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# **Multi-modal approach for investigating brain and behavior changes in an animal model of traumatic brain injury**

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**Running Title:** Imaging biomarker in an animal model of TBI

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## Abstract

Utilization of novel approaches in imaging modalities are needed for enhancing diagnostic and therapeutic outcomes of persons suffering a traumatic brain injury (TBI). This study explored the feasibility of using functional magnetic resonance imaging (fMRI) in conjunction with behavioral measures to target dynamic changes in specific neural circuitries in an animal model of traumatic brain injury. Wistar rats were randomly assigned to one of two groups (traumatic brain injury / sham operation). TBI rats were subjected to the closed head injury (CHI) model. Any observable motor deficits and cognitive deficits associated with the injury were measured using Beam Walk and Morris Water Maze tests, respectively. fMRI was performed to assess the underlying post-traumatic cerebral anatomy and function in acute (24 hours after the injury) and chronic (7 and 21 days after the injury) phases. Beam Walk test results detected no significant differences in motor deficits between groups. Morris Water Maze test indicated that cognitive deficits persisted for the first week following injury and to a large extent, recovered thereafter. Resting state functional connectivity (rsFC) analysis detected initially diminished connectivity between cortical areas involved in cognition for the TBI group; however the connectivity patterns normalized at one week and remained so at three weeks post-injury timepoint. Taken together, we have demonstrated an objective *in vivo* marker for mapping functional brain changes correlated with injury-associated cognitive behavior deficits and offer an animal model for testing potential therapeutic interventions options.

**Keywords:** Traumatic brain injury, animal model, functional connectivity, cognition, imaging

## Introduction

Reports of traumatic brain injuries (TBI) are on the rise, with more than 1.5 million cases per year in the United States alone (Gao and Chen, 2011; Pleasant et al., 2011). Cognitive impairments associated with TBI affect more than 5 million people in the United States (Davis et al., 2010), oftentimes inhibiting a person's basic ability to engage in the simplest cognitive tasks (Van Boven et al., 2009). Traditionally, the challenge to physicians has been to identify the effects of mild TBI patients through a battery of neurological and imaging examinations. Unfortunately, this dual-diagnostic tool set, in its current application, is not sensitive enough to detect milder neurocognitive sequelae of TBI (Newcombe et al., 2011). Presenting additional hardships is the incongruent research between preclinical and clinical investigations of functional outcome. Small animal models have been developed to measure TBI associated cognitive, motor and somatic deficits through behavior tests; while clinical evaluation of TBI relies on structural imaging assessments to determine whether to admit or to safely discharge a patient. However, neither side of the translational paradigm puts a major emphasis on a multi-modal diagnostic approach. This practice can be problematic given that cognitive difficulties can persist despite lack of any observable anatomic change in brain structures related to cognitive functioning (Mayer et al., 2011; Newcombe et al., 2011).

In this study, we have replicated an established animal model of TBI, demonstrated the feasibility of detecting cognitive deficits with behavioral tests, and cortically targeted the functional mechanisms by which the deficits occur. This approach enabled a meaningful correlation of changes in cognitive status and brain function in the acute and subacute period following TBI. Most importantly, we developed a model with strong translational validity. Due to the frequency of closed head injury (CHI) in clinical cases, we utilized a CHI contusion design

in an animal model of TBI, along with behavioral and imaging modalities to evaluate the extent of injury at acute and chronic time points. Following clinical reports of commonly observed cognitive deficits and patients' desires to improve cognition (Corrigan et al., 2004), we have focused our analysis on disturbances within the cognitive domain. Reports of deficits in executive cognitive functioning are prevalent in brain injury cases (Riese H, 1999) and research suggests cognitive dysfunction is most pronounced within the first weeks of injury (Mayer et al., 2011). Aimed at developing a marker to map brain changes associated with behavior, this study was performed within the three weeks following injury. We postulated that if cognitive deficits and neuronal alterations accompany TBI, then non-invasively assessing functional connections between brain regions should be critical in evaluating the extent of injury and, possibly, recovery. In the present study, functional connectivity has been assessed by utilizing a resting-state functional magnetic resonance imaging (rsfMRI) paradigm that has been well established through previous studies (Liang et al., 2011; Liang et al., 2012; Zhang et al., 2010).

