Alterations of Fear-Associated Learning in Mild Traumatic Brain Injury

By John Anthony Greco

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Neuroscience)
in the University of Michigan
2016

Doctoral Committee:

Professor Israel Liberzon – Chair
Associate Professor Gina Poe
Professor Leslie Satin
Associate Professor Chandra Sripada

DEDICATION

I dedicate this thesis to my parents, for all of their care and guidance throughout my life, and to my wife Patricia, whose love and support these past five years has been essential in the completion of this work.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Israel Liberzon, for his contributions throughout the time of my dissertation research. His expertise and assistance were instrumental in the planning and interpretation of these experiments, and his guidance has shaped my development as a scientist. I would also like to thank my committee - Les Satin, Gina Poe and Chandra Sripada - for their support and suggestions throughout my thesis research. I am also extremely thankful for the help provided by Jim Pribble in establishing our collaboration with the Emergency Department.

I am grateful for the contributions of the many members of the Psychiatry Affective Neuroimaging Laboratory and the Trauma, Stress and Anxiety Research Group. Elizabeth Rodriguez was a great help throughout all of the animal behavior experiments and immunohistochemistry. Emi Pop and Jony Sheynin helped tremendously with running and recruiting subjects and helping with often tedious data collection and analysis. Stefanie Block, Liz Duval, Becca Sripada, Tony King, Chieh Chen and Sophie George helped provide feedback and guidance from day one in the lab until today. Xin Wang, Christine Rabinak and Arash Javanbakht were tremendously valuable in the design and programming of my experiments. Hedieh Briggs, Susan Davey and Mala Rajaram were always helpful in navigating any administrative issues I bumped into along the way. Nita Patel ran every subject smoothly and was always receptive and available when implementing and debugging our experiments.

Additionally, the Psychiatry Methods Core, especially Mike Angstadt, was essential in data analysis and solving technical issues.

I would like to thank Ron Koenig in the Medical Scientist Training Program for his support and encouragement and Ellen Elkin, Hilkka Ketola and Laurie Koivapalo for their constant assistance. I would also like to thank Ed Stuenkel, Audrey Seasholtz, Rachel Harbach and Valerie Smith in the Neuroscience Graduate Program for their help and reassurance.

Finally, I would like to thank the Joshua Judson Stern Foundation for its generous financial support throughout my work.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Mild traumatic brain injury (mTBI) and post-traumatic stress disorder (PTSD) are serious pathological conditions that often co-occur following exposure to a traumatic experience. Despite the high comorbidity of mTBI and PTSD, the relationship between these two disorders is poorly understood. One mechanism that may link these two conditions is an alteration in fear-associated learning. Changes in fear-associated learning, specifically in extinction retention, have been reported in PTSD patients and rodent models of PTSD, and we hypothesized that mTBI leads to deficits in fear extinction and extinction retention that predispose patients to PTSD development.

We explored the effect of controlled cortical impact (CCI) and single prolonged stress (SPS), models of mTBI and PTSD, on fear-associated learning and extinction in rats. We observed increased freezing in CCI-injured animals throughout the extinction phase in both experiments, suggesting that the rate of fear extinction is impaired following brain injury in rodents.

We also investigated changes in fear-associated learning in patients with recent mTBI using a previously-validated fear conditioning task with functional magnetic resonance imaging (fMRI). Subjects in the mTBI group demonstrated greater differential skin conductance response (SCR) to conditioned stimuli during fear extinction versus trauma-exposed controls. Additionally, the mTBI group showed greater differential activation versus controls in dorsal anterior cingulate cortex (dACC) and bilateral insula

during late fear extinction and greater activation in dACC and bilateral amygdala during late extinction retention testing. Additionally, differential activation to conditioned stimuli in dACC was directly correlated with differential SCR during late extinction.

Together, these results demonstrate altered extinction learning in an animal model of TBI and in patients with recent mTBI, and these differences are associated with hyperactivation of brain regions known to be involved in extinction learning and the fear response.

CHAPTER I

Introduction

Mild Traumatic Brain Injury and Posttraumatic Stress Disorder

Mild traumatic brain injury (mTBI) is an extremely common consequence of injury that often leads to significant, debilitating symptoms, including headache, fatigue, visual changes and problems with mood, cognition, memory and attention (Corrigan, 2010). It is estimated that 1.5 million Americans seek treatment for TBI every year, with many more undiagnosed cases (National Center for Injury Prevention and Control, 2003). 75% of these TBIs are classified as mild TBI (National Center for Injury Prevention and Control, 2003), which often cannot be detected with conventional computed tomography (CT) and magnetic resonance imaging (MRI) methods (Shenton et al., 2012). Each year, mTBI is estimated to cause $16.5 billion in total lifetime cost in the USA, and this number does not include injuries only treated in EDs and injuries treated in other, non-hospital settings (Thurman, 2001). mTBI is an especially significant in the military, as 19.5% of veterans from Operation Enduring Freedom (OEF) and Operation Iraqi Freedom (OIF) report experiencing an mTBI (Tanielian and Jaycox, 2008).

mTBI is currently defined by the CDC as brain injury with one or more of the following symptoms: confusion or disorientation, loss of consciousness (LOC) for up to 30 minutes, posttraumatic amnesia (PTA) for up to 24 hours and/or transient neurological symptoms (including seizures, irritability, lethargy, vomiting, headache,

dizziness, fatigue or poor concentration) (Carroll et al., 2004). Additionally, patients must have a Glasgow Coma Scale (GCS) score of 13-15 after 30 minutes post-injury, and mTBI symptoms must not be due to drugs, medications, caused by other injuries or problems, or caused by a penetrating head injury (Carroll et al., 2004). By its nature, TBI is a quite heterogeneous injury, in both mechanism and clinical presentation (Rosenbaum and Lipton, 2012). The clinical course following mTBI is extremely variable, as a majority of mTBI patients see their symptoms resolve within two weeks, while approximately 6-15% continue to have symptoms for over a year, or even permanently (Morissette et al., 2011). Additionally, mTBI exposure is associated with an increased incidence of a variety of psychiatric disorders, including bipolar disorder, major depressive disorder, generalized anxiety disorder and posttraumatic stress disorder (PTSD) (Stein et al., 2015, Warren et al., 2015). The relationship between mTBI and PTSD is of particular interest because the development of both disorders requires a traumatic event. Therefore, it is quite possible that they share neurobiological characteristics, although it is unclear whether mTBI is a predisposing factor for the development of posttraumatic stress symptoms. Some early evidence suggested that PTSD could not follow TBI because impaired consciousness prevented trauma patients from encoding memories of the traumatic experience (Sbordone and Liter, 1995). However, a number of subsequent studies have found that PTSD does occur commonly after TBI (Bryant and Harvey, 1998, Hickling et al., 1998, Levin et al., 2001, Hoge et al., 2008), and many studies have even observed PTSD development following severe TBI (McMillan, 1991, 1996, Bryant et al., 2000).

PTSD is a significant public health challenge that affects 7-8% of the general population (Kessler et al., 1995). Like mTBI, these numbers are even higher in veterans; 13.8% of those returning from Operation Enduring Freedom (OEF) and Operation Iraqi Freedom (OIF) developed PTSD (Tanielian and Jaycox, 2008). By definition, PTSD must be preceded by the patient experiencing, witnessing or being confronted by an event or events that involve actual or threatened death or serious injury, or a threat to the physical integrity of oneself or others (American Psychiatric Association, 2013). Patients must have one or more intrusive symptoms, including flashbacks, dreams, recurrent memories of the event, and one or more avoidant symptoms like avoiding talking about event or activities that remind one of the event (American Psychiatric Association, 2013). Two or more hyper-arousal symptoms i.e. sleep disturbance, feeling jittery, problems concentrating and two or more negative alterations in cognition or mood are also required (American Psychiatric Association, 2013). These symptoms must last longer than one month and have functional significance to make a diagnosis of PTSD (American Psychiatric Association, 2013). Like mTBI, PTSD is a costly and debilitating outcome of a traumatic event, and the burden of these two diseases is even greater since they often occur together.

The link between mTBI and PTSD has been well-reported, with a preponderance of epidemiological data showing that people who have sustained mTBIs have a significantly higher rate of PTSD than trauma-matched controls. This includes OEF/OIF veterans (Hoge et al., 2008, Schneiderman et al., 2008) as well as civilian survivors of trauma (Mollica et al., 2002, Bryant, 2010). Three separate studies of military veterans found a strikingly consistent rate of PTSD among OEF/OIF veterans reporting TBI, as

33 to 39% of those surveyed screened positive for PTSD (Hoge et al., 2008, Schneiderman et al., 2008, Tanielian and Jaycox, 2008), compared with just 13.8% of OEF/OIF veterans in general (Vasterling, 2012). Several prospective studies have been published recently that further cement this relationship. One study of 494 hospital-admitted trauma patients found significantly greater rates of PTSD in mTBI patients versus injury-matched controls at both 3-month and 6-month follow up periods (Warren et al., 2015). A similar study of 1,361 injured emergency department patients found that mTBI was a significant predictor of PTSD diagnosis at 3-months post-injury, with an odds ratio of 4.47 (95% CI, 2.38-8.40) (Lagarde et al., 2014). Additionally, a longitudinal study of 4,645 American soldiers deployed to Afghanistan found increased rates of PTSD and posttraumatic stress symptoms (PTSS) in soldiers who reported an mTBI at 3 months and 9 months after their return to the United States (Stein et al., 2015). Despite the numerous large-cohort longitudinal studies that have confirmed the high comorbidity of TBI and PTSD, the relationship between these two disorders is poorly understood. A better comprehension of this relationship is extremely important, as patients with comorbid PTSD and mTBI may be resistant to common treatments for either disorder, including prolonged exposure therapy (due to cognitive deficits commonly found following mTBI). Understanding the mechanisms underlying the relationship between mTBI and PTSD will allow for improved treatments of these common outcomes of trauma.

Fear-Associated Learning

One mechanism that may link mTBI and PTSD is an alteration in fear-related behaviors that can be modeled using fear-associated learning. Fear-associated learning has been used extensively in both animals and humans because it is a convenient, albeit necessarily simplified, model of the acquisition and maintenance of fear responses, and altered fear learning has been hypothesized to play an important role in the development of PTSD and anxiety disorders (Greco and Liberzon, 2016). By definition, patients with PTSD have inappropriately heightened fearful responses (or hyperarousal) to environmental cues and contexts that are in reality safe. These real-world behaviors have been modeled using fear conditioning tasks, and deficits in extinction recall and fear renewal have been observed in PTSD patients in a number of recent studies (Milad et al., 2009, Garfinkel et al., 2014, Shvil et al., 2014). Since fear-associated learning has been used effectively to examine fear-related behaviors in several different patient populations and animal models, it should also be quite effective in investigating whether mTBI causes differences in emotional learning that overlap or potentiate the deficits that are commonly seen in patients with PTSD.

Fear-associated learning (fear conditioning and extinction) has been used for decades to study the neurocircuitry that underlies emotional learning. Animal studies of fear conditioning have established the regions and systems responsible for emotional processing, and these findings have provided a basis for our understanding of the corresponding neurocircuitry in humans. However, it was with the development of functional neuroimaging techniques in the 1990s that researchers have been able to examine whether the regions found to be involved in emotional learning in rodents are

also responsible for similar processes in humans. The classical fear-associated learning paradigms in animals and humans is displayed in Figure I.1.

At its most basic level, fear conditioning involves the association of a neutral conditioned stimulus (CS, often a tone or image) with an aversive unconditioned stimulus (US, often electrical shock or a loud noise). After repetitive presentations of the CS with the US, the CS begins to elicit a conditioned response (CR) that occurs independently of the US. In rats, the CR is typically assessed by measuring freezing or fear-enhanced startle response, and in humans the CR is often assessed via psychophysiological measures such as skin conductance response (SCR), electromyography (EMG) or changes in heart rate. During fear extinction, the previously fear conditioned CS is presented repeatedly, without the US, usually in a different environment (context). Over time, the CS is no longer associated with the conditioned response when presented in extinction context. Fear extinction is thus an effective model of learning establishing that previously "dangerous" stimuli are no longer threatening and are therefore "safe". Furthermore, exposure therapy that is commonly used in anxiety disorders involves components of fear extinction. Finally, during extinction retention (or recall) and fear renewal, the appropriate, context-specific retrieval of previously acquired fear and safety memory traces is tested. Studying extinction retention and fear renewal in humans elucidates how people use contextual processing to disambiguate the various co-occurring environmental cues indicative of both safety and fear.

Animal studies of fear-associated learning have provided an understanding of the brain circuits that govern fear conditioning and extinction, and in general, these findings

have been corroborated by human neuroimaging studies. The neuronal circuitry conserved across species involves the amygdala as a key region involved in acquisition of the fear response in both animals and humans. The amygdala receives inputs from somatosensory cortex, thalamus and hippocampus, which encodes contextual information and compares current contextual cues to previously encoded memories (Maren and Quirk, 2004). Basolateral amygdala (BLA) receives these various sensory and contextual inputs, and a CS-US trace is formed. The centro-medial amygdala receives information from BLA, as well as other direct inputs, and projects to the hypothalamus, periaqueductal grey and other brain stem nuclei, causing the behavioral and physiological changes associated with the conditioned response. Interestingly, it appears that acquisition of the extinction memory also occurs in BLA, while the hippocampus plays an important role in the consolidation of extinction (Quirk and Mueller, 2008), and mPFC seems to be important in the retrieval of the extinction memory (Davis, 1992). Additionally, numerous studies suggest that the central amygdala plays a critical role in fear learning (Li et al., 2013, Penzo et al., 2014, Penzo et al., 2015), and optogenetic studies suggest that different projections to the central amygdala enhance or inhibit fear learning (Tye et al., 2011). These complementary pathways could explain how the central amygdala appears to be involved in both fear acquisition and extinction, as separate inputs to this region both increase and decrease the fear response.

To date, the majority of human neuroimaging studies of fear conditioning and extinction have been performed using functional magnetic resonance imaging (fMRI). FMRI allows researchers to identify the neuronal circuitry involved in performing various

tasks by measuring blood-oxygen level dependent (BOLD) effects. More recent work has used functional connectivity measures that analyze the time courses from the BOLD fMRI signal to glean information about how various regions in the brain communicate with one another. FMRI has often been paired with psychophysiological measures such as skin conductance response (SCR), fear-potentiated startle response or changes in heart rate in order to assess the connection between brain activity and conditioned response in humans.

A variety of stimuli have been used and validated in human studies of fear conditioning, with the CS+ typically a distinct visual cue or sound, and the US a mild electrical shock, an aversive loud noise or less frequently, an unpleasant smell or an uncomfortable visceral stimulus (Kattoor et al., 2013, Gramsch et al., 2014, Kattoor et al., 2014). Human fear conditioning studies typically include a second cue (CS-) that is not associated with the US, allowing for a comparison of the CS+ and CS- responses to isolate the conditioned response using psychophysiological measures and the neural correlates of fear conditioning using neuroimaging techniques.

