1248} = 22.31$, $p < .0001$) and Group x Time Interaction ($F_{96,1248} = 10.32$, $p < .0001$). Tukey's post-hoc tests revealed multiple significant between group differences as detailed in Figure 3.

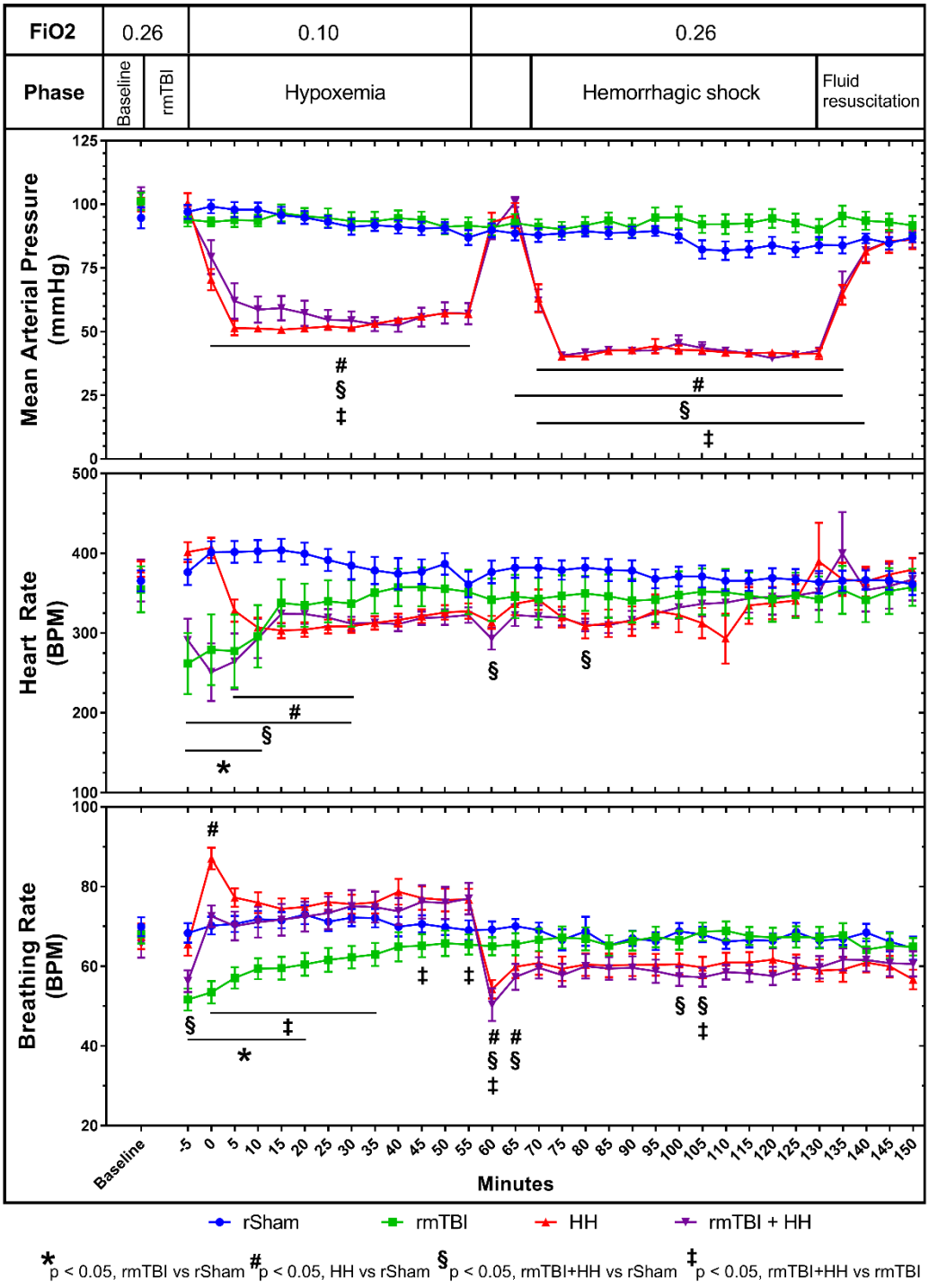


Figure 3. Mean arterial pressure, heart rate, and breathing rate following experimental procedures. Data points represent average values over 2 min at 5 min intervals. Physiology recordings could not be obtained during brain injury procedures. Two way Repeated Measures ANOVA with Tukey's posttest. rSham = repeat sham procedure; rmTBI = repeat mild traumatic brain injury; HH = hypoxemia + hemorrhagic shock.