## Methods

### *Animals*

Male Wistar rats, weighing 275-300g, were divided into two groups (traumatic brain injury, n=7; sham operation, n=7) and housed in pairs. The housing environment was maintained at 22-24 °C with a 12 hour light/dark schedule (lights on at 06:00 and off at 18:00). Food and water were provided ad libitum. All rats were acquired and cared for in accordance with the guidelines published in the NIH Guide for the Care and Use of Laboratory Animals.

### *Closed Head TBI Model*

Closed head TBI was produced using a weight drop device, as previously described in detail (Henninger et al., 2005). Briefly, the skull was exposed and the animal's head fixed laterally between adjustable rubber coated Plexiglas blocks to prevent head movement at the moment of impact. An incision exposing bregma was required to maintain precision and reproducibility across animals, as well as to visually assess for skull fractures at the site of impact. A weight (175 g) was freely dropped 114 cm to strike a cylindrical polyacetal transducer rod (Delrin, tip-diameter 10 mm, 32.6 g, e-module 29.000) placed with its tip directly on the rat's skull, posterior to bregma. The central region between bregma and lambda was aligned under the tip at an angle of 90°. The transducer rod was held to prevent rebound impact immediately following the initial weight drop. Sham rats were anesthetized and surgically prepared, but were not subjected to TBI. After trauma or sham procedure, the incision was sutured and animals were administered warm Ringer's Lactate solution (5 mL, S.C.) and buprenorphine (0.05 mg/kg, S.C.) to relieve pain. Post-procedure exclusion criteria included the presence of skull fractures and lesions seen on visual assessment or in MR images.

### *Behavioral Measurements*

Motor deficits were evaluated using the Beam Walk test. Measurements of hindlimb coordination (the count of hindlimb slips off the beam surface) and navigational speed (the time lapsed from the start to end location) were recorded as the animal traversed an elevated wooden beam, 100cm in length. A safe location (i.e. an enclosed box) was placed at one end of the beam as motivation for the animal to navigate across (Virley et al., 2000). Trained twice daily for a maximum of 3 minutes per trial, each animal was tested prior to the injury/sham procedure, as well as 24 hours and 7 days post-CHI. Pre-procedure testing provided baseline performance data and habituated animals to the apparatus.

To focus on impairments in memory, cognitive abilities were measured in the Morris Water Maze (Davis et al., 2010). The apparatus (Morris, 1984) and procedures (Vorhees and Williams, 2006) were modified from previously established studies. To provide animals with indicators for spatial acquisition, the testing environment incorporated salient visual cues placed outside the apparatus. Animals retained information regarding the fixed location of the submerged platform with respect to the external spatial cues. Latency to the platform was recorded. Accounting for natural swimming patterns and testing bias, entry points into the pool were randomized for each trial. Four daily trials were performed for five consecutive days. Spatial acquisition learning was measured from day 2 to day 6 post-procedure. One week following the initial spatial acquisition, the Morris Water Maze was repeated in a reversal learning phase; however, the platform was relocated to the opposite quadrant of the apparatus from initial testing (Vorhees and Williams, 2006). This reversal learning phase was performed on days 15-19 post-procedure and all methods remained consistent with the spatial acquisition phase, with the exception of the platform location.

### *Magnetic Resonance Imaging (MRI)*

Eleven control animals and seven TBI animals were imaged to evaluate resting state functional connectivity. MRI experiments were performed on a 4.7T/40cm horizontal magnet equipped with a Biospec Bruker console (inner diameter 12cm). TBI animals were imaged at 0 (i.e. within 24 hours after the injury), 7 and 21 days after TBI. Isoflurane was delivered at 2% with ambient air to keep animals anesthetized during imaging. T<sub>2</sub>-weighted high resolution anatomic images were acquired using Rapid Acquisition Relaxation Enhanced (RARE) sequence with relaxation time TR=2.0s, echo time TE=12ms, resolution matrix=256x256, field of view (FOV)=30mmx30mm, slice number=18, and slice thickness=1mm. Functional magnetic resonance imaging (fMRI) incorporated multi-slice T<sub>2</sub>\*-weighted Echo-Planar Images (EPI), acquired while at rest, to estimate resting state functional connectivity (rsFC). The functional imaging acquisition parameters were: TR=1000ms, TE=30ms, matrix size=64×64×18, and FOV=30mm×30mm×18mm. 200 volumes were acquired each run and 6 runs were repeated per imaging session.