Early studies using fMRI found differential responses (to CS+ vs CS-) in ACC, anterior insula, hippocampus and amygdala using both trace and delay conditioning paradigms (Buchel et al., 1998, LaBar et al., 1998, Buchel et al., 1999). These findings have been consistently replicated by other research using fMRI (Armony and Dolan, 2002, Critchley et al., 2002, Knight et al., 2005, Marschner et al., 2008, Knight et al., 2009, Reinhardt et al., 2010, Bach et al., 2011, Andreatta et al., 2012, Pohlack et al., 2012b, van Well et al., 2012), as well as PET (Furmark et al., 1997) and MEG (Moses et al., 2007, Balderston et al., 2014). Activation in amygdala, anterior insula, ACC and left

PFC has also been observed while subjects viewed a movie of someone else undergoing fear conditioning (Olsson et al., 2007, Ma et al., 2013), suggesting similar processes of fear learning can be elicited without directly experiencing the US. Over the course of fear conditioning amygdala activation typically decreases, as does hippocampal activation during trace conditioning (Buchel et al., 1998, LaBar et al., 1998, Buchel et al., 1999, Reinhardt et al., 2010). Meanwhile, ACC and insula activation remain more consistent (Buchel et al., 1999), suggesting that amygdala and hippocampus may play a key role during acquisition of conditioned response, while ACC and insula are more involved in CR expression. These results closely resemble findings observed in animal models of fear conditioning, with additional cortical regions implicated mainly in human neuroimaging experiments. One recent large meta-analysis of fear conditioning experiments (Fullana et al., 2015) involving 677 subjects further confirmed large-scale activations in ACC/mPFC and the anterior insula, as well as additional cortical regions (supplementary motor area, dorsolateral PFC and precuneus), ventral striatum and midbrain. These were interpreted as representing a "central autonomic-interoceptive" network. Interestingly, amygdala signal was not found in this analysis, and the authors interpret these negative findings (and the strong ACC and anterior insula) signals in light of the fact that human studies primarily involve conscious fear processing. However, the authors also acknowledge that the meta-analytical approach is subject to false negative errors, as well as specific technical difficulties in imaging small structures.

Among the regions implicated by neuroimaging studies of fear-associated learning, the roles of mPFC and, to some degree, the dACC in fear conditioning have

been debated, with some studies suggesting that these regions are involved specifically in instructed fear learning. However, a meta-analysis of previous work and a study using both instructed fear and Pavlovian conditioning showed that these regions are activated across a variety of different fear conditioning paradigms (Mechias et al., 2010, Maier et al., 2012). DACC thickness also directly correlates with SCR during conditioning (Milad et al., 2007). Thus, dACC and dmPFC appear to play a role in general threat appraisal across a variety of fear conditioning paradigms. The mPFC also appears to be involved in fear acquisition in rodents, but it is not required for fear learning (Etkin et al., 2011). Ventral infralimbic (IL) and dorsal prelimbic (PL) regions that are analogous to the mPFC in humans project to neurons in both central (Quirk et al., 2003) and basolateral (Rosenkranz et al., 2003) amygdala, with IL seemingly more important in inhibitory innervation to central amygdala (Vertes, 2004). These findings, and in particular the direct connections between mPFC and amygdala, support the notion that dmPFC might play a modulatory or supportive role during fear acquisition. Varying the contingency rate between the CS and US in human studies have implicated additional candidate regions in fear conditioning. In addition, human studies that varied the contingency rate between the CS and US have implicated additional cortical regions in fear conditioning. Studies using high contingency rates show that dlPFC is commonly activated when the threat is highly predictable (Eippert et al., 2012, Wheelock et al., 2014). This raises the possibility that that working memory processes in dlPFC become involved once the CS-US pairing has been explicitly learned. The use of a partial reinforcement schedule eliminates this cognitive expectancy effect and limits the role of explicit, declarative memory in the process. Amygdala activation has been observed independent of

contingency awareness (Tabbert et al., 2011), while interestingly, increased CS-US contingency and US expectancy has been associated with decreased activation in regions implicated in fear learning, including amygdala, insula, ACC, and vmPFC (Dunsmoor et al., 2008, Knight et al., 2010, Wood et al., 2012, Wood et al., 2013). The aforementioned meta-analysis (Fullana et al., 2015) also found that higher reinforcement rates were associated with lower activation in rostral dACC/dmPFC, ventral anterior insula and right secondary somatosensory cortex. This potentially suggests that when implicit learning has taken place, there is less robust activation of the basic fear-associated learning circuitry described above.

Structural studies have also linked increased SCR during fear acquisition to some of the same key regions implicated by functional neuroimaging studies, particularly amygdala (Cacciaglia et al., 2014, Winkelmann et al., 2015). Increased insula thickness was also associated with a greater conditioned response during conditioning (Hartley et al., 2011). However, positive correlations between conditioned response to CS+ during conditioning and regional thickness found in the amygdala and insula have not been universally observed when the association between hippocampal volume and fear acquisition has been examined. Larger hippocampal volumes have been associated with greater ability to discriminate between contexts (Pohlack et al., 2012a), and smaller hippocampal volumes have been associated with decreased conscious cue discrimination but not differences in SCR (Cacciaglia et al., 2014).

In agreement with animal studies, contextual fear conditioning studies (where the US is delivered in a danger context without a CS) in humans have also implicated the amygdala, hippocampus and mPFC as key regions in this process, although the

number of studies that have used contextual conditioning in humans is quite limited. One study analyzing differential responses during contextual conditioning found that activation in medial amygdala was coupled with activation in several important fear learning regions, including hippocampus, anterior insula, parahippocampal gyrus, subgenual ACC and OFC (Alvarez et al., 2008). This analysis fits with findings from cued conditioning that identified these regions as areas involved in either fear learning or the recognition of fearful stimuli. Importantly, the hippocampus plays a central role during contextual conditioning, as several studies have established (Alvarez et al., 2008, Andreatta et al., 2015). One recent study also reported that initial activity in contextual conditioning was found in dorsomedial PFC, dorsolateral PFC and OFC (Andreatta et al., 2015). These results corroborate the current understanding of prefrontal-hippocampal circuitry and connectivity as the key network responsible for contextual processing (Maren et al., 2013, Preston and Eichenbaum, 2013).

Functional connectivity analyses have been used more recently to examine the connectivity between brain regions during fear conditioning tasks. Analyzing the time courses of data collected with BOLD fMRI allows for the identification of regions that are activated and deactivated simultaneously, suggesting that these areas are functionally connected. Additionally, resting state fMRI has been used before and after fear conditioning in an attempt to measure the connections between brain regions at rest and how differences in this connectivity might correlate with changes in fear conditioning. Studies during or immediately following fear acquisition have found that several regions implicated by previous neuroimaging studies of fear conditioning also show functional connectivity with each other. During conditioning, increased connectivity

between amygdala and a variety of regions, including hippocampus, vmPFC, dlPFC and ACC was associated with higher conditionability as well as neuroticism (Tzschoppe et al., 2014). Immediately following fear acquisition, subjects have shown enhanced amygdala-dACC and hippocampus-insula functional connectivity, as well as less amygdala-mPFC functional coupling (Feng et al., 2014), although a different study reported greater amygdala-dmPFC functional connectivity (Schultz et al., 2012). In another study, resting dACC metabolism positively predicted differential SCR response and SCR performance was positively correlated with the amygdala-ACC connectivity (Linnman et al., 2012b). These results support the hypothesis that fear conditioning involves communication and modification of the connections between the same regions implicated in previous neuroimaging studies and studies using rodent models, but the specific mechanism and the exact changes have to be further elucidated.

Neuroimaging studies of fear extinction have provided insight into how humans associate previously aversive stimuli, with safety. In concert with the animal literature, amygdala activation has also been observed during extinction learning in humans, and similarly to the observation in the amygdala during fear acquisition, this effect is often graded through the course of extinction (LaBar et al., 1998, Gottfried and Dolan, 2004, Phelps et al., 2004). BLA is activated when CS-US associations become more predictable (Boll et al., 2013) suggesting that BLA encodes information about the relationship between the CS and the US. This is in concert with our understanding of the role of BLA in extinction learning from animal studies (Quirk and Mueller, 2008). Interestingly, a meta-analysis that combined fear extinction with additional “emotional

regulation" experiments confirmed amygdala activation across these types of studies (Diekhof et al., 2011) with the vmPFC activated specifically in fear extinction studies.

The role of vmPFC in extinction learning has been discussed extensively in recent years. Although these findings have been reported by fewer studies than findings in the amygdala, several studies have replicated findings in rodents suggesting that vmPFC plays a role in extinction learning (Gottfried and Dolan, 2004, Phelps et al., 2004). vmPFC activation is also observed in fear reversal tasks (a modified acquisition task that flips the CS+ and CS- halfway through the run) when a CS that used to be associated with fear is now safe (Schiller et al., 2008). Evidence from the animal literature (Quirk and Mueller, 2008) shows that lesions of the vmPFC cause problems with extinction recall, but it is unclear whether this was indicative of a role of vmPFC in actual extinction learning, the consolidation of the extinction memory, or extinction recall. However, as more neuroimaging studies have included the fear extinction retention phase, evidence is accumulating that vmPFC is particularly important in the recall of extinction in subsequent testing.

More recently, studies have started to examine the extinction retention (or recall) and the fear renewal phases, which are usually tested 24 hours following the extinction phase. Both examine responses to the extinguished cue (CS+E); however, in extinction retention this cue is presented in the extinction (safe) context, and in fear renewal it is presented in the original fear acquisition (danger) context or a novel context. It has been reported that vmPFC plays an important role in extinction retention (Kalisch et al., 2006), corroborating animal work implicating the region in the recall of the extinction memory. Activity in vmPFC has also been found to be highly correlated with

hippocampal activation during extinction retention, and this activation was also negatively correlated with skin conductance response indicative of the expression of the extinction memory (Milad et al., 2007). Further studies have also found that the degree of fear extinction retention is positively correlated with vmPFC thickness (Milad et al., 2005, Hartley et al., 2011, Winkelmann et al., 2015). The role of vmPFC in modulating response during extinction recall has also been observed using EEG (Mueller et al., 2014), although the limited spatial resolution of EEG makes it difficult to pinpoint activity specifically to the vmPFC. The hypothesis that vmPFC modulates amygdala activation during this process has been recently supported by fMRI findings in patients with vmPFC damage (Motzkin et al., 2015, and baseline amygdala metabolism has been negatively correlated with activation in vmPFC during extinction recall (Linnman et al., 2012a). All of this evidence points to the critical role of vmPFC in extinction recall, and its close relationship with hippocampus suggests it may be involved in the context-dependent expression of the extinction memory.

Fear-Associated Learning in PTSD

Among various psychiatric disorders, fear conditioning and extinction research has been particularly salient to our understanding of PTSD, phobias and other anxiety disorders. With respect to PTSD, fear-associated learning has been utilized both in studies of PTSD patients and in studies using animal models of PTSD with remarkably converging findings. A summary of findings in fear-associated learning studies of PTSD patients is listed in Table I.1. While alterations during fear acquisition (Linnman et al., 2011) or extinction (Fani et al., 2012) in PTSD patients have been occasionally

reported, in general the preponderance of the findings suggest that fear acquisition and extinction are overall preserved in PTSD patients (Milad et al., 2009, Garfinkel et al., 2014, Shvil et al., 2014) as well as in animal models of PTSD (Knox et al., 2012a).

In contrast, abnormalities in extinction retention and fear renewal have been consistently demonstrated both in an animal model of PTSD (Knox et al., 2012a) and in PTSD patients, with associated changes in regional brain activation and volume. Briefly, fear renewal involves a previously extinguished CS that is presented either in the fear context (the context that was used for fear acquisition) or a novel context, and no US is presented. Similar to extinction retention testing, the context triggers recall of CS memory; however, here it triggers the fear trace rather than the safety trace. PTSD patients have demonstrated lower activation in hippocampus and vmPFC, and greater activation in dACC during extinction recall (Milad et al., 2009, Rougemont-Bucking et al., 2011), changes that have also been associated with higher SCR to CS+E signifying impaired recall (Milad et al., 2009). Our laboratory demonstrated higher SCR and greater amygdala activity to the CS+E during extinction recall in PTSD patients (Garfinkel et al., 2014). We also observed altered fear renewal in PTSD patients, with lower SCR to the CS+E, and lower activity in amygdala and vmPFC as compared with combat controls. Greater activation in insula during extinction recall and fear renewal was also observed. Since both extinction recall and fear renewal are dependent on contextual disambiguation of the CS, the deficits in both processes strongly implicate abnormalities in contextual processing during fear-associated learning in PTSD. This is also very consistent with the abnormalities observed in hippocampal-mPFC circuitry, as

these structures play a key role in the contextualization of memory (Maren et al., 2013, Preston and Eichenbaum, 2013).

In concert, in an animal model of PTSD developed in our laboratory, single prolonged stress (SPS) (Liberzon et al., 1997), both abnormal fear renewal (Knox et al., 2012a) and changes in hippocampus and mPFC (Knox et al., 2012b) have been consistently demonstrated. Volumetric findings of reduced hippocampal volume, reduced vmPFC volume, and reduced gray matter density in dACC are also associated with PTSD symptomology (Gilbertson et al., 2007, Bonne et al., 2008, Rogers et al., 2009, Kuhn and Gallinat, 2013), further implicating hippocampal-prefrontal involvement. It should be noted that one study of men and women with PTSD found that only men showed deficient extinction recall, and men also showed greater activation in ACC compared to women during recall (Shvil et al., 2014). These results might reflect findings in studies performed in healthy subjects that demonstrate gender differences in fear-associated learning. In sum, patients with PTSD show impairments in the contextual modulation of both fear extinction and enhancement, and these deficits may directly contribute to the development of PTSD symptoms (Milad et al., 2008).

Fear-Associated Learning in mTBI

Fear-associated learning in animal models of TBI is a rapidly expanding literature, but a variety of different models and no generally-accepted parameters within those models has led to quite inconsistent findings. Studies using lateral fluid percussion (LFP), weight drop and blast models of injury (Elder et al., 2012, Meyer et al., 2012, Reger et al., 2012) first suggested that these injuries led to enhanced fear

acquisition. However, a subsequent study using a blast injury model found a decreased conditioned fear response in injured animals (Genovese et al., 2013), and a recent study using the LFP model found decreased freezing behavior during fear acquisition testing (Palmer et al., 2016). These studies did not examine freezing behavior over time during their acquisition testing, in which animals were re-exposed to the danger context without presentation of the CS (in the case of contextual conditioning) or with presentation of the CS (cued conditioning). One possible explanation for the results observed in these studies is that increases in freezing behavior during so-called acquisition testing are deficits in fear extinction that have been collapsed across the course of the presentation of US or context. The acquisition testing lasted anywhere from 3 to 8 minutes, and depending on the study, could be long enough to start the process of extinction in some animals. In paradigms that have a short acquisition phase, it may take only a few minutes of cue or context presentation for animals to begin learning safety; in fact, in previous studies in our lab, decreased freezing behavior indicative of extinction learning typically starts after three CS trials (that last a total of three minutes) (Knox et al., 2012). By collapsing freezing behavior across the first 8 minutes of the acquisition test, differences in the way that animals are learning safety may confound measures of the retrieval of the acquisition memory.