Voxel-based analysis of changes in ^{18}[F]FDG Uptake

Changes in regional brain glucose metabolism were detected between all groups (rmTBI, rSham + HH, and rmTBI + HH compared to Sham) at day 1 post injury (Table 1 and Figure 4). In the rmTBI group, significantly increased uptake was observed in a cluster centered on the caudate putamen relative to shams (p = .006). In the rSham + HH group, a cluster of significantly increased uptake was observed in a region centered on the mammillary bodies (p = .001), while clusters of decreased uptake were observed in multiple regions of primary somatosensory cortex (p = .005 and .008). In the combined rmTBI + HH group, a large and significant cluster of increased uptake was observed in multiple regions including olfactory bulb, internal capsule, and medial entorhinal cortex (p < .001), while clusters of significantly decreased uptake were observed in two regions of primary somatosensory cortex (p < .001), as well as a cluster in the retrosplenial cortex (p = .035). By day 7 post injury, no significant differences were observed between rmTBI, rSham + HH, and rmTBI + HH groups when compared with shams.

Table 1. Voxel-based SPM analysis of [^{18}F] FDG uptake between experimental groups and sham day 1 post-injury

	Cluster Level		Peak Level		Region at Center of Cluster	Change	d
	p	k_E	p	T			
rmTBI vs. rSham	0.006	1995	0.000	5.82	Caudate Putamen	↑	0.90
rSham + HH vs. rSham	0.001	3139	0.000	7.24	Mammilary Nucleus	↑	1.02
	0.005	2093	0.000	5.07	Primary Somatosensory Cortex	↓	-0.90
	0.008	1804	0.000	4.43	Primary Somatosensory Cortex	↓	-0.86
rmTBI + HH vs. rSham	0.000	13158	0.000	8.53	Olfactory Bulb	↑	0.95
			0.000	5.53	Internal Capsule	↑	
			0.000	5.23	Medial Entorhinal Cortex	↑	
	0.032	1086	0.000	4.81	Cingulate Cortex	↑	0.90
	0.000	4967	0.000	6.73	Primary Somatosensory Cortex	↓	-0.95
	0.000	3878	0.000	6.11	Primary Somatosensory Cortex	↓	-0.98
	0.035	1035	0.000	5.77	Retrosplenial Cortex	↓	-1.01

SPM = statistical parametric mapping; rmTBI = repeat mild traumatic brain injury; rSHAM = repeat sham; HH = hypoxemia + hemorrhagic shock; d, Cohen's d. Arrows in the change column represent either an increase or decrease in FDG uptake. Height threshold: T = 3.21, p = .001. Extent threshold: k = 1000 voxels, p = .038. Expected voxels per cluster, <k> = 225.487. Expected number of clusters, <c> = .21.