### *MRI Data Analysis*

Functional connectivity (i.e. temporal coherence in spontaneous BOLD response between different brain regions) was estimated using Medical Image Visualization and Analysis (MIVA, <http://ccni.wpi.edu/miva.html>) and Matlab (The Mathworks Inc., Natick, MA, USA), following a well-established computational work-flow (Liang et al., 2011; Liang et al., 2012; Zhang et al., 2010). Co-registration, based on anatomical images, was performed to a fully segmented rat brain atlas implemented in MIVA for each rat's data set.

All raw fMRI images were motion corrected using Statistical Parametric Mapping (SPM8) software (Wellcome Department of Cognitive Neurology, London, UK), and spatially filtered using a Gaussian function (FWHM = 1mm). All time courses were then 0.01-0.1Hz band-pass filtered. The time course for each individual voxel was further corrected for head movement by regressing out the six motion parameters (translations and rotations) estimated within the motion correction step. The signal from the ventricles and white matter was estimated by averaging the time courses of all voxels within the ventricles and white matter and then was regressed out from the time courses.

The cross-correlation (CC) coefficient between regionally averaged time course of the predetermined seed region and the time course of each individual voxel was calculated. Cortical seeds were selected based on an a priori hypothesis supporting the relevance to the acquired cognitive behavioral measures. Selected anatomical seed regions included the somatosensory cortex and the infralimbic cortex. CC coefficients were transformed to z scores using Fisher's transformation. A connectivity map for each seed region was created for each fMRI run and maps corresponding to the same seed across multiple runs were averaged to create the connectivity map for each animal.

### *Statistics*

Single factor ANOVA was performed on each animal to produce comprehensive group results for all behavior measurements. Significance was determined to be  $p < 0.05$ .

For each seed region, a composite connectivity map was generated using a one sample t-test across subjects, to create group result maps of functional connectivity, thresholded at  $p < 0.05$ . False positives were corrected using FDR (false discovery rate) in SPM8 at  $p < 0.05$ .

In addition, average CC coefficients in different regions of interest (ROIs) were calculated in order to quantify regional connectivity change. Group difference of regional averaged CC coefficients was evaluated using two-sample t-tests.

## Results

### *Beam Walk Test*

Average time to traverse the beam did not reveal significant inter-group differences at any of the three time points (Baseline, [F(1,12)=0.04, p>0.5]; 24 hours post-injury, [F(1,12)=2.99, p>0.1]; 7 days post-injury, [F(1,12)=0.02, p>0.5]). Likewise, hindlimb slips were statistically similar between groups when measured at either post-injury time point (Baseline, [F(1,12)=2.4, p>0.1]; 24 hours post-injury, [F(1,12)=4.29, p>0.05]; 7 days post-injury, [F(1,12)=0.37, p>0.5]). The results detected no significant motor deficits associated with the brain injury.

### *Morris Water Maze Test*

Single factor ANOVA revealed significant inter-group differences as demonstrated in Figure 1a. Shorter latency to the platform was observed in control animals, relative to TBI counterparts, during spatial acquisition testing on day 2 [F(1,12)=5.17, p=0.04], day 4 [F(1,12)=5.72, p=0.03], day 5 [F(1,12)=4.84, p=0.04], and day 6 [F(1,12)=32.06, p=0.0001] post-surgery. Results of day 3 testing yielded a trend towards significance in behavioral performance [F(1,12)=3.69, p=0.07]. Initial differences seen between groups on post-surgery day 2 are representative of cognitive deficits 48 hours post-injury and cannot be explained as differences in motor abilities given results of the beam walk test. Analysis of reversal learning (Figure 1b) revealed significant inter-group difference on the last day of testing [F(1,12)=7.36,

p=0.01], with slower performance by TBI animals; but did not reach statistical significance on the first four days.

### *Functional Connectivity*

Functional connectivity patterns did not demonstrate significant differences between the TBI and the control groups, 24 hours, 7 days and 21 days post-surgery, when the seed was placed at the somatosensory cortex. This result was consistent with the Beam Walk test, suggesting there was no motor deficit in TBI animals.