Several studies have looked at trial-by-trial freezing behavior, and the results of these experiments have also been mixed. One study using the controlled cortical impact (CCI) model in mice before and after conditioning found no effect of injury on freezing behavior during acquisition, extinction or extinction recall, in spite of the fact that the parameters used in the experiment were quite severe for mice (3 mm diameter impact,

impact velocity of 6 m/s, 100 ms duration, 0.6 mm depth), causing a significant lesion in cortex and ipsilateral hippocampus (Sierra-Mercado et al., 2015). Also, mice that underwent a repetitive TBI injury, in which two external impacts were delivered with a rubber-tipped CCI device, showed no differences in freezing behavior during cued or contextual conditioning and extinction testing (Klemenhagen et al., 2013).

Nonetheless, three recent publications in three separate models of TBI suggest that extinction, in fact, might be impaired in models of TBI. Heldt et al. (2014) observed increased freezing behavior in mice throughout cued extinction on consecutive days with a blast model, and Schneider et al. (2016) observed increased freezing behavior in contextual fear extinction in a CCI model of mice. Finally, a recent study combined a stress model (social defeat) with a closed-head weight drop model of TBI and found increased freezing during contextual extinction in social defeat/injury and no stress / injury groups versus the control / sham group (Davies et al., 2016).

Two recent experiments have combined stress models with TBI models and fear conditioning with inconsistent results. As mentioned above, Davies et al. (2016) used a model of social defeat stress and found increased freezing during contextual extinction testing in both injured rats and stress rats versus controls. They tested fear extinction on 2 more consecutive days, and found that only the combined stress and injury group showed impairments in two subsequent rounds of extinction testing (similar to extinction retention testing), suggesting that stress and injury together may cause deficits in extinction retention. Additionally, Ojo et al. (2014) combined an external CCI model with a 21-day unpredictable stress paradigm with predatory odor and footshock. Using contextual and cued fear acquisition testing nine days after fear conditioning, they found

increased freezing in the stress and stress-mTBI groups versus controls (Ojo et al., 2014). Extinction was not tested. More investigation clearly needs to be done on the combinatorial effects of stress and brain injury, especially in a model of PTSD with cross-validation to behavioral differences in human PTSD patients and with a fear-associated learning paradigm that has been shown to properly assess these differences.

Neural Circuits and Regions Implicated in mTBI, PTSD and Fear Conditioning

Several brain regions that have been implicated in fear-associated learning in healthy subjects have been found to be affected in mTBI and PTSD patients. As established earlier, dACC and insula are integral regions in the production and expression of the fear response, and differences in both of these regions have been observed in fear-associated learning studies in PTSD patients (Linnman et al., 2011, Sripada et al., 2013, Garfinkel et al., 2014, Shvil et al., 2014). Additionally, dACC and insula are commonly implicated in fMRI studies of mTBI (Smits et al., 2009, Eierud et al., 2014), and measures of brain damage and altered connectivity following mTBI most often pinpoint frontal tracts nearby these regions, including the genu of the corpus callosum and cingulum tracts (Eierud et al., 2014, Mayer et al., 2015).

Amygdala has been shown to be involved in both fear expression throughout fear associated learning, as well as encoding of CS-US contingency in both fear acquisition and extinction (Maren et al., 2013, Greco and Liberzon, 2016). Reduced amygdalar volume has been observed in studies of mTBI patients (Depue et al., 2014, Zagorchev et al., 2016), and a variety of neurobiological and neurophysiological changes have

been reported in amygdala in animal models of TBI. Unfortunately, these changes are inconsistent, with studies finding increased NMDA receptor expression in BLA (Reger et al., 2012) and increased neuronal cell numbers in BLA (Meyer et al., 2012) while others observed decreased numbers of excitatory neurons in BLA (Heldt et al., 2014) and decreased excitability in BLA (Palmer et al., 2016). Nonetheless, the implication of amygdala in studies of mTBI, as well as numerous studies of PTSD (Milad et al., 2009, Linnman et al., 2011, Sripada et al., 2013, Garfinkel et al., 2014), suggest that changes in amygdala following mTBI may be associated with differences in fear learning.

Hippocampus is another key node in the fear-associated learning circuitry that has been implicated in PTSD, animal models of mTBI and limited studies of patients with mTBI. Like the amygdala, the hippocampus has been hypothesized to be involved throughout fear-associated learning, particularly in the encoding of the context (both danger context in acquisition and safety context in extinction) and later retrieval of context-dependent information (Maren et al., 2013). The hippocampus is intimately linked with amygdala in fear-associated learning, and contextual comparisons made in the hippocampus are thought to activate the CS+/US and CS-/no US associations in amygdala (Maren et al., 2013). Like dACC, insula and amygdala, differences in BOLD signal in hippocampus have been observed in fear-associated learning studies in PTSD patients (Milad et al., 2009, Linnman et al., 2011, Sripada et al., 2013). mTBI has been associated with decreased hippocampal volume (Zagorchev et al., 2016), and interestingly, one study found that TBI patients who developed mood disorders had smaller hippocampal volumes than TBI patients who did not have mood disturbances (Jorge et al., 2007). In animal models of TBI, a number of different changes have been

observed in hippocampus, including neuronal cell loss (Colicos et al., 1996, Kim et al., 2001, Meyer et al., 2012, Aungst et al., 2014), deficient long-term potentiation (Schwarzbach et al., 2006, Atkins, 2011, Aungst et al., 2014) and decreased glucocorticoid receptor concentration (Gao et al., 2012, Griesbach et al., 2012). Changes in hippocampal neurogenesis and differences in hippocampal activation may be associated with deficient contextual encoding in mTBI and fear-associated learning deficits that could lead to increased posttraumatic stress symptoms.

Hypothesis and Aims

Based on the evidence presented above and the strong links between the mTBI and PTSD reviewed earlier, we hypothesized that mTBI leads to deficits in fear-associated learning that predispose patients to PTSD development following brain injury. We particularly anticipated deficits in extinction retention, as this process is most commonly affected in PTSD patients. We were interested in studying all phases of fear learning, as no previous work had been performed in human mTBI subjects and, as shown above, results in animal models of brain injury have not been entirely inconsistent. We thus allowed for the possibility that differences in other aspects of fear-associated learning (i.e. impaired fear extinction or strengthened fear acquisition) could contribute to the increased posttraumatic symptoms seen in patients with mild traumatic brain injury.

Additionally, we were interested in whether combining models of PTSD and TBI would lead to synergistic effects that further impact fear-associated learning, especially extinction retention. At the time that these experiments were planned, no other research

had been published combining stress models with fear conditioning, and even now, the results from the two experiments using stress and TBI together have been inconsistent. More investigation clearly needs to be done on the combinatorial effects of stress and brain injury, especially in a model of PTSD with cross-validation to behavioral differences in human PTSD patients and with a fear-associated learning paradigm that has been shown to properly assess these differences.

Further, we hypothesized that changes in brain activation, histological measures of trauma and inflammation, and changes in neurogenesis, particularly in dACC, insula, amygdala and HC would underlie these dysfunctions in fear-associated learning. We assessed these alterations in rodent models of PTSD and mTBI, and in patients with a recent history of brain injury. Over a decade of work in our laboratory has been devoted to the SPS model of PTSD (Liberzon et al., 1997, Liberzon et al., 1999, Yamamoto et al., 2009), and in the completed studies, we combined SPS with a well-established model of mTBI, controlled cortical impact (CCI) (Dixon et al., 1991). In CCI, a craniotomy is performed to temporarily open the skull of the animal, and the brain is directly impacted using a pneumatic or electromagnetic impactor. We selected CCI as our model of brain injury because it allowed precise control over injury severity and consistency in injury between animals, and CCI is a well-tolerated surgery (Xiong et al., 2013). Mortality rates and rates of neurological symptoms like seizures are higher with other models like LFP and weight-drop models, and we wanted to reduce these effects, especially when combining brain injury with a model of PTSD in a novel paradigm. In the planning of our experiments, we made sure to use parameters known to cause no permanent lesions or complete neuronal loss. High severity of injury may eliminate fear-

associated learning deficits in animal models, which could explain negative results seen in previous research (Sierra-Mercado et al., 2015). There were concerns about using a model with a fairly localized impact over the parietal cortex, but using established parameters was prioritized over moving the impact point slightly more frontally. Finally, data from previous experiments in varying injury models showed that no model had consistent results, and thus the variability in the data from fear learning could not solely be linked to differences between models.

In Experiment 1, which employed animal models, we investigated whether animals with mild brain injury produced by CCI showed changes in conditioned freezing behavior during fear conditioning and extinction. Based upon our hypothesis, we predicted that brain-injured rodents would exhibit deficits throughout the extinction phase and in extinction retention. In Experiment 2, we investigated the combined effects of CCI and SPS on fear learning and extinction, again in animals. We predicted that CCI exposure would increase freezing behavior during extinction and extinction recall, and we expected that CCI and SPS would have synergistic effects, with CCI and SPS inducing larger extinction recall deficits than CCI or SPS alone. Additionally, we characterized the injury, including measures of neuronal cell loss, inflammatory response and changes in hippocampal neurogenesis associated with the CCI model (with and without SPS). We wanted to ensure that our level of injury was appropriate for a model of mTBI, as well as determining whether differences in inflammatory response to the injury were associated with behavioral differences. We also correlated changes in hippocampal neurogenesis with behavioral freezing measures of fear-associated

learning to test whether there is a change in contextual encoding or retrieval associated with the numerous hippocampal deficits previously observed in animal models of mTBI.

Next, we tested our hypotheses in human subjects with a recent history of mTBI. We used an in-scanner fear conditioning, extinction and extinction recall paradigm and included expectancy scores and skin conductance response (SCR) as behavioral measures of fear-associated learning. Human subjects were recruited in the emergency department and assessed within two weeks in order to determine whether patients with recent mTBI showed behavioral differences indicative of differences in fear-associated learning. We expected that recent head injury would lead to increased SCR during extinction and extinction retention. Further, fMRI was used to establish neural correlates of fear-associated learning deficits in the same sample of mTBI patients. The in-scanner fear conditioning, extinction and extinction recall tests were used to assess changes in BOLD signal in mTBI subjects and controls. Based upon our understanding of the fear-associated learning neurocircuitry and regions commonly affected by mTBI, we expected that patients with recent mTBI would show greater activation in dACC, insula and amygdala versus controls during extinction and extinction recall. These findings would be indicative of an increased fear response during periods of fear-associated learning that should be associated with safety. We also expected less hippocampal activation during extinction recall in these patients, as previous research suggests that the hippocampus is involved in the retrieval of the extinction memory. We included the multisource interference task (MSIT), a standard, validated task that reliably activates the cingulofrontoparietal attention network (Bush and Shin, 2006), in order to assess attentional deficits in our sample and determine if differences in attention could be

partially responsible for any deficits in fear-associated learning we might observe, as well as differences in corresponding activation.

With the knowledge gained through these experiments, we hope to inform future molecular biology and electrophysiology experiments in rodent models and identify aspects of fear learning and extinction to examine in future studies of patients with both mTBI and PTSD. Future experiments building upon the findings observed here should aid in the development of therapeutic and preventative strategies for the significant public health challenge of mTBI and the associated development of PTSD.

References

Alvarez RP, Biggs A, Chen G, Pine DS, Grillon C (2008) Contextual fear conditioning in humans: cortical-hippocampal and amygdala contributions. *J Neurosci* **28**:6211-6219.

American Psychiatric Association (2013) *Diagnostic and statistical manual of mental disorders* (5th ed.).

Andreatta M, Fendt M, Muhlberger A, Wieser MJ, Imobersteg S, Yarali A, Gerber B, Pauli P (2012) Onset and offset of aversive events establish distinct memories requiring fear and reward networks. *Learn Mem* **19**:518-526.

Andreatta M, Glotzbach-Schoon E, Muhlberger A, Schulz SM, Wiemer J, Pauli P (2015) Initial and sustained brain responses to contextual conditioned anxiety in humans. *Cortex* **63**:352-363.

Armony JL, Dolan RJ (2002) Modulation of spatial attention by fear-conditioned stimuli: an event-related fMRI study. *Neuropsychologia* **40**:817-826.

Atkins CM (2011) Decoding Hippocampal Signaling Deficits after Traumatic Brain Injury. *Transl Stroke Res* **2**:546-555.

Aungst SL, Kabadi SV, Thompson SM, Stoica BA, Faden AI (2014) Repeated mild traumatic brain injury causes chronic neuroinflammation, changes in hippocampal synaptic plasticity, and associated cognitive deficits. *J Cereb Blood Flow Metab* **34**:1223-1232.

Bach DR, Weiskopf N, Dolan RJ (2011) A stable sparse fear memory trace in human amygdala. *J Neurosci* **31**:9383-9389.

Balderston NL, Schultz DH, Baillet S, Helmstetter FJ (2014) Rapid amygdala responses during trace fear conditioning without awareness. *PLoS One* **9**:e96803.

Boll S, Gamer M, Gluth S, Finsterbusch J, Buchel C (2013) Separate amygdala subregions signal surprise and predictiveness during associative fear learning in humans. *Eur J Neurosci* **37**:758-767.

Bonne O, Vythilingam M, Inagaki M, Wood S, Neumeister A, Nugent AC, Snow J, Luckenbaugh DA, Bain EE, Drevets WC, Charney DS (2008) Reduced posterior hippocampal volume in posttraumatic stress disorder. *J Clin Psychiatry* **69**:1087-1091.

Bryant RA, Harvey AG (1998) Relationship between acute stress disorder and posttraumatic stress disorder following mild traumatic brain injury. Am J Psychiatry 155:625-629.

Bryant RA, Marosszeky JE, Crooks J, Gurka JA (2000) Posttraumatic stress disorder after severe traumatic brain injury. Am J Psychiatry 157:629-631.

Bryant RA, O'Donnell, M.L., Creamer, M., McFarlane, A.C., Clark, C.R., Silove, D. (2010) The Psychiatric Sequelae of Traumatic Injury. *Am J Psychiatry* **167:**312-320.

Buchel C, Dolan RJ, Armony JL, Friston KJ (1999) Amygdala-hippocampal involvement in human aversive trace conditioning revealed through event-related functional magnetic resonance imaging. *J Neurosci* **19**:10869-10876.

Buchel C, Morris J, Dolan RJ, Friston KJ (1998) Brain systems mediating aversive conditioning: an event-related fMRI study. *Neuron* **20**:947-957.

Bush G, Shin LM (2006) The Multi-Source Interference Task: an fMRI task that reliably activates the cingulo-frontal-parietal cognitive/attention network. *Nat Protoc* **1**:308-313.

Cacciaglia R, Pohlack ST, Flor H, Nees F (2014) Dissociable roles for hippocampal and amygdalar volume in human fear conditioning. *Brain Struct Funct* **220**:2575-86.