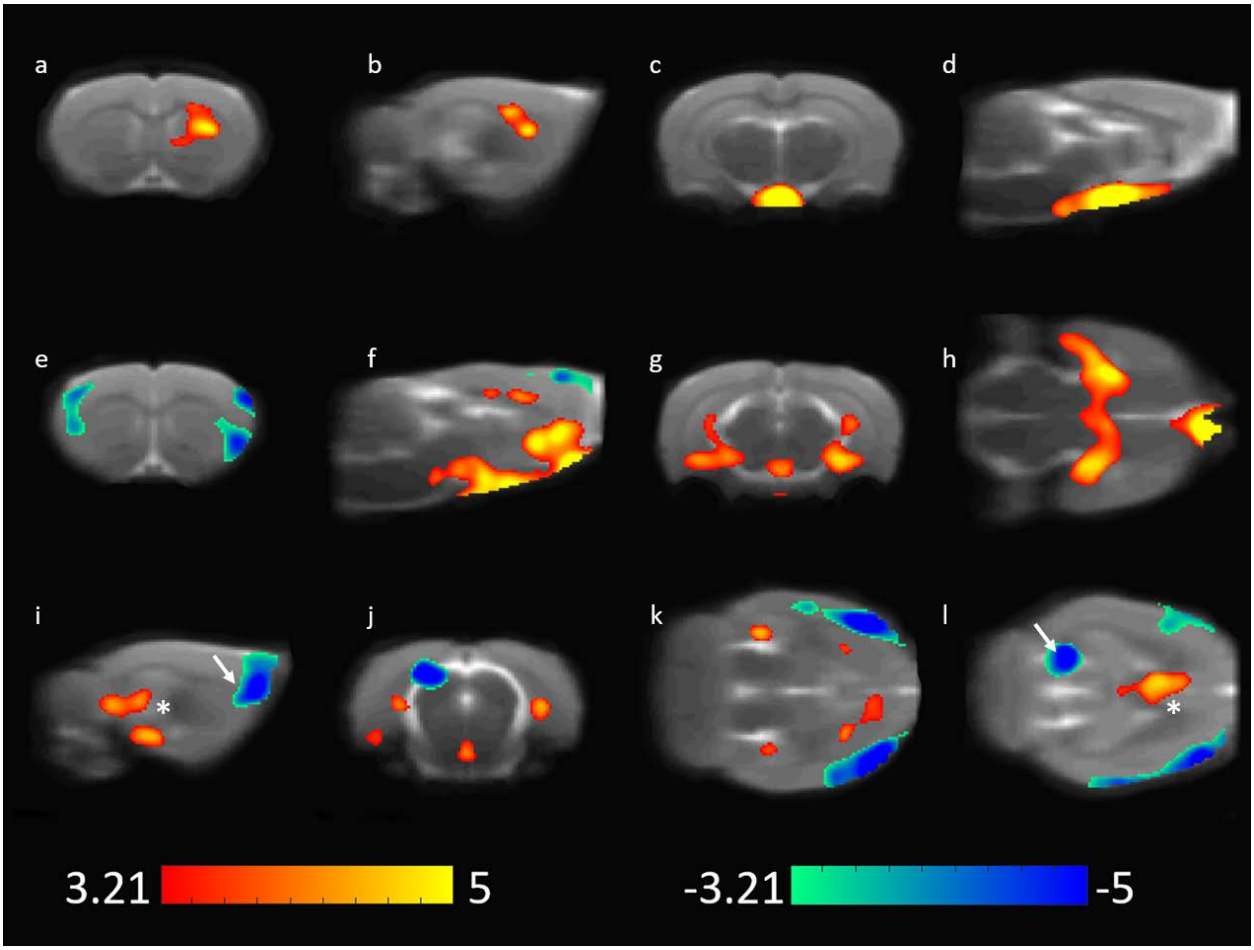


Figure 4. Representative images of increases (red-orange) and decreases (green-blue) in ^{18}F-fluorodeoxyglucose ([^{18}F]FDG) on day 1 following repeat mild traumatic brain injury (rmTBI) (panels a-b), hypoxemia and hemorrhagic shock (panels c-e), and rmTBI combined with hypoxemia and hemorrhagic shock (panels f-l). Animals in the rmTBI only group displayed a single significant cluster of increased [^{18}F]FDG uptake centered in the caudate putamen (coronal (a) and sagittal (b) views). Animals in the hypoxemia + hemorrhagic shock group displayed significant clusters of increased [^{18}F]FDG uptake centered in the mammillary nucleus (coronal (c) and sagittal (d) views) and significant decreases in [^{18}F]FDG in multiple areas of anterior primary somatosensory cortex (panel e, coronal view). Animals with combined rmTBI + hypoxemia and hemorrhagic shock showed the greatest changes in [^{18}F]FDG on day 1 following injury, with significant clusters of increased uptake observed centered on olfactory bulb (panel f, sagittal view), internal capsule (coronal (g) and horizontal (h) views), medial entorhinal cortex (panel i, asterisk, sagittal view), and cingulate cortex (panel l, asterisk, horizontal view); significant clusters of decreased [^{18}F]FDG uptake were observed in multiple areas of primary somatosensory cortex (panel i, arrow, sagittal and panel k, horizontal view) as well as retrosplenial cortex (panel j, coronal view and panel l, arrow, horizontal view).