Figure 2 shows the composite functional connectivity maps using infralimbic cortex as the seed, for the control group (Figure 2a) as well as the TBI group 24 hours (Figure 2b), 7 days (Figure 2c) and 21 days (Figure 2d) post-surgery. Compared to controls, diminished rsFC in TBI animals, particularly between infralimbic and the anterior cingulate cortex (ACC), was observed at the initial imaging time point (24 hours post-surgery) and normalized thereafter to a similar strength as controls by day 7 post-surgery. This normalization in connectivity remained until day 21.

Figure 3 illustrates the average CC coefficient between the infralimbic cortex and the ACC, characterizing the strength of the connectivity, thresholded with CC of 0.2. Compared with the control group, connectivity between the infralimbic cortex and ACC was impaired in the TBI group only for the first 24 hours following injury ( $t(16)=4.18$ ,  $p=0.001$ ). TBI intra-group comparison showed significant alterations in connectivity on day 7 ( $t(12)=-4.1$ ,  $p=0.006$ ) compared to day 1 post-injury, and remained similar to control level on day 21.

## Discussion

Standard neuroradiological techniques have the potential to detect anatomical injuries associated with traumatic brain injury; however, many patients suffering from mild or moderate TBI experience functional deficits in cognition, regardless of the existence or absence of structural lesions. For this reason, we chose an animal model of mild TBI without any observable abnormalities in serial MR images, but displayed behavioral, specifically cognitive, challenges (Henninger et al., 2005). In the present study, our TBI animal model demonstrated cognitive deficits that persisted up to one week post-injury, with a relative disappearance of inter-group difference over the subsequent two weeks. Additionally, functional connectivity analysis detected diminished correlation between time courses from different cortical areas 24 hours post-surgery. However, significant improvement in connectivity returned these measures to the control level at day 7 and day 21 post-surgery. This multi-modal approach enabled us to identify functional brain changes associated with quantified behavioral deficits, in an injury model absent of any anatomical abnormalities.

Despite the absence of deficits in behavior performance of TBI animals from one week to three weeks post-injury, modest differences in the reversal learning task were exhibited at the end of testing. In clinical cases, regardless of the existence or absence of structural lesions, TBI patients often report cognitive difficulties, especially in circumstances requiring them to remain on-task for an extended period of time. Referred to as “cognitive fatigue” or deficits in cognitive endurance, one hypothesis for this phenomenon is that patients do not experience significant decreased cognitive performance over time, but instead, experience persistent fatigue during a task requiring sustained cognitive effort for a prolonged period (Kohl et al., 2009; Lux, 2007). When an extended task coincides with a distracting environment, fatigue is thought to be

intensified (Lux, 2007). The five-day reversal learning phase in rats may serve as a proxy for a prolonged cognitive task in humans. Therefore, the theory of cognitive fatigue may provide a mechanistic basis for the significant inter-group difference observed on only the last day of testing. Given our data, we speculate that TBI rats potentially began cognitive recovery in the week between spatial acquisition and reversal learning, as explained by the lack of significance in behavioral measures on days 15-18, but showed a deficit in cognitive endurance by the fifth day of consecutive testing. The requirement to consistently recall information of the platform location based on numerous salient visual cues may have impacted the cognitive endurance of the recovering TBI animals.

As an addendum onto the traditional behavior measurements, we utilized rsFC to assess deficits in intrinsic brain connectivity, a technique that is novel to animal model investigations of TBI. Typically, functional imaging investigations of traumatic brain injuries study widespread deficits in evoked neuronal activity (McAllister et al., 1999; Perlstein et al., 2004). Notwithstanding the importance of targeting these general task-related activations, prior techniques were restricted to providing information on cortical activations within, rather than between, particular brain regions. A series of functional connectivity studies have demonstrated that patterned communications exist within various brain networks during resting and passive task states (Gusnard et al., 2001). During these states, distributed brain regions within functional-anatomic networks spontaneously increase and decrease intrinsic activity together (Fox et al., 2005). Temporally correlated, these spontaneous fluctuations map intrinsic brain activity within and between different brain networks. Recently added (Bonnelle et al., 2011; Hillary et al., 2011; Mayer et al., 2011) to the conventional MRI techniques used in clinical TBI studies (Beauchamp et al., 2011; Belanger et al., 2007; Johnston et al., 2001; Scheid et al., 2003); functional

connectivity has just begun to investigate potential damage to specific inter-regional neural connections generated by mild to moderate TBI (Mayer et al., 2011; Slobounov et al., 2011; Stevens et al., 2012).