Carroll LJ, Cassidy JD, Holm L, Kraus J, Coronado VG (2004) Methodological issues and research recommendations for mild traumatic brain injury: the WHO Collaborating Centre Task Force on Mild Traumatic Brain Injury. *J Rehabil Med* 113-125.

Colicos MA, Dixon CE, Dash PK (1996) Delayed, selective neuronal death following experimental cortical impact injury in rats: possible role in memory deficits. *Brain Res* **739**:111-119.

Corrigan JD, Anbesaw, W.S., Orman, J.A. (2010) The Epidemiology of Traumatic Brain Injury. *J Head Trauma Rehabil* **25**:72–80.

Critchley HD, Mathias CJ, Dolan RJ (2002) Fear conditioning in humans: the influence of awareness and autonomic arousal on functional neuroanatomy. *Neuron* **33**:653-663.

Davies DR, Olson D, Meyer DL, Scholl JL, Watt MJ, Manzerra P, Renner KJ, Forster GL (2016) Mild Traumatic Brain Injury with Social Defeat Stress Alters Anxiety, Contextual Fear Extinction, and Limbic Monoamines in Adult Rats. *Front Behav Neurosci* **10**.

Davis M (1992) The role of the amygdala in fear and anxiety. *Annual review of neuroscience* **15**:353-375.

Depue BE, Olson-Madden JH, Smolker HR, Rajamani M, Brenner LA, Banich MT (2014) Reduced amygdala volume is associated with deficits in inhibitory control: a voxel- and surface-based morphometric analysis of comorbid PTSD/mild TBI. *Biomed Res Int* **2014**:691505.

Diekhof EK, Geier K, Falkai P, Gruber O (2011) Fear is only as deep as the mind allows: A coordinate-based meta-analysis of neuroimaging studies on the regulation of negative affect. *NeuroImage* **58**:275-285.

Dixon CE, Clifton GL, Lighthall JW, Yaghmai AA, Hayes RL (1991) A controlled cortical impact model of traumatic brain injury in the rat. *J Neurosci Methods* **39**:253-262.

Dunsmoor JE, Bandettini PA, Knight DC (2008) Neural correlates of unconditioned response diminution during Pavlovian conditioning. *Neuroimage* **40**:811-817.

Eierud C, Craddock RC, Fletcher S, Aulakh M, King-Casas B, Kuehl D, LaConte SM (2014) Neuroimaging after mild traumatic brain injury: Review and meta-analysis(). *NeuroImage: Clinical* **4**:283-294.

Eippert F, Gamer M, Buchel C (2012) Neurobiological mechanisms underlying the blocking effect in aversive learning. *J Neurosci* **32**:13164-13176.

Elder GA, Dorr NP, De Gasperi R, Gama Sosa MA, Shaughness MC, Maudlin-Jeronimo E, Hall AA, McCarron RM, Ahlers ST (2012) Blast exposure induces post-traumatic stress disorder-related traits in a rat model of mild traumatic brain injury. *J Neurotrauma* **29**:2564-2575.

Etkin A, Egner T, Kalisch R (2011) Emotional processing in anterior cingulate and medial prefrontal cortex. *Trends in cognitive sciences* **15**:85-93.

Fani N, Tone EB, Phifer J, Norrholm SD, Bradley B, Ressler KJ, Kamkwalala A, Jovanovic T (2012) Attention bias toward threat is associated with exaggerated fear expression and impaired extinction in PTSD. *Psychol Med* **42**:533-543.

Feng P, Feng T, Chen Z, Lei X (2014) Memory consolidation of fear conditioning: bi-stable amygdala connectivity with dorsal anterior cingulate and medial prefrontal cortex. *Soc Cogn Affect Neurosci* **9**:1730-1737.

Fullana MA, Harrison BJ, Soriano-Mas C, Vervliet B, Cardoner N, Avila-Parcet A, Radua J (2015) Neural signatures of human fear conditioning: an updated and extended meta-analysis of fMRI studies. *Mol Psychiatry* **21**: 500-508.

Furmark T, Fischer H, Wik G, Larsson M, Fredrikson M (1997) The amygdala and individual differences in human fear conditioning. *Neuroreport* **8**:3957-3960.

Gao W, Xu H, Liang M, Huang JH, He X (2012) Association between reduced expression of hippocampal glucocorticoid receptors and cognitive dysfunction in a rat model of traumatic brain injury due to lateral head acceleration. *Neurosci Lett* **533**: 50-54.

Garfinkel SN, Abelson JL, King AP, Sripada RK, Wang X, Gaines LM, Liberzon I (2014) Impaired contextual modulation of memories in PTSD: an fMRI and psychophysiological study of extinction retention and fear renewal. *J Neurosci* **34**:13435-13443.

Genovese RF, Simmons LP, Ahlers ST, Maudlin-Jeronimo E, Dave JR, Boutte AM (2013) Effects of mild TBI from repeated blast overpressure on the expression and extinction of conditioned fear in rats. *Neuroscience* **254**:120-129.

Gilbertson MW, Williston SK, Paulus LA, Lasko NB, Gurvits TV, Shenton ME, Pitman RK, Orr SP (2007) Configural cue performance in identical twins discordant for posttraumatic stress disorder: theoretical implications for the role of hippocampal function. *Biol Psychiatry* **62**:513-520.

Gottfried JA, Dolan RJ (2004) Human orbitofrontal cortex mediates extinction learning while accessing conditioned representations of value. *Nat Neurosci* **7**:1144-1152.

Gramsch C, Kattoor J, Icenhour A, Forsting M, Schedlowski M, Gizewski ER, Elsenbruch S (2014) Learning pain-related fear: neural mechanisms mediating rapid differential conditioning, extinction and reinstatement processes in human visceral pain. *Neurobiol Learn Mem* **116**:36-45.

Greco JA, Liberzon I (2016) Neuroimaging of Fear-Associated Learning. *Neuropsychopharmacology* **41**:320-334.

Griesbach GS, Tio DL, Vincelli J, McArthur DL, Taylor AN (2012) Differential effects of voluntary and forced exercise on stress responses after traumatic brain injury. *J Neurotrauma* **29**:1426-1433.

Hartley CA, Fischl B, Phelps EA (2011) Brain structure correlates of individual differences in the acquisition and inhibition of conditioned fear. *Cereb Cortex* **21**:1954-1962.

Heldt SA, Elberger AJ, Deng Y, Guley NH, Del Mar N, Rogers J, Choi GW, Ferrell J, Rex TS, Honig MG, Reiner A (2014) A novel closed-head model of mild traumatic brain injury caused by primary overpressure blast to the cranium produces sustained emotional deficits in mice. *Front Neurol* **5**:2.

Hickling EJ, Gillen R, Blanchard EB, Buckley T, Taylor A (1998) Traumatic brain injury and posttraumatic stress disorder: a preliminary investigation of neuropsychological test results in PTSD secondary to motor vehicle accidents. Brain Inj 12:265-274.

Hoge CW, McGurk D, Thomas JL, Cox AL, Engel CC, Castro CA (2008) Mild Traumatic Brain Injury in U.S. Soldiers Returning from Iraq. *New England Journal of Medicine* **358**:453-463.

Jorge RE, Acion L, Starkstein SE, Magnotta V (2007) Hippocampal volume and mood disorders after traumatic brain injury. *Biol Psychiatry* **62**:332-338.

Kalisch R, Korenfeld E, Stephan KE, Weiskopf N, Seymour B, Dolan RJ (2006) Context-dependent human extinction memory is mediated by a ventromedial prefrontal and hippocampal network. *J Neurosci* **26**:9503-9511.

Kattoor J, Gizewski ER, Kotsis V, Benson S, Gramsch C, Theysohn N, Maderwald S, Forsting M, Schedlowski M, Elsenbruch S (2013) Fear conditioning in an abdominal pain model: neural responses during associative learning and extinction in healthy subjects. *PLoS One* **8**:e51149.

Kattoor J, Thurling M, Gizewski ER, Forsting M, Timmann D, Elsenbruch S (2014) Cerebellar contributions to different phases of visceral aversive extinction learning. *Cerebellum* **13**:1-8.

Kessler RC, Sonnega A, Bromet E, Hughes M, Nelson CB (1995) Posttraumatic stress disorder in the National Comorbidity Survey. *Arch Gen Psychiatry* **52**:1048-1060.

Kim BT, Rao VL, Sailor KA, Bowen KK, Dempsey RJ (2001) Protective effects of glial cell line-derived neurotrophic factor on hippocampal neurons after traumatic brain injury in rats. *J Neurosurg* **95**:674-679.

Klemenhagen KC, O'Brien SP, Brody DL (2013) Repetitive concussive traumatic brain injury interacts with post-injury foot shock stress to worsen social and depression-like behavior in mice. *PLoS One* **8**:e74510.

Knight DC, Nguyen HT, Bandettini PA (2005) The role of the human amygdala in the production of conditioned fear responses. *Neuroimage* **26**:1193-1200.

Knight DC, Waters NS, Bandettini PA (2009) Neural substrates of explicit and implicit fear memory. *Neuroimage* **45**:208-214.

Knight DC, Waters NS, King MK, Bandettini PA (2010) Learning-related diminution of unconditioned SCR and fMRI signal responses. *Neuroimage* **49**:843-848.

Knox D, George SA, Fitzpatrick CJ, Rabinak CA, Maren S, Liberzon I (2012a) Single prolonged stress disrupts retention of extinguished fear in rats. *Learning & Memory* **19**:43-49.

Knox D, Nault T, Henderson C, Liberzon I (2012b) Glucocorticoid receptors and extinction retention deficits in the single prolonged stress model. *Neuroscience* **223**:163-173.

Koenigs M, Huey ED, Raymont V, Cheon B, Solomon J, Wassermann EM, Grafman J (2008) Focal brain damage protects against post-traumatic stress disorder in combat veterans. *Nat Neurosci* **11**:232-237.

Kuhn S, Gallinat J (2013) Gray matter correlates of posttraumatic stress disorder: a quantitative meta-analysis. *Biol Psychiatry* **73**:70-74.

LaBar KS, Gatenby JC, Gore JC, LeDoux JE, Phelps EA (1998) Human amygdala activation during conditioned fear acquisition and extinction: a mixed-trial fMRI study. *Neuron* **20**:937-945.

Lagarde E, Salmi L, Holm LW, et al. (2014) ASsociation of symptoms following mild traumatic brain injury with posttraumatic stress disorder vs postconcussion syndrome. *JAMA Psychiatry* **71**:1032-1040.

Levin HS, Brown SA, Song JX, McCauley SR, Boake C, Contant CF, Goodman H, Kotrla KJ (2001) Depression and posttraumatic stress disorder at three months after mild to moderate traumatic brain injury. J Clin Exp Neuropsychol 23:754-769.

Li H, Penzo MA, Taniguchi H, Kopec CD, Huang ZJ, Li B (2013) Experience-dependent modification of a central amygdala fear circuit. *Nat Neurosci* **16**:332-339.

Liberzon I, Krstov M, Young EA (1997) Stress-restress: effects on ACTH and fast feedback. *Psychoneuroendocrinology* **22**:443-453.

Liberzon I, Lopez JF, Flagel SB, Vazquez DM, Young EA (1999) Differential regulation of hippocampal glucocorticoid receptors mRNA and fast feedback: relevance to post-traumatic stress disorder. *J Neuroendocrinol* **11**:11-17.

Linnman C, Zeffiro TA, Pitman RK, Milad MR (2011) An fMRI study of unconditioned responses in post-traumatic stress disorder. *Biol Mood Anxiety Disord* **1**:8.

Linnman C, Zeidan MA, Furtak SC, Pitman RK, Quirk GJ, Milad MR (2012a) Resting amygdala and medial prefrontal metabolism predicts functional activation of the fear extinction circuit. *Am J Psychiatry* **169**:415-423.

Linnman C, Zeidan MA, Pitman RK, Milad MR (2012b) Resting cerebral metabolism correlates with skin conductance and functional brain activation during fear conditioning. *Biol Psychol* **89**:450-459.

Ma Q, Huang Y, Wang L (2013) Left prefrontal activity reflects the ability of vicarious fear learning: a functional near-infrared spectroscopy study. *ScientificWorldJournal* **2013**:652542.

Maier S, Szalkowski A, Kamphausen S, Perlov E, Feige B, Blechert J, Philipsen A, van Elst LT, Kalisch R, Tuscher O (2012) Clarifying the role of the rostral dmPFC/dACC in fear/anxiety: learning, appraisal or expression? *PLoS One* **7**:e50120.

Maren S, Phan KL, Liberzon I (2013) The contextual brain: implications for fear conditioning, extinction and psychopathology. *Nat Rev Neurosci* **14**:417-428.

Maren S, Quirk GJ (2004) Neuronal signalling of fear memory. *Nat Rev Neurosci* **5**:844-852.

Marschner A, Kalisch R, Vervliet B, Vansteenwegen D, Buchel C (2008) Dissociable roles for the hippocampus and the amygdala in human cued versus context fear conditioning. *J Neurosci* **28**:9030-9036.

Mayer AR, Bellgowan PSF, Hanlon FM (2015) Functional magnetic resonance imaging of mild traumatic brain injury. *Neuroscience & Biobehavioral Reviews* **49**:8-18.

McMillan TM (1991) Post-traumatic stress disorder and severe head injury. Br J Psychiatry 159:431-433.

McMillan TM (1996) Post-traumatic stress disorder following minor and severe closed head injury: 10 single cases. Brain Inj 10:749-758.

Mechias ML, Etkin A, Kalisch R (2010) A meta-analysis of instructed fear studies: implications for conscious appraisal of threat. *Neuroimage* **49**:1760-1768.

Meyer DL, Davies DR, Barr JL, Manzerra P, Forster GL (2012) Mild traumatic brain injury in the rat alters neuronal number in the limbic system and increases conditioned fear and anxiety-like behaviors. *Exp Neurol* **235**:574-587.

Milad MR, Orr SP, Lasko NB, Chang Y, Rauch SL, Pitman RK (2008) Presence and acquired origin of reduced recall for fear extinction in PTSD: results of a twin study. *J Psychiatr Res* **42**:515-520.

Milad MR, Pitman RK, Ellis CB, Gold AL, Shin LM, Lasko NB, Zeidan MA, Handwerger K, Orr SP, Rauch SL (2009) Neurobiological basis of failure to recall extinction memory in posttraumatic stress disorder. *Biol Psychiatry* **66**:1075-1082.

Milad MR, Quinn BT, Pitman RK, Orr SP, Fischl B, Rauch SL (2005) Thickness of ventromedial prefrontal cortex in humans is correlated with extinction memory. *Proc Natl Acad Sci U S A* **102**:10706-10711.

Milad MR, Quirk GJ, Pitman RK, Orr SP, Fischl B, Rauch SL (2007) A role for the human dorsal anterior cingulate cortex in fear expression. *Biol Psychiatry* **62**:1191-1194.

Mollica RF, Henderson DC, Tor S (2002) Psychiatric effects of traumatic brain injury events in Cambodian survivors of mass violence. *Br J Psychiatry* **181**:339-347.