Discussion

In this study, we examined the effects of rmTBI, with or without combined hypoxemia and HS, on acute post-traumatic physiology and brain metabolism as measured by changes in [^{18}F]FDG uptake with PET imaging. The primary findings of this study indicate that: 1) rmTBI, hypoxemia and HS, and rmTBI + hypoxemia and HS all result in transient physiological disruptions relative to controls, although there appeared

to be no synergistic effects of rmTBI combined with hypoxemia and HS on physiological outcome measures, as these groups showed similar physiological responses during the acute post-injury period, and 2) rmTBI, hypoxemia and HS, and rmTBI combined with hypoxemia and HS alter glucose metabolism which can be detected with FDG-PET imaging 1 day post-injury, and the most widespread alterations in glucose metabolism were seen in the combined injury group; by day 7 post-injury, changes in glucose metabolism were not detected using this method of FDG-PET imaging. These data indicate that early FDG-PET imaging is capable of detecting acute, transient changes in brain glucose metabolism following repeat concussion, with or without combined hypoxemia and HS. Overall, the results suggest a possible synergistic effect of rmTBI combined with hypoxemia and HS on cerebral glucose uptake.

Previous research has demonstrated the utility of PET imaging in human TBI as well as in experimental models. Regional glucose hypometabolism has been observed in veterans exposed to repetitive blast TBI[16] as well as in boxers with chronic, repetitive TBI[23]. A number of other studies point to a period of chronic hypometabolism in multiple brain regions that can be detected with PET in the months to years following TBI, and some of these changes have been linked with outcome (for a review, see Byrnes et al. [24]). In contrast, preclinical models of TBI have uncovered a more complex metabolic response to trauma, with acute and sub-acute dynamic (e.g., both increased and decreased) metabolic changes that are region specific.[25–27] In animal models of TBI, a number of studies have also found that extracranial insults, including hypoxemia and HS, may exacerbate both functional and neuropathological outcome. However, the effects of TBI combined with extracranial injury on brain metabolism have not been previously investigated. The present study suggests that acute post-injury brain metabolism is significantly altered by rmTBI, hypoxemia and HS, and rmTBI combined with hypoxemia and HS, with the latter showing the greatest alterations in glucose metabolism, evidenced by the highest number of regions with significant increases and decreases in [^{18}F]FDG uptake. It is noteworthy that the magnitude of rmTBI used produced minimal changes in brain metabolism measured acutely after injury when administered as an isolated injury, with only a single region demonstrating significant increases in [^{18}F]FDG uptake. However, when combined with hypoxemia and HS, this magnitude of rmTBI produced significant changes in brain metabolism across multiple brain regions. However, none of the experimental

groups displayed alterations in brain metabolism at 7 days post-injury, indicating that the dynamic changes in glucose metabolism within the parameters used in this study are transient in nature.

The long-term clinical significance of acute metabolic changes following exposure to TBI, with or without extracranial injury, have not been fully elucidated. However, many studies suggest that TBI-induced metabolic changes may be reflective of underlying secondary injury progression, and rmTBI is known to produce metabolic vulnerability early following injury.[28] TBI-induced changes in brain metabolic activity detected with FDG-PET imaging have been correlated with neuroinflammatory processes[19], glial activation[26], and behavioral changes.[27] As TBI is known to produce dramatic alterations in ionic flux soon after injury,[29,30] acute hypermetabolism observed with FDG-PET in rmTBI may reflect the increased energy demands to restore ionic homeostasis. Similarly, post-injury hypometabolism has been observed within hours of injury in animal models and is generally assumed to occur after a period of hypermetabolism.[31] In the current study, it is unclear whether the regions showing decreased glucose uptake at day 1 have undergone a transition from hyper- to hypometabolism. Further studies are needed to fully characterize the time course and regional distribution of such metabolic changes. However, it is clear that concomitant extracranial injury can exacerbate the acute metabolic dysfunction seen after TBI. Collectively, these and other findings indicate that acute regional changes in brain glucose metabolism, the so-called metabolic crisis, may represent an important therapeutic target in TBI,[32] including rmTBI combined with extracranial trauma. Studies such as the present one, using highly sensitive FDG-PET techniques capable of detecting changes soon after injury, are critical to understanding the dynamic interplay between rmTBI, extracranial injury, metabolic demand, and eventual outcome.