rsFC imaging, in the present study, detected discrete differences in connectivity strength between selective brain areas of the TBI rats from the time of initial injury to the study endpoint. Consistent with our cognitive behavioral measurement, disrupted connectivity was observed immediately following injury. Interestingly, results at the three week time point showed normalized cortical connectivity in the TBI rats to that of the control animals, whereas behavior results still showed slowed escape latency. Similarities in post-surgery days 7 and 21 imaging results suggest that neuroadaptation in TBI rats can restore similar connection strength in brain regions involved in cognitive tasks (Van Boven et al., 2009), despite performance differences, relative to controls, on the first four days of each testing phase.

Taken together, we speculate that the return to baseline (control) connectivity between cortical regions at later imaging time points, sans any external therapeutic intervention, may be indicative of neuroplasticity changes post-injury. That is, the observed dynamic changes, from weakened to normalized connectivity in comparison to control animals and slowed to almost normalized behavior performance, may be the result of neuroplastic processes responsible for restoring disrupted patterns of communication between neural circuits, as early as one week post-injury. Indeed, rsFC may serve as a valuable biomarker of injury and restoration of function in a manner similar to that noted in the reorganization of certain connections following peripheral nerve injury (Pawela et al., 2010).

There are several limitations to this study. While our concussion device was guided but not mechanistically controlled, thus presenting a potential constraint to the intra-group reproducibility, we did not observe significant intra-group differences in any of the acquired measures. With regard to inter-group comparison, control animals were imaged once based on the assumption that consistent results will be obtained by repeated imaging, since the reliability of rsFC measurement over time has been widely confirmed with test-retest designs. Furthermore, the reliability of rsFC in anesthetized animals is confirmed by Liu and colleagues (Liu et al., 2011) reported a strong neurovascular coupling in isoflurane-anesthetized rats, suggesting rsFC measured by fMRI in isoflurane anesthetized rats was of neural origin. Isoflurane is a vasodilator that may change the baseline blood flow level in the brain; however, resting-state fMRI measurement is not sensitive to baseline blood flow level, as it assesses the temporal coherence of spontaneous fluctuations of the fMRI signal, rather than its absolute amplitude. Therefore, the use of isoflurane should not have significant impact on resting-state fMRI measurement and the imaging of control animals only once should not change rsFC measurements. Finally, since we do not have the statistical power to perform statistical comparison for individual voxels and the main objective of this study was to examine changes of rsFC between brain regions (i.e ROIs), data was statistically compared on the ROI level and not voxel-by-voxel basis.

In conclusion, we examined a CHI animal model in order to establish the feasibility of utilizing behavior and functional connectivity assessments as a biomarker for dynamic changes associated with cognitive progression in TBI. Two-fold in relevance, our findings present an objective *in vivo* marker for mapping functional brain changes correlated with injury-associated cognitive behavior deficits and offer an animal model, strong in translational validity, for testing potential therapeutic interventions options targeted at preclinical TBI.

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## **Author Disclosure Statement**

For each author, no competing financial interests exist.

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## Figure Captions:

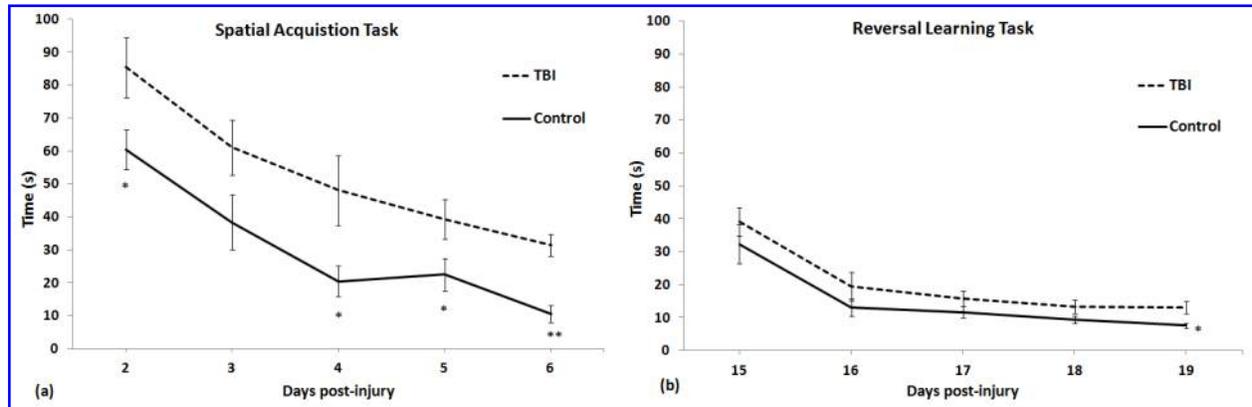


Figure 1: Behavioral measurements, i.e. time taken to find the platform in seconds, from Morris Water Maze test. (1a) Spatial acquisition task was performed with the TBI and control groups for 5 days, starting post-surgery day 2. Significant intergroup differences were observed on all days, with the exception of the second day of testing. (1b) Two weeks post-injury, reversal learning task was performed with the same rats. Significant inter-group difference was observed on the last day of testing.