Morissette SB, Woodward M, Kimbrel NA, Meyer EC, Kruse MI, Dolan S, Gulliver SB (2011) Deployment-related TBI, persistent postconcussive symptoms, PTSD, and depression in OEF/OIF veterans. *Rehabil Psychol* **56**:340-350.

Moses SN, Houck JM, Martin T, Hanlon FM, Ryan JD, Thoma RJ, Weisend MP, Jackson EM, Pekkonen E, Tesche CD (2007) Dynamic neural activity recorded from human amygdala during fear conditioning using magnetoencephalography. *Brain Res Bull* **71**:452-460.

Motzkin JC, Philippi CL, Wolf RC, Baskaya MK, Koenigs M (2015) Ventromedial prefrontal cortex is critical for the regulation of amygdala activity in humans. *Biol Psychiatry* **77**:276-284.

Mueller EM, Panitz C, Hermann C, Pizzagalli DA (2014) Prefrontal oscillations during recall of conditioned and extinguished fear in humans. *J Neurosci* **34**:7059-7066.

National Center for Injury Prevention and Control (2003) *Report to Congress on Mild Traumatic Brain Injury in the United States: Steps to Prevent a Serious Public Health Problem*. Atlanta, GA: Centers for Disease Control and Prevention.

Ojo JO, Greenberg MB, Leary P, Mouzon B, Bachmeier C, Mullan M, Diamond DM, Crawford F (2014) Neurobehavioral, neuropathological and biochemical profiles in a novel mouse model of co-morbid post-traumatic stress disorder and mild traumatic brain injury. *Front Behav Neurosci* **8**:213.

Olsson A, Nearing KI, Phelps EA (2007) Learning fears by observing others: the neural systems of social fear transmission. *Soc Cogn Affect Neurosci* **2**:3-11.

Palmer CP, Metheny HE, Elkind JA, Cohen AS (2016) Diminished amygdala activation and behavioral threat response following traumatic brain injury. *Experimental Neurology* **277**:215-226.

Penzo MA, Robert V, Li B (2014) Fear conditioning potentiates synaptic transmission onto long-range projection neurons in the lateral subdivision of central amygdala. *J Neurosci* **34**:2432-2437.

Penzo MA, Robert V, Tucciarone J, De Bundel D, Wang M, Van Aelst L, Darvas M, Parada LF, Palmiter RD, He M, Huang ZJ, Li B (2015) The paraventricular thalamus controls a central amygdala fear circuit. *Nature* **519**:455-459.

Phelps EA, Delgado MR, Nearing KI, LeDoux JE (2004) Extinction learning in humans: role of the amygdala and vmPFC. *Neuron* **43**:897-905.

Pohlack ST, Nees F, Liebscher C, Cacciaglia R, Diener SJ, Ridder S, Woermann FG, Flor H (2012a) Hippocampal but not amygdalar volume affects contextual fear conditioning in humans. *Hum Brain Mapp* **33**:478-488.

Pohlack ST, Nees F, Ruttorf M, Schad LR, Flor H (2012b) Activation of the ventral striatum during aversive contextual conditioning in humans. *Biol Psychol* **91**:74-80.

Preston AR, Eichenbaum H (2013) Interplay of hippocampus and prefrontal cortex in memory. *Curr Biol* **23**:R764-773.

Quirk GJ, Likhtik E, Pelletier JG, Paré D (2003) Stimulation of medial prefrontal cortex decreases the responsiveness of central amygdala output neurons. *The Journal of Neuroscience* **23**:8800-8807.

Quirk GJ, Mueller D (2008) Neural Mechanisms of Extinction Learning and Retrieval. *Neuropsychopharmacology* **33**:56-72.

Reger ML, Poulos AM, Buen F, Giza CC, Hovda DA, Fanselow MS (2012) Concussive brain injury enhances fear learning and excitatory processes in the amygdala. *Biol Psychiatry* **71**:335-343.

Reinhardt I, Jansen A, Kellermann T, Schuppen A, Kohn N, Gerlach AL, Kircher T (2010) Neural correlates of aversive conditioning: development of a functional imaging paradigm for the investigation of anxiety disorders. *Eur Arch Psychiatry Clin Neurosci* **260**:443-453.

Rogers MA, Yamasue H, Abe O, Yamada H, Ohtani T, Iwanami A, Aoki S, Kato N, Kasai K (2009) Smaller amygdala volume and reduced anterior cingulate gray matter density associated with history of post-traumatic stress disorder. *Psychiatry Res* **174**:210-216.

Rosenbaum SB, Lipton ML (2012) Embracing chaos: the scope and importance of clinical and pathological heterogeneity in mTBI. *Brain Imaging and Behavior* **6**:255-282.

Rosenkranz JA, Moore H, Grace AA (2003) The prefrontal cortex regulates lateral amygdala neuronal plasticity and responses to previously conditioned stimuli. *The Journal of Neuroscience* **23**:11054-11064.

Rougemont-Bucking A, Linnman C, Zeffiro TA, Zeidan MA, Lebron-Milad K, Rodriguez-Romaguera J, Rauch SL, Pitman RK, Milad MR (2011) Altered processing of contextual information during fear extinction in PTSD: an fMRI study. *CNS Neurosci Ther* **17**:227-236.

Sbordone RJ, Liter JC (1995) Mild traumatic brain injury does not produce post-traumatic stress disorder. Brain Inj 9:405-412.

Schiller D, Levy I, Niv Y, LeDoux JE, Phelps EA (2008) From fear to safety and back: reversal of fear in the human brain. *J Neurosci* **28**:11517-11525.

Schneider BL, Ghoddoussi F, Charlton JL, Kohler RJ, Galloway MP, Perrine SA, Conti AC (2016) Increased Cortical Gamma-Aminobutyric Acid Precedes Incomplete Extinction of Conditioned Fear and Increased Hippocampal Excitatory Tone in a Mouse Model of Mild Traumatic Brain Injury. *J Neurotrauma*.

Schneiderman AI, Braver ER, Kang HK (2008) Understanding sequelae of injury mechanisms and mild traumatic brain injury incurred during the conflicts in Iraq and Afghanistan: persistent postconcussive symptoms and posttraumatic stress disorder. *Am J Epidemiol* **167**:1446-1452.

Schultz DH, Balderston NL, Helmstetter FJ (2012) Resting-state connectivity of the amygdala is altered following Pavlovian fear conditioning. *Front Hum Neurosci* **6**:242.

Schwarzbach E, Bonislawski D, Xiong G, Cohen A (2006) Mechanisms Underlying the Inability to Induce Area CA1 LTP in the Mouse After Traumatic Brain Injury. *Hippocampus* **16**:541-550.

Shenton ME, Hamoda HM, Schneiderman JS, Bouix S, Pasternak O, Rathi Y, Vu MA, Purohit MP, Helmer K, Koerte I, Lin AP, Westin CF, Kikinis R, Kubicki M, Stern RA, Zafonte R (2012) A review of magnetic resonance imaging and diffusion tensor imaging findings in mild traumatic brain injury. *Brain Imaging Behav* **6**:137-192.

Shvil E, Sullivan GM, Schafer S, Markowitz JC, Campeas M, Wager TD, Milad MR, Neria Y (2014) Sex differences in extinction recall in posttraumatic stress disorder: a pilot fMRI study. *Neurobiol Learn Mem* **113**:101-108.

Sierra-Mercado D, McAllister LM, Lee CC, Milad MR, Eskandar EN, Whalen MJ (2015) Controlled cortical impact before or after fear conditioning does not affect fear extinction in mice. *Brain Res* **1606**:133-141.

Smits M, Dippel DW, Houston GC, Wielopolski PA, Koudstaal PJ, Hunink MG, van der Lugt A (2009) Postconcussion syndrome after minor head injury: brain activation of working memory and attention. *Hum Brain Mapp* **30**:2789-2803.

Sripada RK, Garfinkel SN, Liberzon I (2013) Avoidant symptoms in PTSD predict fear circuit activation during multimodal fear extinction. *Front Hum Neurosci* **7**:672.

Stein MB, Kessler RC, Heeringa SG, Jain S, Campbell-Sills L, Colpe LJ, Fullerton CS, Nock MK, Sampson NA, Schoenbaum M, Sun X, Thomas ML, Ursano RJ, collaborators ObotAS (2015) Prospective Longitudinal Evaluation of the Effect of Deployment-Acquired Traumatic Brain Injury on Posttraumatic Stress and Related Disorders: Results From the Army Study to Assess Risk and Resilience in Servicemembers (Army STARRS). *American Journal of Psychiatry* **172**:1101-1111.

Tabbert K, Merz CJ, Klucken T, Schweckendiek J, Vaitl D, Wolf OT, Stark R (2011) Influence of contingency awareness on neural, electrodermal and evaluative responses during fear conditioning. *Soc Cogn Affect Neurosci* **6**:495-506.

Tanielian T, Jaycox LH (2008) Invisible wounds of war: Psychological and cognitive injuries, their consequences, and services to assist recovery. Santa Monica, CA: RAND Corporation.

Thurman DJ (2001) The epidemiology and economics of head trauma. In: Head Trauma: Basic, Preclinical, and Clinical Directions (Miller L, H. R., ed) New York: John Wiley and Sons.

Tye KM, Prakash R, Kim S-Y, Fenno LE, Grosenick L, Zarabi H, Thompson KR, Gradinaru V, Ramakrishnan C, Deisseroth K (2011) Amygdala circuitry mediating reversible and bidirectional control of anxiety. *Nature* **471**:358-362.

Tzschoppe J, Nees F, Banaschewski T, Barker GJ, Buchel C, Conrod PJ, Garavan H, Heinz A, Loth E, Mann K, Martinot JL, Smolka MN, Gallinat J, Strohle A, Struve M, Rietschel M, Schumann G, Flor H (2014) Aversive learning in adolescents: modulation by amygdala-prefrontal and amygdala-hippocampal connectivity and neuroticism. *Neuropsychopharmacology* **39**:875-884.

van Well S, Visser RM, Scholte HS, Kindt M (2012) Neural substrates of individual differences in human fear learning: evidence from concurrent fMRI, fear-potentiated startle, and US-expectancy data. *Cogn Affect Behav Neurosci* **12**:499-512.

Vasterling JJ, Bryant, R.A., Keane, T.M. (2012) Understanding the Interface of Traumatic Stress and Mild Traumatic Brain Injury. In: PTSD and Mild Traumatic Brain Injury (Vasterling, J. J., Bryant, R.A., Keane, T.M., ed) New York: The Guilford Press.

Vertes RP (2004) Differential projections of the infralimbic and prelimbic cortex in the rat. *Synapse* **51**:32-58.

Warren AM, Boals A, Elliott TR, Reynolds M, Weddle RJ, Holtz P, Trost Z, Foreman ML (2015) Mild traumatic brain injury increases risk for the development of posttraumatic stress disorder. *J Trauma Acute Care Surg* **79**:1062-1066.

Wheelock MD, Sreenivasan KR, Wood KH, Ver Hoef LW, Deshpande G, Knight DC (2014) Threat-related learning relies on distinct dorsal prefrontal cortex network connectivity. *Neuroimage* **102 Pt 2**:904-912.

Winkelmann T, Grimm O, Pohlack ST, Nees F, Cacciaglia R, Dinu-Biringer R, Steiger F, Wicking M, Ruttorf M, Schad LR, Flor H (2015) Brain morphology correlates of interindividual differences in conditioned fear acquisition and extinction learning. *Brain Struct Funct* **221**: 1927-1937.

Wood KH, Kuykendall D, Ver Hoef LW, Knight DC (2013) Neural substrates underlying learning-related changes of the unconditioned fear response. *Open Neuroimag J* **7**:41-52.

Wood KH, Ver Hoef LW, Knight DC (2012) Neural mechanisms underlying the conditioned diminution of the unconditioned fear response. *Neuroimage* **60**:787-799.

Xiong Y, Mahmood A, Chopp M (2013) Animal models of traumatic brain injury. *Nat Rev Neurosci* **14**:128-142.

Yamamoto S, Morinobu S, Takei S, Fuchikami M, Matsuki A, Yamawaki S, Liberzon I (2009) Single prolonged stress: toward an animal model of posttraumatic stress disorder. *Depress Anxiety* **26**:1110-1117.

Zagorchev L, Meyer C, Stehle T, Wenzel F, Young S, Peters J, Weese J, Paulsen K, Garlinghouse M, Ford J, Roth R, Flashman L, McAllister T (2016) Differences in Regional Brain Volumes Two Months and One Year after Mild Traumatic Brain Injury. *J Neurotrauma* **33**:29-34.

Phase of Fear Learning	Reference	Subjects	Key brain regions implicated
Acquisition	Linnman *et al*, 2011	19 PTSD patients, 24 trauma-exposed controls	Amygdala, dACC, HC, insula
Acquisition and extinction	Sripada *et al*, 2013	15 PTSD patients	Amygdala, HC, insula, OFG, superior MFG
[a]Acquisition and extinction	Fani *et al*, 2015	48 African-American females	ACC, cingulum, HC
Acquisition, extinction, recall	Milad *et al*, 2009	16 PTSD patients, 15 trauma-exposed controls	Amygdala, cerebellum, dACC, HC, vmPFC
Acquisition, extinction, recall	Rougemont-Bucking *et al*, 2011	18 PTSD patients, 16 trauma-exposed controls	dACC, MFG, PCC, vmPFC
[b]Acquisition, extinction, recall	Shvil *et al*, 2014	31 PTSD patients, 25 trauma-exposed controls	dACC, insula, vmPFC
Acquisition, extinction, recall, renewal	Garfinkel *et al*, 2014	14 PTSD patients, 14 combat-exposed controls	Amygdala, insula, thalamus, vmPFC

Table I.1: Results from studies of fear-associative learning in patients with posttraumatic stress disorder (PTSD). [a]Examined structural connectivity, [b]Examined sex differences. Abbreviations: ACC, anterior cingulate cortex; dACC, dorsal anterior cingulate cortex; HC, hippocampus; MFG, medial frontal gyrus; OFG, orbital frontal gyrus; PCC, posterior cingulate cortex; vmPFC, ventromedial prefrontal cortex.