There are some limitations to this study. While we examined brain metabolism with FDG-PET at days 1 and 7 post-injury, more timepoints are necessary to fully characterize the brains metabolic response to rmTBI combined with extracranial injury. Both human and experimental studies of TBI suggest that chronic glucose hypometabolism may be a key signature of the injured brain. Although this was not observed in the current study, it is possible that a shift from acute, dynamic glucose metabolism to a more sustained period of hypometabolism occurs beyond the 7 day timepoint used in this study. The inter-injury interval used in the current study also limits the generalizability of these findings. While this study provides evidence of the effects

of rmTBI at acute, 5 min intervals, it is possible that a different pattern of metabolic dysfunction would be observed with a different inter-injury interval. Indeed, previous work indicates that the metabolic state of the brain at the moment of injury may be critical for determining how repetitive, mild injuries influence outcome.[18,33,34] Lastly, as this study was focused on the metabolic response of the brain to rmTBI combined with extracranial injury, future studies are needed to determine if the synergistic effects of these injuries also lead to worse neuropathological and functional outcome.

In conclusion, these results suggest a complex metabolic response to rmTBI that is worsened by extracranial injury. Using a military-relevant model of blunt force head injury, we have demonstrated that hypoxemia and HS administered after rmTBI increases the metabolic vulnerability of the brain. Interventions that target these early metabolic changes may be beneficial in rmTBI.

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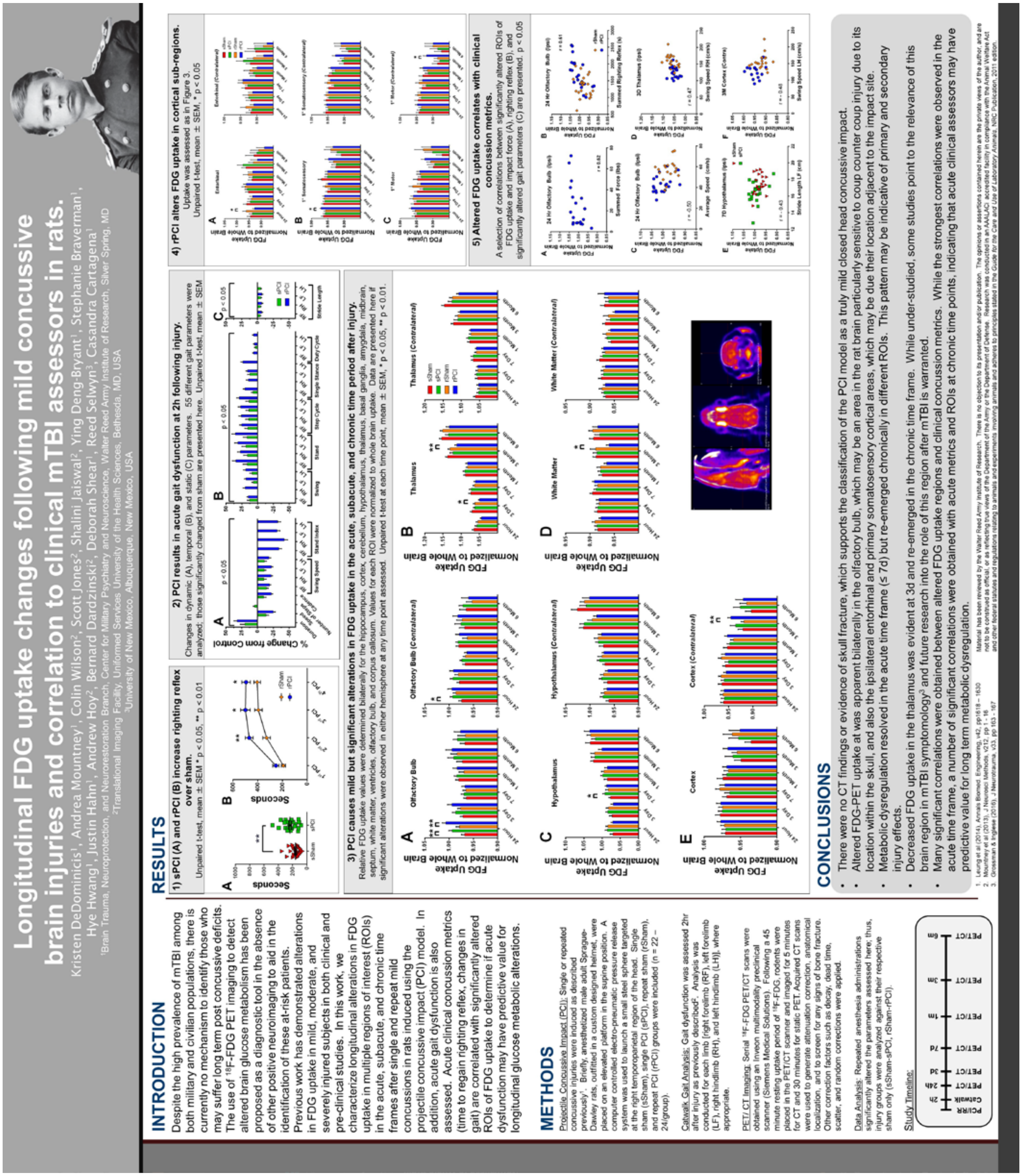
APPENDIX B: POSTER PRESENTATIONS