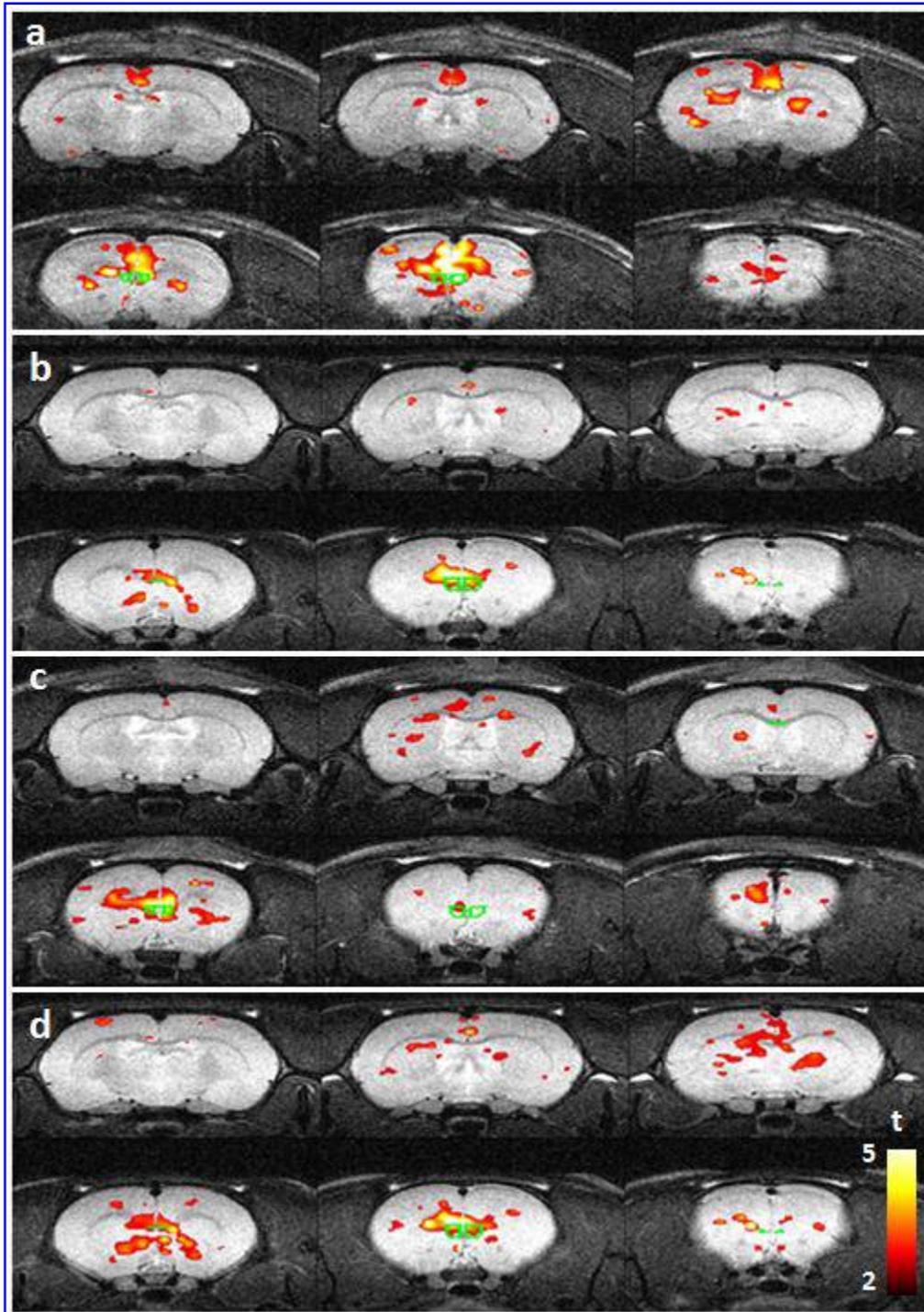


Figure 2: The functional connectivity map from the seed of infralimbic cortex. (2a) Control group within 24 hours post-surgery; (2b) TBI group within 24 hours post-injury; (2c) TBI group 7 days post-injury; (2d) TBI group 21 days post-injury. The green outline denotes the location of the seed region. The connectivity maps were normalized 7 days post-injury to the level observed in the control group.

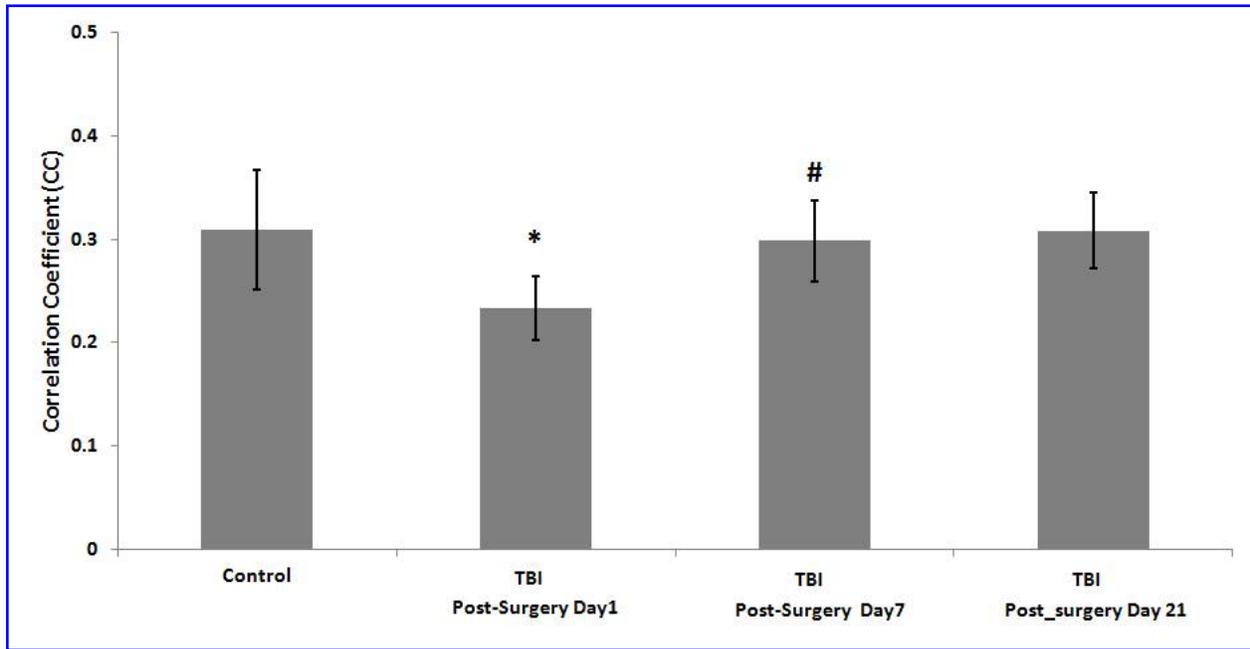


Figure 3: Average correlation coefficients across 11 control rats and 7 TBI rats, representing the strength of functional connections between the seed region, i.e. infralimbic cortex, and the anterior cingulate cortex (ACC). Threshold CC was set to 0.2. Compared with control group, connectivity between the infralimbic cortex and ACC significantly weakened in the TBI group within 24 hours following injury ( $t(16)=4.18, p=0.001$ ). TBI intra-group comparison between days 1 and 7 showed statistically different connectivity ( $t(12)=-4.1, p=0.006$ ) and on day 21 post-injury remained normalized to the level of the control group.