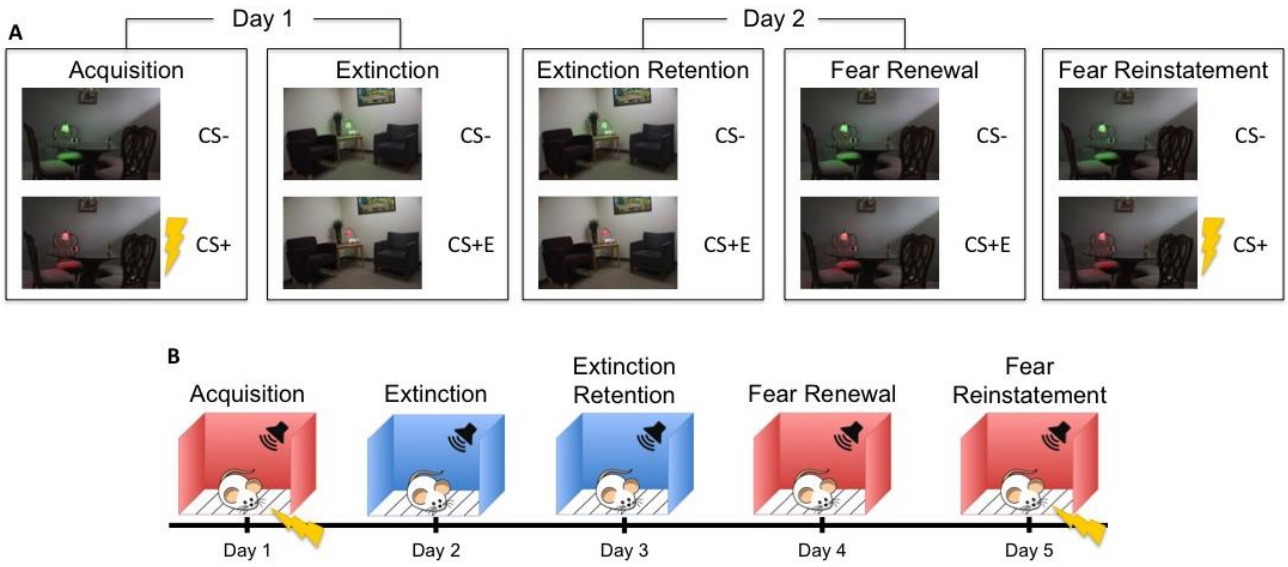

Figure I.1: Fear-associated learning paradigms in human neuroimaging (A) and rodents (B).

CHAPTER II

Impaired rates of fear extinction in a rodent model of traumatic brain injury and posttraumatic stress disorder

Introduction

The psychiatric effects of trauma are wide-ranging and considerably impact quality of life for civilian and military populations around the world. Recently, significant public attention has been drawn to traumatic brain injury (TBI), both in veterans returning from Operation Enduring Freedom and Operation Iraqi Freedom, as well as in the general population. TBI is a substantial public health challenge, annually accounting for over 1.5 million hospital visits and a lifetime cost of over 16.5 billion dollars in the United States alone (Thurman, 2001, National Center for Injury Prevention and Control, 2003). Although at least 75% of TBIs are categorized as mild traumatic brain injuries (mTBIs) that usually do not produce abnormalities that can be identified with neuroimaging, mTBI can cause lasting, debilitating neurological symptoms and has been associated with increased rates of numerous psychiatric disorders (National Center for Injury Prevention and Control, 2003, Morissette et al., 2011). These include depression, schizophrenia, bipolar disorder and posttraumatic stress disorder (PTSD) (Orlovska et al., 2014, Stein et al., 2015). The relationship between mTBI and PTSD is particularly interesting since both disorders result from exposure to a traumatic event, and may share similar etiological characteristics. Rates of PTSD are significantly higher in civilians and veterans with a history of mTBI, a relationship that has been well-

established through epidemiological research, including several major longitudinal studies (Lagarde et al., 2014, Stein et al., 2015, Warren et al., 2015).

Despite the clear association between mTBI and PTSD, the mechanism underlying this interaction is still uncertain. One possible mechanism that may link these two conditions is an alteration in fear-associated learning. Fear-associated learning (or fear conditioning, extinction, extinction retention / recall and fear renewal) has been used extensively in both animal studies and humans because it is an effective model of the acquisition and maintenance of fear responses, and altered fear learning has been hypothesized to play an important role in the development of PTSD and anxiety disorders (Greco and Liberzon, 2016). Fear-associated learning research in both animal models of PTSD (Knox et al., 2012a) and PTSD patients (Milad et al., 2009, Garfinkel et al., 2014) has particularly pinpointed impairments in extinction retention (or extinction recall).

A number of fear-associated learning studies have recently been performed in animal models of TBI, with varying results. Some studies have found no differences in fear-associated learning across both fear conditioning and extinction (Klemenhagen et al., 2013, Sierra-Mercado et al., 2015), enhanced fear acquisition has been observed in others (Elder et al., 2012, Meyer et al., 2012, Reger et al., 2012), and two studies (in the same models) have actually observed impairments in fear acquisition (Genovese et al., 2013, Palmer et al., 2016). However, three recent studies in weight drop, controlled cortical impact (CCI) and blast models of TBI found increased freezing behavior throughout fear extinction (Heldt et al., 2014, Davies et al., 2016, Schneider et al., 2016). Limited studies in combined models of stress and TBI have also produced

inconsistent results, with one study observing increased freezing during acquisition testing in animals receiving unpredictable stress with CCI (Ojo et al., 2014), and another study observing increased freezing in animals exposed to weight drop and social defeat stress during three consecutive days of extinction testing (Davies et al., 2016). These varying results do suggest that fear-associated learning is affected by mTBI, but it is unclear which aspect of learning is impaired.

The experiments described in this manuscript aimed to identify changes in fear-associated learning following the CCI model of TBI and a model of PTSD, single prolonged stress (SPS), that has produced behavioral and neurobiological changes (Knox et al., 2012a, Knox et al., 2012b) similar to those seen in PTSD patients (Milad et al., 2009, Garfinkel et al., 2014). Based upon previous findings in animal models of TBI and PTSD, we hypothesized that animals with brain injury produced by CCI would demonstrate increased freezing during extinction learning and extinction retention testing, with animals receiving both CCI and SPS showing further enhanced freezing during extinction recall. Additionally, we aimed to characterize underlying neurophysiological and inflammatory changes in these animals. We were particularly interested in whether inflammatory changes and deficits in hippocampal neurogenesis were associated with hypothesized changes in fear-associated learning. Differences in hippocampal neurogenesis that are associated with differential fear-associated learning may suggest a potential mechanism underlying these behavioral changes, and measures of the inflammatory response allow us to determine whether increased injury severity is associated with greater fear-associated learning deficits. We expected that injury would lead to increased microglial activation that would be directly correlated with

freezing behavior during extinction retention testing, and that hippocampal neurogenesis following fear-associated learning would be diminished in injured rats, with further reductions in rats who received SPS and CCI.

Methods

Subjects

Sixty-four adult male Sprague Dawley rats (42–45 days old; 150 g), were obtained from Charles River (Wilmington, MA). All rats were pair-housed and acclimated for a minimum of 5 days and were then individually housed after the CCI or sham craniotomy procedure. All rats had ad libitum access to water and standard rat chow. All experimental procedures were approved by the VA Ann Arbor Healthcare System Institutional Animal Care Usage Committee.

Controlled Cortical Impact

All animals were assigned to either an injury or sham procedure following acclimation. Rats in the injury group were anesthetized using isoflurane and remained unconscious throughout the CCI procedure. The animal was positioned in a stereotaxic frame, and a 7 mm diameter craniotomy was performed (using aseptic techniques) over the site of impact, midway between bregma and lambda, with the medial edge of the craniotomy 1 mm right of the midline (Dixon et al., 1991, Brody et al., 2007, Yu et al., 2009). A unilateral brain injury consisting of a single impact of a 5 mm impactor tip to the exposed dura mater of the animal at an angle of 15° from the vertical plane was delivered using the Impact One Head Impactor (Leica Biosystems, Buffalo Grove, IL). The impact was delivered with a 1.5 mm depth, 3.0 m/s velocity and 100 ms dwell time.

The injury parameters were selected based upon previous studies using CCI in rats and mice that delivered a mild injury (Dixon et al., 1991, Brody et al., 2007). Following surgery, the removed segment of the skull was replaced and secured, and the scalp was sutured. Rats were returned to their cages, singly housed, and monitored for any signs of pain, distress or neurological symptoms. Rats in the sham injury groups were given the same anesthesia and craniotomies, but no injury was induced. No animals exhibited neurological symptoms following surgery. Animals in both the injury and sham groups recovered in their cages, singly housed, for 7 days post-injury.

Single Prolonged Stress

All rats in Experiment 2 were assigned to a stress or control procedure before fear conditioning. The SPS procedure has been described previously (Liberzon et al., 1997, Knox et al., 2012a) and is shown in Figure II.1. Rats in the stress group were exposed to restraint for 2 hours, followed immediately by a 20 minute forced swim. Forced swimming occurred in a plastic tub (55.6-cm diameter, 45.4-cm height) filled two-thirds from the bottom with water (20–24°C). Fifteen minutes after the forced swim, rats were exposed to ether (75 mL) in a glass desiccator until full anesthesia, tested using the the toe or tail pinch response (approximately 5 min of ether exposure). Immediately after the induction of general anesthesia, rats were removed from the desiccator, housed singly, and left undisturbed for 7 days. Rats assigned to the control group were housed singly, left undisturbed, and remained in the housing colony until experimental procedures commenced.

Behavioral apparatus

All sessions were conducted in eight identical rodent observation chambers constructed of aluminum and Plexiglas (30 × 24 × 21 cm; Med Associates, Fairfax, VT), situated in sound-attenuating boxes in an isolated room. The floor of each chamber consisted of 19 stainless steel rods (4 mm in diameter) spaced 1.5 cm apart (center to center). The grid floor was connected to a shock source and a solid-state grid scrambler (Med Associates) which delivered the footshock unconditioned stimulus (US). Mounted on one wall of the chamber was a speaker to provide a distinct auditory conditioned stimulus (CS); on the opposite wall was a 15-W house light and a fan, which provided background noise (65 dB). Cameras mounted to the ceiling of the sound-attenuating chambers were used to record behavior, which was scored offline automatically using Anymaze (Stoelting Co, Wood Dale, IL).

Two unique contexts were created by manipulating auditory, visual, and olfactory cues: Context A was comprised of a 1% ammonium hydroxide solution in chambers, red light on, chamber doors open, and fans off; Context B was comprised of a 1% acetic acid solution placed in trays at the bottom of the chambers, the house light on, chamber doors closed, and fans on in the chambers.

Cued fear conditioning, extinction and extinction retention testing

On the first day of the fear conditioning paradigm, rats were transported from their home cages and placed in the conditioning context (Context A). During fear acquisition, rats received five paired presentations of a tone (10 s, 2 kHz, 80 dB) that co-terminated with a footshock (1.0 mA, 1 s) beginning 180 s after being placed in Context A (Knox et al., 2012b, (George et al., 2015). One day after conditioning, all rats underwent fear extinction. The animals were placed into a novel context (Context B)

and were presented with 30 tone presentations (10 s, 2 kHz, 80 dB, 60-s ITI), in the absence of footshock, beginning 180 s after being placed into the chambers in order to extinguish fear responding to the tone (i.e., extinction training). Two days after conditioning (post-injury day 9), all rats were placed into Context B and were presented with 10 tones beginning 180 s after being placed into the chambers in order to assess extinction retention.

Experiment 1: Controlled cortical impact, cued fear conditioning, extinction and extinction retention testing

On post-injury day 7, 16 rats (injury = 8; control = 8) were transported from their home cages in groups of eight and underwent the fear conditioning paradigm described above. 24 hours following the completion of extinction retention testing, all 16 animals were euthanized. The procedure for Experiment 1 is shown in Figure II.2A.

Experiment 2: Controlled cortical impact, single prolonged stress, cued fear conditioning, extinction and extinction retention testing

A separate group of 48 rats (control/control = 12, injury/control = 12, control/SPS = 12, injury/SPS = 12) received the stress or control procedure on post-injury day 7. One animal in the control/SPS group died during ether exposure in the SPS protocol. On post-injury day 15 (following the 7-day quiescent period of the stress or control procedure), all animals were transported from their home cages in groups of seven or eight and underwent the fear conditioning paradigm described above. 24 hours following the completion of extinction retention testing, all 47 animals were euthanized. The procedure for Experiment 2 is shown in Figure II.2B.

Histological Preparation

Animals were anesthetized and then euthanized through incision of the chest cavity to create a pneumothorax, immediately followed by cardiovascular perfusion (Gage et al., 2012). After flushing the circulatory system with physiological saline, all animals were perfused with 4% paraformaldehyde (PFA). Brains were harvested from each animal and stored in 4% PFA overnight. Brains were then stored in 30% sucrose until they sank (for cryoprotection), approximately 3 days. Brains were then cryostat sectioned at a thickness of 50 μm, from 1 mm post-bregma to 1 mm pre-lambda, over the area of the impact. Eight sets of sections were taken from each animal, so there was a distance of 400 μm between sections in each histology protocol.

Cresyl violet staining

After mounting on Trubond 380 slides (Tru Scientific, Bellingham, WA), sections were dried overnight. Nissl substance in brain sections was then stained with cresyl violet (Shitaka et al., 2011), and double-blinded scorers assessed contusion volume and neuronal tissue loss in the stained sections.

Doublecortin staining

Changes in hippocampal neurogenesis following CCI and SPS were also assessed, using doublecortin (DCX) staining. Free-floating sections were washed in Tris buffer, quenched with hydrogen peroxide, and incubated with rabbit anti-doublecortin antibody overnight (Abcam, Cambridge, MA). Sections were then washed, incubated with anti-rabbit secondary antibody, amplified with the Vectastain avidin-biotin complex (ABC) kit (Vector Laboratories, Burlingame, CA), and stained with 3,3'-diaminobenzidine (DAB). After mounting, drying and cover-slipping, pictures were taken in the dentate gyrus (DG) of the hippocampus in all animals at 40x magnification using

an Olympus CX41 optical microscope and an INFINITY 1-2 digital camera. Images were then auto-leveled in Adobe Photoshop, and numbers of DCX-positive hippocampal neurons were counted by double-blinded scorers.

Iba1 staining

Inflammatory microglial response following CCI and SPS was assessed using Iba1 (ionized calcium-binding adapter molecule-1) staining. Similar techniques were used as in the DCX protocol, using rabbit anti-Iba1 antibody (Wako Pure Chemical Industries, Ltd, Osaka, Japan). Pictures were captured at 10x magnification in ipsilateral hippocampus and thalamus, as well as bilateral cortex of all animals. After auto-leveling in Adobe Photoshop and auto-thresholding in ImageJ, the area of the frame that consisted of Iba1-positive microglia was automatically calculated using ImageJ.

Data analysis and statistical analysis

Freezing was defined as the absence of movement, except that necessary for breathing, for greater than 2 seconds and quantified as a percentage of the total time recorded (Knox et al., 2012a). Trials were organized into blocks of three during extinction and blocks of two during extinction recall. These values were analyzed using repeated-measures ANOVA, and post-hoc comparisons using *t*-tests, with Bonferroni corrections, were performed when significant overall *F*-ratios were obtained. Additionally, an extinction index was calculated during extinction recall by calculating the percent of time spent freezing during all CS trials and subtracting the percent of time spent freezing at baseline. Group differences in these values were analyzed with independent-samples *t*-tests.