Acute changes in FDG PET after single and repeat mTBI in rats correlate with clinically relevant symptoms of concussion.

Casandra M. Cartagena[1], Scott Jones[2], Andrea Mountney[1], Deborah A. Shear[1], Stephanie Braverman[1], Colin Wilson[2], Shalini Jaiswal[2], Frank C. Tortella[1], Reed Selwyn[2]

[1] Brain Trauma Neuroprotection and Neurorestoration, Walter Reed Army Institute of Research, Silver Spring MD, USA

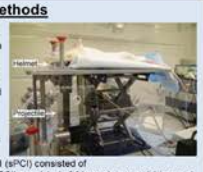
[2] Translational Imaging Facility, Uniformed Services University Health Sciences, Bethesda MD, USA

Introduction

Mild traumatic brain injury (mTBI) can lead to immediate symptoms (headache, dizziness, confusion, loss of consciousness) but lack gross pathology on computerized tomography (CT). Most patients recover within 10 days but a minority have continued deficits. Change in 18F-fluorodeoxyglucose (FDG), a glucose analog, measured with positron emission topography (PET) has been suggested as a potential prognostic indicator of this minority. Reduced brain glucose metabolism as indicated by FDG uptake is well studied among moderate to severe TBI patients but less so in mTBI patients (Bouma 1991, Bergsneider 2000, 2001, Hattori 2003, Vespa 2003, 2006, Kato 2007, Lull 2010). Preclinical studies of FDG uptake in fluid percussion indicate reduced glucose metabolism in several regions following injury, although there may be an acute increase observed (Byrnes 2014, Selwyn 2013). Here we examine changes in glucose metabolism in specific regions of interest (ROIs) at 24h and 3d post single and repeat mTBI using the projectile concussive impact model (PCI) and look to determine whether acute changes in brain glucose metabolism correlate with clinically relevant deficits relating to levels of consciousness, time to regain the righting reflex (RR) , and gait abnormalities (swing speed, step cycle, and stand index).

Methods

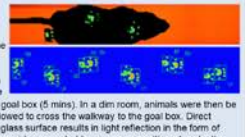
Projectile Concussive Impact (PCI): The PCI injury apparatus consists of an elevated platform and a computer-controlled electro-pneumatic pressure release system used to launch a small projectile (3.52 g steel sphere) targeted at the rat's head (Chen 2012, Leung 2014). Following anesthetization with 4% isoflurane, a custom-designed helmet (Army Research Lab, Aberdeen Proving Ground, MD) was securely fastened onto the rat's head. Single PCI (sPCI) consisted of a single PCI impact while repeat PCI (rPCI) consisted of 4 impacts spaced 1 hr apart. Shams animals underwent equivalent procedures and anesthesia without impact and included single Sham (sSham) and repeat Sham (rSham) (N=11-16 per group).