Rats that did not show a conditioned freezing response > 30% at the start of the fear extinction session were excluded from final analyses. In addition, rats exhibiting freezing levels ±2 standard deviations from a group mean were removed from the analyses. Three rats (18.75%) were excluded from Experiment 1, leaving 6 rats in the Control group and 7 rats in the Injury group. Eleven rats (23.4%) were excluded from Experiment 2, leaving 7 rats in the Control/Control group, 10 rats in the Control/SPS group, 10 rats in the Injury/Control group and 9 rats in the Injury/SPS group. The number of animals excluded due to absent freezing response is in line with previous studies of fear-associated learning in rats.

Histological data was analyzed using independent samples t-tests and two-way ANOVA. Correlations were also calculated between histologic measures and freezing behavior using bivariate regression. The criterion for significance was set at $p < 0.05$. All data are represented as means ± SEM.

Results

Freezing Behavior

Experiment 1

Graphs of freezing behavior from experiment 1 are displayed in Figure II.3. There was a main effect of trial on freezing during fear acquisition across both groups ($F(2.067, 22.739)=37.408$, $p<.001$). We did not observe a significant difference between groups in freezing behavior during fear acquisition.

There was a main effect of block on freezing during fear extinction across both groups ($F(10,110)=6.575$, $p<.001$). There was also a significant interaction between

block and injury during fear extinction (F(10,110)=2.302, p<0.05). After adjusting for multiple comparisons, *post hoc* tests showed no significant effects for individual blocks.

There was a main effect of block on freezing during extinction retention testing (F(5,55)=3.767, p<.01). There was no significant difference between groups in freezing behavior during fear extinction recall.

Experiment 2

Freezing behavior from experiment 1 is displayed in Figure II.4. We observed a main effect of trial on freezing during fear acquisition across all groups (F(3.654,116.937)=100.203, p<.001). There was no significant difference of injury or SPS in freezing behavior during fear acquisition.

There was a main effect of trial on freezing during fear extinction across all groups (F(3.660, 117.134)=14.851, p<.001), and there was no main effect of injury or SPS on freezing behavior during fear extinction. There was a significant interaction between block and injury during fear extinction (F(3.660, 117.134) = 2.719, p<0.05). After correcting for multiple comparisons, post hoc tests found that there was significantly greater time spent freezing in injured versus control animals during block 8 (50.12% ± 13.85 vs 32.98% ± 12.54, F(1,35)=15.018, p<0.001).

There was also a main effect of block on freezing during extinction retention across all groups (F(5,160)=11.67, p<.001), but there was no main effect of injury or SPS. A trending interaction was observed in freezing behavior between block, injury and SPS (F(5,160)=2.210, p=.056). Additionally, there was a significant interaction between time and injury in freezing behavior when comparing the first and second halves of extinction retention testing (F(1,34)=9.639, p<.05).

Cresyl Violet Staining

Nissl staining revealed no evidence of neuronal cell loss or contusions at 10 days or 18 days following brain injury. Representative images from each experimental group are shown in Figure II.5.

Immunohistochemistry

Iba1 expression

At ten days post-CCI surgery, there was a significantly greater area of Iba1 positive cells in injured animals vs control animals in ipsilateral thalamus (21.67 $\pm$ 1.01 vs 15.19 $\pm$ 1.70, t=3.138, p<.01) and ipsilateral cortex (25.94 $\pm$ 0.87 vs 19.87 $\pm$ 2.06, t=2.560, p<.05), but there was no significant difference in ipsilateral hippocampus or contralateral cortex. Additionally, there was a trending relationship in the CCI group between area of Iba1 positive cells in ipsilateral thalamus and extinction index (r(4)=.810, p=.0508). These data, as well as representative images from ipsilateral cortex in control and injured animals are shown in Figure II.6. There were no other significant or trend-level relationships between freezing behavior and Iba1 positive cells. No differences were seen between groups in the area of Iba1 positive cells at 18 days post-injury.

DCX expression

At 10 days post-CCI surgery, there were no significant differences between injury and control groups in the number of DCX-positive neural progenitors in ipsilateral or contralateral HC. There were also no significant differences between injury and control groups in the ratio between the number of DCX-positive neural progenitors in ipsilateral versus contralateral HC.

At 18 days post-CCI surgery, there were no significant effects of injury or SPS on the number of DCX-positive neural progenitors in ipsilateral or contralateral HC. There was also no significant interaction between injury and SPS on the number of DCX-positive cells. There were also no significant differences between injury and control groups in the ratio between DCX-positive cells in ipsilateral HC versus contralateral HC. There was, however, a trend-level effect of SPS on the number of DCX-positive cells in ipsilateral HC versus contralateral HC ($t(26)=1.877$, $p=.0717$).

There were no significant correlations between freezing behavior and the number of DCX-positive cells in either experiment.

Discussion

Findings in both experiments demonstrate an effect of CCI injury on fear extinction. Particularly, we observed a significant interaction between block and injury in freezing behavior in both Experiment 1 and Experiment 2 that signifies an impaired rate of fear extinction. Fear extinction deficits have been reported in several models of TBI, including blast, weight drop and CCI (Heldt et al., 2014, Davies et al., 2016, Schneider et al., 2016). Negative results in other studies may be explained by examining differences in injury and conditioning protocols. Meyer, et al (2012) did not report differential extinction effects following weight-drop injury, but their extinction procedures occurred in the same context as conditioning. Retaining the same visual, olfactory and auditory contextual inputs significantly impacts extinction processing, as there is far more overlap between the contextual information that is encoded in the safety trace and the contextual information that was previously encoded in the fear trace during

acquisition. Another experiment that did not find differences in freezing during extinction used a level of injury that caused a significant lesion in the ipsilateral cortex and hippocampus (Sierra-Mercado et al., 2015). Therefore, it is difficult to compare the results of these two studies, as the injury that was induced in our study had quite different characteristics.

In addition to differences in levels of injury, it has been suggested that injury mechanism may explain some of the variability in these findings. However, conflicting outcomes in fear-associated learning have been reported within all three of the same models of injury (lateral fluid percussion, CCI, weight drop, blast), suggesting that differences in injury might not account for all the variability. There is tremendous value in using different models of injury in animals, as mTBI in humans is an extremely heterogeneous disorder, with widely varying injury mechanisms, severities of impact and clinical presentations (Rosenbaum and Lipton, 2012). Nonetheless, cross-validation of behavioral results and neurobiological differences with findings in mTBI patients is tremendously important to determine which of the varying results seen in animal TBI models are carried over into humans.

In contrast to our expectation, the deficits observed during the extinction phase did not manifest during extinction retention testing, as the injured rats did not show any differences in freezing that were indicative of an impairment in extinction retention. The significant difference in freezing behavior found during extinction retention testing was an interaction in freezing between time (first and second halves) and injury. This is not indicative of an extinction retention deficit, but rather an impairment in extinction itself, as the later period of extinction retention testing essentially consists of another round of

extinction learning. However, the trending interaction between block, SPS and injury in freezing during extinction recall suggests a combined, deleterious effect of SPS and injury on extinction retention. Davies et al. (2016) found increased freezing behavior during the second and third days of three consecutive extinction learning in animals that exposed to social defeat and weight drop injury. We did not observe such a strong effect, and this could be due to lower power that prevented us from observing the effect, differences in timing or other aspects of the injury protocol.

The freezing behavior observed during fear conditioning in both experiments suggests that there is no difference in the process of fear acquisition in injured animals. In both experiments, animals learned to associate US with CS, a necessary process in order to test differences in extinction learning. There was no significant difference in freezing behavior at the beginning of fear extinction in either experiment. These findings are in line with previous research suggesting that fear acquisition remains intact in animals that have received brain injury (Klemenhagen et al., 2013, Sierra-Mercado et al., 2015). However, similarly to a number of previous studies (Klemenhagen et al., 2013, Heldt et al., 2014, Sierra-Mercado et al., 2015, Davies et al., 2016, Schneider et al., 2016), we did not find any evidence of enhanced fear acquisition, something that has been reported previously in the literature (Elder et al., 2012, Meyer et al., 2012, Reger et al., 2012). These studies used acquisition testing to test levels of fear conditioning, and collapsed freezing behavior in response to context or cues across a period of three to eight minutes. Extinction learning may occur within this time period, and plotting freezing behavior in trials or blocks allows for more specific interpretation of results.

The level of injury that was used in our experiments was specifically set to provide mild impact that did not cause significant lesions or neuronal death. Nissl staining at both 10 and 18 days post-injury showed that there was no noticeable neuronal cell loss or contusion following CCI. We anticipated this from previous experiments, and this models well what is seen in human mTBI, which usually cannot be detected with conventional computed tomography (CT) and magnetic resonance imaging (MRI) methods (Shenton et al., 2012). However, findings in immunohistochemistry experiments confirmed that there was an increased inflammatory response in ipsilateral cortex and thalamus at 10 days post-injury, which was similar to differences seen in other animal models of TBI (Shitaka et al., 2011, Hylin et al., 2013, Mannix et al., 2016).

The trend-level interaction between Iba1 expression in ipsilateral thalamus and freezing during extinction recall within injured rats is intriguing, as epidemiological data suggests that increased severity of injury (while still categorized at mild or moderate TBI) is associated with increased prevalence of PTSD (Stein et al., 2015). Measures of activated microglia in thalamus were mainly included because previous research showed that significant differences in Iba1 expression were observed in thalamus for CCI-injured animals versus controls for at least 28 days post-injury (Shitaka et al., 2011). However, as this was the only region where inflammation was directly related to increased freezing behavior, we may consider it as a region of interest in future studies. Thalamus is involved in the fear response circuitry, but its role is typically defined as an intermediate node between sensory cortex and amygdala (Maren and Quirk, 2004). More research investigating changes within thalamus after TBI are necessary in order to

determine whether the relationship observed in this study was simply coincidental (thalamus as a marker of brain-wide inflammation post-CCI) or direct.

DCX immunohistochemistry did not demonstrate any effects of CCI injury on hippocampal neurogenesis at 10 or 18 days post-injury. This is similar to other findings in rodent models of TBI. Studies have demonstrated no differences in hippocampal neurogenesis at 7 and 10 days (Yu et al., 2008, Carlson et al., 2014), two weeks (Wang et al., 2016) and eight weeks after mild CCI injury (Acosta et al., 2013). It is possible that changes in HC do not underlie the alterations in fear extinction that we observed, but it is also likely that the timing of our experiments might not have captured injury-induced alterations in hippocampal neurogenesis that may have occurred. Other experiments have shown reduced numbers of neural progenitors in ipsilateral HC at 3 days post-injury (Yu et al., 2008, Carlson et al., 2014). The trend-level effect of SPS on ipsilateral HC versus contralateral HC suggests that SPS affects the recovery process following injury (as well as the craniotomy). Animals exposed to unavoidable shock have been shown to have decreased hippocampal neurogenesis at 30 days after this stress procedure (Kim and Seo, 2013). Further experiments investigating the effect of SPS on hippocampal neurogenesis are necessary, and the timecourse of these effects particularly needs to be investigated.

There were several limitations that require consideration. 18.75% of the animals in Experiment 1 and 23.4% of the animals in Experiment 2 were excluded because they failed to acquire the fear response. While there was no significant difference between groups in the numbers of animals excluded from analyses, we might have too low power to detect some of the hypothesized changes. Additional animals in future

experiments may provide the power necessary to find significant effects where we observed trend-level associations. Also, condensing the time period between different aspects of the protocol may strengthen behavioral effects and cause neurobiological changes in areas more commonly associated with damage in mTBI patients. For instance, the CCI procedure was very well-tolerated in animals, and the seven-day recovery period is probably longer than necessary. Starting the SPS procedure more promptly after CCI may potentiate combinatorial effects during extinction retention testing; however, there is a concern that stress from both the CCI and sham injury procedures may wash out the effect of stress in SPS. Using an impact to the skull without a craniotomy may reduce stress to the animal during the injury procedure and uncover SPS effects that we did not observe in these experiments. Additionally, impact to a more frontal region of the brain may be more representative of human mTBI, as neuroimaging suggests that frontal regions are most commonly affected in mTBI patients (Eierud et al., 2014, Mayer et al., 2015).

Together, the differences observed in freezing behavior suggest that alterations in extinction learning occur following brain injury in rats. Although we did not observe problems in extinction retention (characteristic of PTSD) following this slower or deficient extinction learning, it is possible that changes in the fear-associated learning paradigm, increased injury severity, or closer temporal proximity between the induction of injury and fear conditioning may uncover problems in extinction recall. We did find some evidence of a combinatory effect of single prolonged stress and injury, and the effect of stress may impair contextual processing and retrieval of an improperly-coded safety memory, potentiating the effect of the extinction deficit. As extinction deficits have

now been observed in models of both mTBI and PTSD, this aspect of fear learning may represent a shared process affected in both disorders. More research must be performed in order to understand the mechanism underlying these problems in fear extinction. The combined CCI / SPS protocol was well-tolerated and represents a model that can be used to investigate neurobiological differences in rodents that may translate to a better understanding of how changes in the brain following stress and TBI correlate with symptomatology. Additionally, the model may be useful in examining the effects of potential treatments. However, the most important concern in the area of fear-associated learning and mTBI is collecting data from human subjects that validates findings in animal models.

References

Acosta SA, Diamond DM, Wolfe S, Tajiri N, Shinozuka K, Ishikawa H, Hernandez DG, Sanberg PR, Kaneko Y, Borlongan CV (2013) Influence of Post-Traumatic Stress Disorder on Neuroinflammation and Cell Proliferation in a Rat Model of Traumatic Brain Injury. *PLoS One* **8:**e81585.

Brody DL, Donald C, Kessens CC, Yuede C, Parsadanian M, Spinner M, Kim E, Schwetye KE, Holtzman DM, Bayly PV (2007) Electromagnetic Controlled Cortical Impact Device for Precise, Graded Experimental Traumatic Brain Injury. J Neurotrauma 24:657-673.

Carlson SW, Madathil SK, Sama DM, Gao X, Chen J, Saatman KE (2014) Conditional Overexpression of Insulin-Like Growth Factor-1 Enhances Hippocampal Neurogenesis and Restores Immature Neuron Dendritic Processes After Traumatic Brain Injury. *J Neuropathol Exp Neurol* **73**:734-746.

Davies DR, Olson D, Meyer DL, Scholl JL, Watt MJ, Manzerra P, Renner KJ, Forster GL (2016) Mild Traumatic Brain Injury with Social Defeat Stress Alters Anxiety, Contextual Fear Extinction, and Limbic Monoamines in Adult Rats. *Front Behav Neurosci* **10**.

Dixon CE, Clifton GL, Lighthall JW, Yaghmai AA, Hayes RL (1991) A controlled cortical impact model of traumatic brain injury in the rat. J Neurosci Methods 39:253-262.

Eierud C, Craddock RC, Fletcher S, Aulakh M, King-Casas B, Kuehl D, LaConte SM (2014) Neuroimaging after mild traumatic brain injury: Review and meta-analysis(). *NeuroImage : Clinical* **4**:283-294.

Elder GA, Dorr NP, De Gasperi R, Gama Sosa MA, Shaughness MC, Maudlin-Jeronimo E, Hall AA, McCarron RM, Ahlers ST (2012) Blast exposure induces post-traumatic stress disorder-related traits in a rat model of mild traumatic brain injury. *J Neurotrauma* **29**:2564-2575.