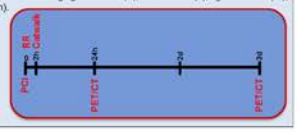
PET/ CT imaging: Serial ^{18}F-FDG PET/CT scans were obtained using an Inveon multimodality preclinical scanner (Siemens Medical Solutions) 24h and 3d post-injury. Prior to imaging, 1.5 - 2 mCi of ^{18}F-FDG were administered by tail vein injection. Static PET was conducted with 45 minutes of uptake followed by 30 minutes of PET imaging. The animals were maintained under anesthesia and kept warm during the 45-minute uptake period. Anesthesia was maintained by isoflurane at 1-2%. Following the resting uptake period, rodents were placed in the PET/CT scanner and imaged for 5 minutes for CT and 30 minutes for PET. Acquired CT scans were used to generate attenuation correction, anatomical localization, and to screen for any signs of bone fracture. Other correction factors such as decay, dead time, scatter, and random corrections were applied. We calculated a relative uptake value (RUV). This calculation was determined for each animal by first normalizing whole brain. ROIs analyzed included right (rt) and left (lt) cortex (CX), hypothalamus (Hyp), amygdala (Amy), and olfactory bulb (OB) as well as others. Only ROIs showing some effect on at least one measure are presented.

Righting Reflex (RR): Following sham procedure or PCI injury, the animal was placed on its back (supine position) in its home cage. The time to right was calculated in seconds from the time of impact to the time the animal rotates its body into the upright (prone) position.

CatWalk Gait Analysis: Catwalk gait analysis was conducted 2 hr after injury. The CatWalk consisted of a 1.3 m long glass plate (walkway) illuminated on the side by dim fluorescent lighting. Rats were first acclimated to a darkened goal box (5 mins). In a dim room, animals were then be placed on the walkway and allowed to cross the walkway to the goal box. Direct contact between the paw and glass surface results in light reflection in the form of illuminated footprints which were video-recorded by a camera positioned under the walkway. The images from each trial were converted into digital signature and processed using CatWalk XT 9.1 software Currently the CatWalk software analyzes over 250 different gait parameters. Three parameters were selected here for comprehensive analysis (swing speed, step cycle, and stand index). Analysis was conducted for each limb including right forelimb (rf), left forelimb (lf), right hindlimb (rh), and left hindlimb (lh).



Timeline:



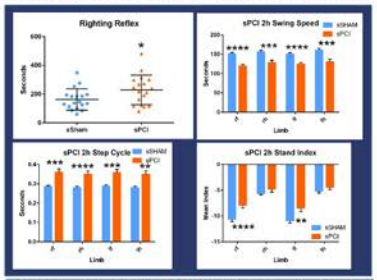


Figure 1. Increased time to regain Righting Reflex immediately after and altered gait 2h after single PCI, t-test *p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001 error bars: SEM.

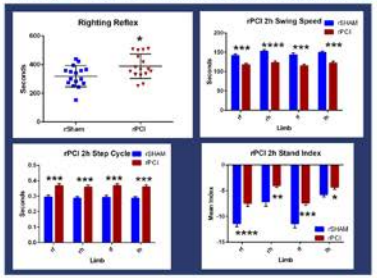


Figure 2. Increased time to regain Righting Reflex immediately after and altered gait parameters 2h after repeat PCI, t-test *p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001 error bars: SEM.

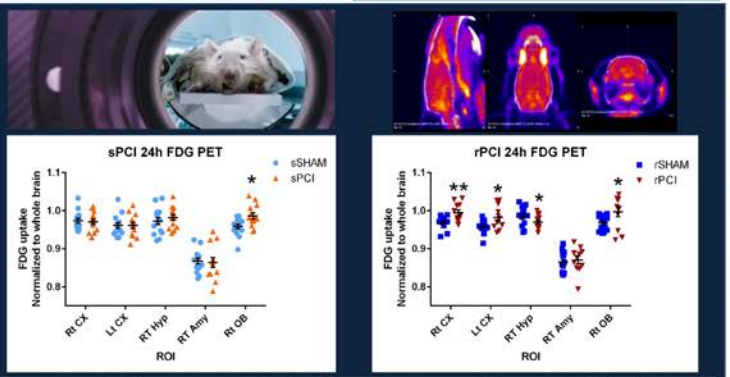


Figure 3. Altered FDG uptake in brain ROIs 24h after single and repeat PCI, t-test *p<0.05; ** p<0.01, error bars: SEM.

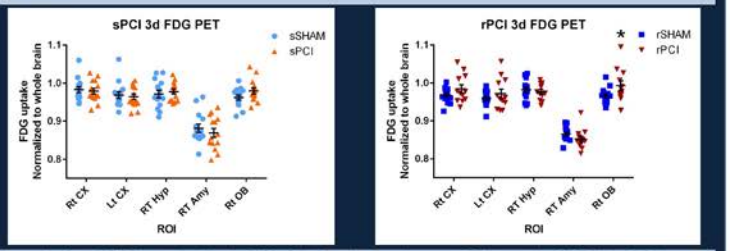


Figure 4. Altered FDG uptake in brain ROIs 3d after single and repeat PCI, t-test *p<0.05; ** p<0.01, error bars: SEM.

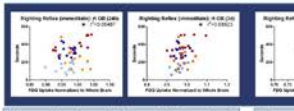


Figure 5. Correlation of RR and rt OB at 24h and 3d post-injury. Spearman (n.s.) Pearson *p<0.05.

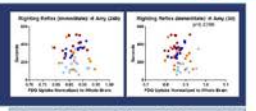


Figure 6. Correlation of RR and rt Amy at 24h and 3d post-injury. Spearman (n.s.) Pearson *p<0.05.

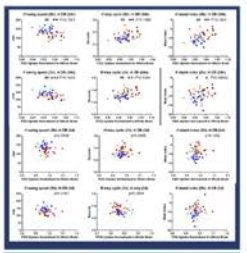


Figure 7. Correlation of gait parameters and rt OB at 24h and 3d post-injury. Pearson *p<0.05; ** p<0.01; **** p<0.001 Spearman # p<0.05, ## p<0.01.

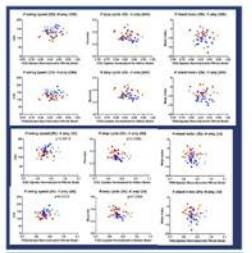


Figure 8. Correlation of gait parameters and rt amy at 24h and 3d post-injury. Pearson (n.s.) Spearman # p<0.05.

Results

- All rats were negative for CT findings including skull fracture.
- RR time was increased and gait altered after both sPCI and rPCI
- 24h post-sPCI or rPCI FDG uptake was significantly increased in right OB. At 3d post-rPCI FDG levels remained high in OB.
- 24 hr post rPCI FG uptake was increased in right and left CX and decreased in right Hyp. By 3d post-injury these changes were no longer significant.
- Increased FDG uptake in OB at 24 or at 3d post-injury correlated with RR immediately after injury. In addition, at 3d post-injury FDG uptake in Amy showed a correlation trend with RR.
- Altered gate (decreased swing speed, increased step cycle time and stand index) 2 h post-injury correlated with FDG uptake in OB 24 hr post-injury with some significance or trends remaining at 3d.
- Although altered gait did not correlate with FDG uptake in Amy at 24h, there were strong trends with FDG uptake at 3 d post-injury.

Conclusions

- Although previous studies of FDG PET have indicated overall decreased FDG uptake following TBI this may be related to the injury severity. Increases in energy utilization seen here with FDG PET may indicate cells in these regions have initiated recovery processes rather than committing to cell death. Changes in OB and correlation to clinically relevant deficits suggesting the OB area may be an acute marker of injury severity in the absence of gross lesions or positive CT. Although not well studied, damage to the OB and smell dysfunction have been reported following mTBI (Charland-Verville 2012). Given the proximity of the OB to the skull, this area may be particularly sensitive to coup counter coup injury following concussion.
- Changes in Amy may be indicative of delayed changes to ROIs involved in motivation and mood and warrant continued study.
- Ongoing studies will determine whether FDG-PET alterations continue, or have prognostic value, for long-term deficits.

References