Gage GJ, Kipke DR, Shain W (2012) Whole Animal Perfusion Fixation for Rodents. J Vis Exp.

Garfinkel SN, Abelson JL, King AP, Sripada RK, Wang X, Gaines LM, Liberzon I (2014) Impaired contextual modulation of memories in PTSD: an fMRI and psychophysiological study of extinction retention and fear renewal. *J Neurosci* **34**:13435-13443.

Genovese RF, Simmons LP, Ahlers ST, Maudlin-Jeronimo E, Dave JR, Boutte AM (2013) Effects of mild TBI from repeated blast overpressure on the expression and extinction of conditioned fear in rats. *Neuroscience* **254**:120-129.

George SA, Rodriguez-Santiago M, Riley J, Rodriguez E, Liberzon I (2015) The effect of chronic phenytoin administration on single prolonged stress induced extinction

retention deficits and glucocorticoid upregulation in the rat medial prefrontal cortex. Psychopharmacology (Berl) 232:47-56.

Greco JA, Liberzon I (2016) Neuroimaging of Fear-Associated Learning. *Neuropsychopharmacology* **41**:320-334.

Heldt SA, Elberger AJ, Deng Y, Guley NH, Del Mar N, Rogers J, Choi GW, Ferrell J, Rex TS, Honig MG, Reiner A (2014) A novel closed-head model of mild traumatic brain injury caused by primary overpressure blast to the cranium produces sustained emotional deficits in mice. *Front Neurol* **5**:2.

Hylin MJ, Orsi SA, Zhao J, Bockhorst K, Perez A, Moore AN, Dash PK (2013) Behavioral and histopathological alterations resulting from mild fluid percussion injury. *J Neurotrauma* **30**:702-715.

Kim B-K, Seo J-H (2013) Treadmill exercise alleviates post-traumatic stress disorder-induced impairment of spatial learning memory in rats. *J Exerc Rehabil* **9**:413-419.

Klemenhagen KC, O'Brien SP, Brody DL (2013) Repetitive concussive traumatic brain injury interacts with post-injury foot shock stress to worsen social and depression-like behavior in mice. *PLoS One* **8**:e74510.

Knox D, George SA, Fitzpatrick CJ, Rabinak CA, Maren S, Liberzon I (2012a) Single prolonged stress disrupts retention of extinguished fear in rats. *Learn Mem* **19**:43-49.

Knox D, Nault T, Henderson C, Liberzon I (2012b) Glucocorticoid receptors and extinction retention deficits in the single prolonged stress model. *Neuroscience* **223**:163-173.

Lagarde E, Salmi L, Holm LW, et al. (2014) ASsociation of symptoms following mild traumatic brain injury with posttraumatic stress disorder vs postconcussion syndrome. *JAMA Psychiatry* **71**:1032-1040.

Liberzon I, Krstov M, Young EA (1997) Stress-restress: effects on ACTH and fast feedback. Psychoneuroendocrinology 22:443-453.

Mannix R, Berkner J, Mei Z, Alcon S, Hashim J, Robinson S, Jantzie L, Meehan W, Qiu J (2016) Adolescent Mice Demonstrate a Distinct Pattern of Injury after Repetitive Mild Traumatic Brain Injury. *J Neurotrauma*.

Maren S, Quirk GJ (2004) Neuronal signalling of fear memory. *Nat Rev Neurosci* **5**:844-852.

Mayer AR, Bellgowan PSF, Hanlon FM (2015) Functional magnetic resonance imaging of mild traumatic brain injury. *Neuroscience & Biobehavioral Reviews* **49**:8-18.

Meyer DL, Davies DR, Barr JL, Manzerra P, Forster GL (2012) Mild traumatic brain injury in the rat alters neuronal number in the limbic system and increases conditioned fear and anxiety-like behaviors. *Exp Neurol* **235**:574-587.

Milad MR, Pitman RK, Ellis CB, Gold AL, Shin LM, Lasko NB, Zeidan MA, Handwerger K, Orr SP, Rauch SL (2009) Neurobiological basis of failure to recall extinction memory in posttraumatic stress disorder. *Biol Psychiatry* **66**:1075-1082.

Morissette SB, Woodward M, Kimbrel NA, Meyer EC, Kruse MI, Dolan S, Gulliver SB (2011) Deployment-related TBI, persistent postconcussive symptoms, PTSD, and depression in OEF/OIF veterans. *Rehabil Psychol* **56**:340-350.

National Center for Injury Prevention and Control (2003) *Report to Congress on Mild Traumatic Brain Injury in the United States: Steps to Prevent a Serious Public Health Problem*. Atlanta, GA: Centers for Disease Control and Prevention.

Ojo JO, Greenberg MB, Leary P, Mouzon B, Bachmeier C, Mullan M, Diamond DM, Crawford F (2014) Neurobehavioral, neuropathological and biochemical profiles in a novel mouse model of co-morbid post-traumatic stress disorder and mild traumatic brain injury. *Front Behav Neurosci* **8**:213.

Orlovska S, Pedersen MS, Benros ME, Mortensen PB, Agerbo E, Nordentoft M (2014) Head injury as risk factor for psychiatric disorders: a nationwide register-based follow-up study of 113,906 persons with head injury. *Am J Psychiatry* **171**:463-469.

Palmer CP, Metheny HE, Elkind JA, Cohen AS (2016) Diminished amygdala activation and behavioral threat response following traumatic brain injury. *Experimental Neurology* **277**:215-226.

Reger ML, Poulos AM, Buen F, Giza CC, Hovda DA, Fanselow MS (2012) Concussive brain injury enhances fear learning and excitatory processes in the amygdala. *Biol Psychiatry* **71**:335-343.

Rosenbaum SB, Lipton ML (2012) Embracing chaos: the scope and importance of clinical and pathological heterogeneity in mTBI. *Brain Imaging and Behavior* **6**:255-282.

Schneider BL, Ghoddoussi F, Charlton JL, Kohler RJ, Galloway MP, Perrine SA, Conti AC (2016) Increased Cortical Gamma-Aminobutyric Acid Precedes Incomplete Extinction of Conditioned Fear and Increased Hippocampal Excitatory Tone in a Mouse Model of Mild Traumatic Brain Injury. *J Neurotrauma*.

Shenton ME, Hamoda HM, Schneiderman JS, Bouix S, Pasternak O, Rathi Y, Vu MA, Purohit MP, Helmer K, Koerte I, Lin AP, Westin CF, Kikinis R, Kubicki M, Stern RA, Zafonte R (2012) A review of magnetic resonance imaging and diffusion tensor imaging findings in mild traumatic brain injury. *Brain Imaging Behav* **6**:137-192.

Shitaka Y, Tran HT, Bennett RE, Sanchez L, Levy MA, Dikranian K, Brody DL (2011) Repetitive Closed-Skull Traumatic Brain Injury in Mice Causes Persistent Multifocal Axonal Injury and Microglial Reactivity. *J Neuropathol Exp Neurol* **70**:551-567.

Sierra-Mercado D, McAllister LM, Lee CC, Milad MR, Eskandar EN, Whalen MJ (2015) Controlled cortical impact before or after fear conditioning does not affect fear extinction in mice. *Brain Res* **1606**:133-141.

Stein MB, Kessler RC, Heeringa SG, Jain S, Campbell-Sills L, Colpe LJ, Fullerton CS, Nock MK, Sampson NA, Schoenbaum M, Sun X, Thomas ML, Ursano RJ, collaborators ObotAS (2015) Prospective Longitudinal Evaluation of the Effect of Deployment-Acquired Traumatic Brain Injury on Posttraumatic Stress and Related Disorders: Results From the Army Study to Assess Risk and Resilience in Servicemembers (Army STARRS). *American Journal of Psychiatry* **172**:1101-1111.

Thurman DJ (2001) The epidemiology and economics of head trauma. In: Head Trauma: Basic, Preclinical, and Clinical Directions (Miller L, H. R., ed) New York: John Wiley and Sons.

Wang X, Gao X, Michalski S, Zhao S, Chen J (2016) Traumatic Brain Injury Severity Affects Neurogenesis in Adult Mouse Hippocampus. *J Neurotrauma* **33**:721-733.

Warren AM, Boals A, Elliott TR, Reynolds M, Weddle RJ, Holtz P, Trost Z, Foreman ML (2015) Mild traumatic brain injury increases risk for the development of posttraumatic stress disorder. *J Trauma Acute Care Surg* **79**:1062-1066.

Yu S, Kaneko Y, Bae E, Stahl CE, Wang Y, van Loveren H, Sanberg PR, Borlongan CV (2009) Severity of controlled cortical impact traumatic brain injury in rats and mice dictates degree of behavioral deficits. Brain Research 1287:157-163.

Yu T-S, Zhang G, Liebl DJ, Kernie SG (2008) Traumatic brain injury-induced hippocampal neurogenesis requires activation of early nestin-expressing progenitors. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **28**:12901-12912.

Figure II.1: Single prolonged stress (SPS) paradigm. Animals are first exposed to (A) two hours of prolonged physical restraint, followed immediately by (B) twenty minutes forced swim. After a 15-minute recuperation period, animals are (C) exposed to ether until induction of general anesthesia and then (D) returned to their cages and left undisturbed for a 7-day quiescent period. Control animals remain in their cages for the entirety of the SPS protocol.

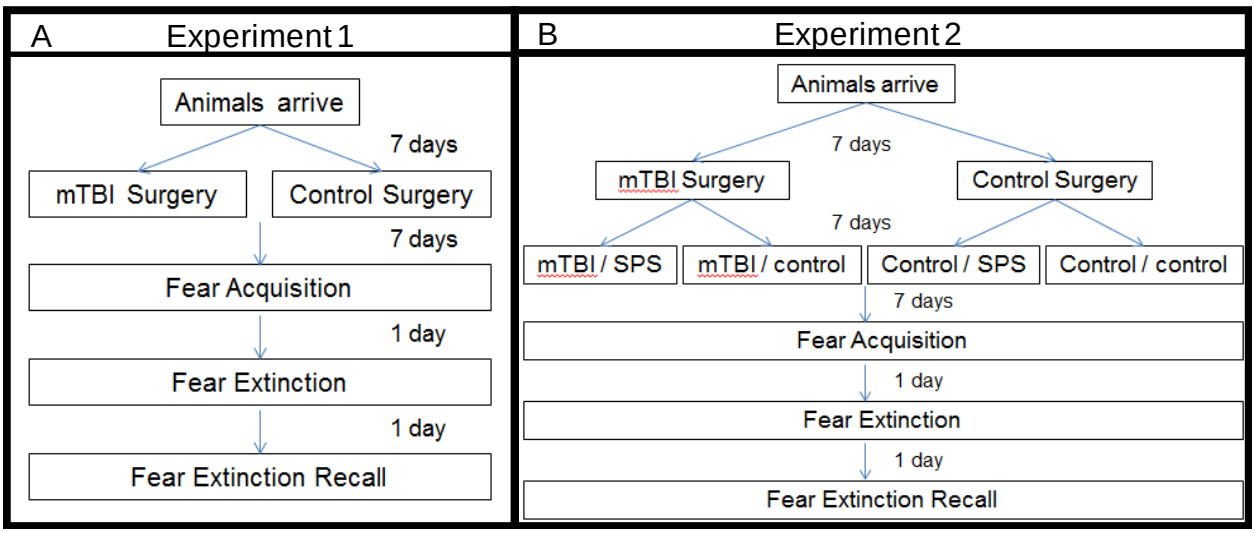

Figure II.2: Graphical representation of protocols. (A) Experiment 1 and (B) Experiment 2.

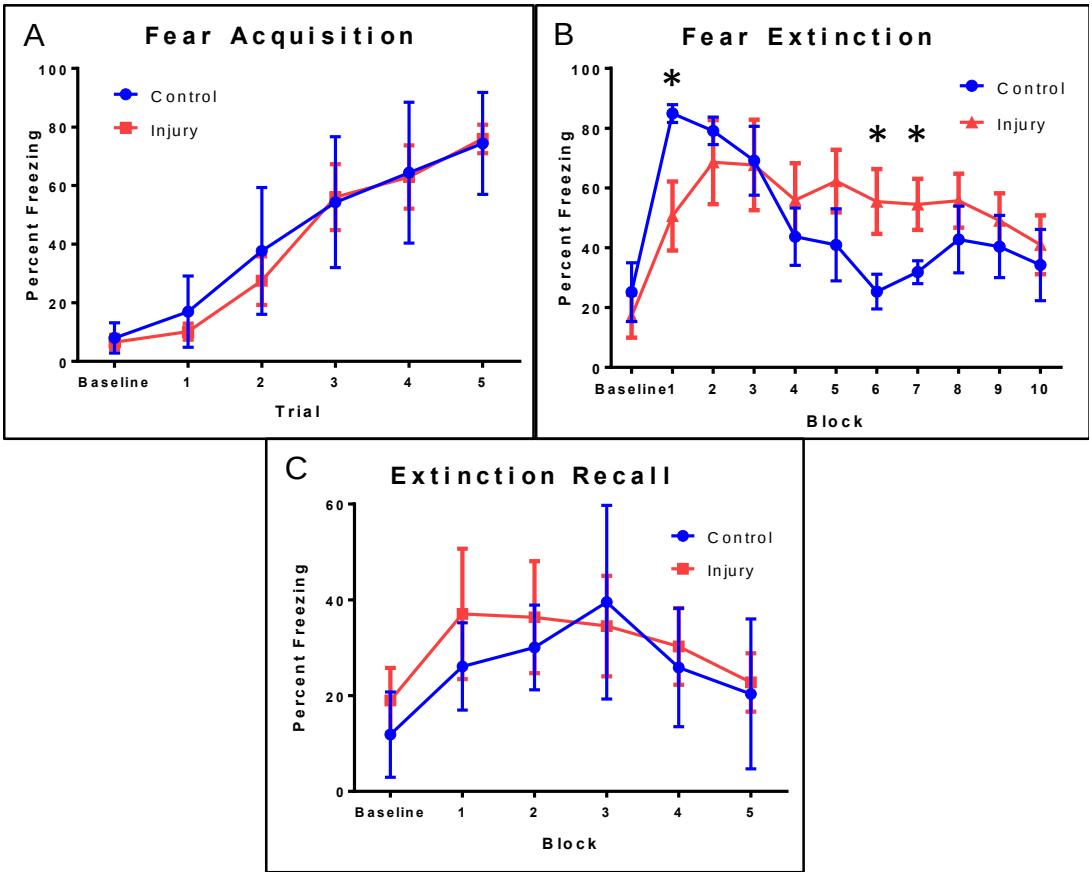

Figure II.3: Differences in freezing behavior during Experiment 1. (A) There were no differences between groups in freezing behavior during fear acquisition. (B) There was a significant interaction between group and block in freezing behavior during fear extinction indicative of impaired extinction learning in the CCI-injured animals. (C) There were no differences between groups in freezing behavior during extinction recall.

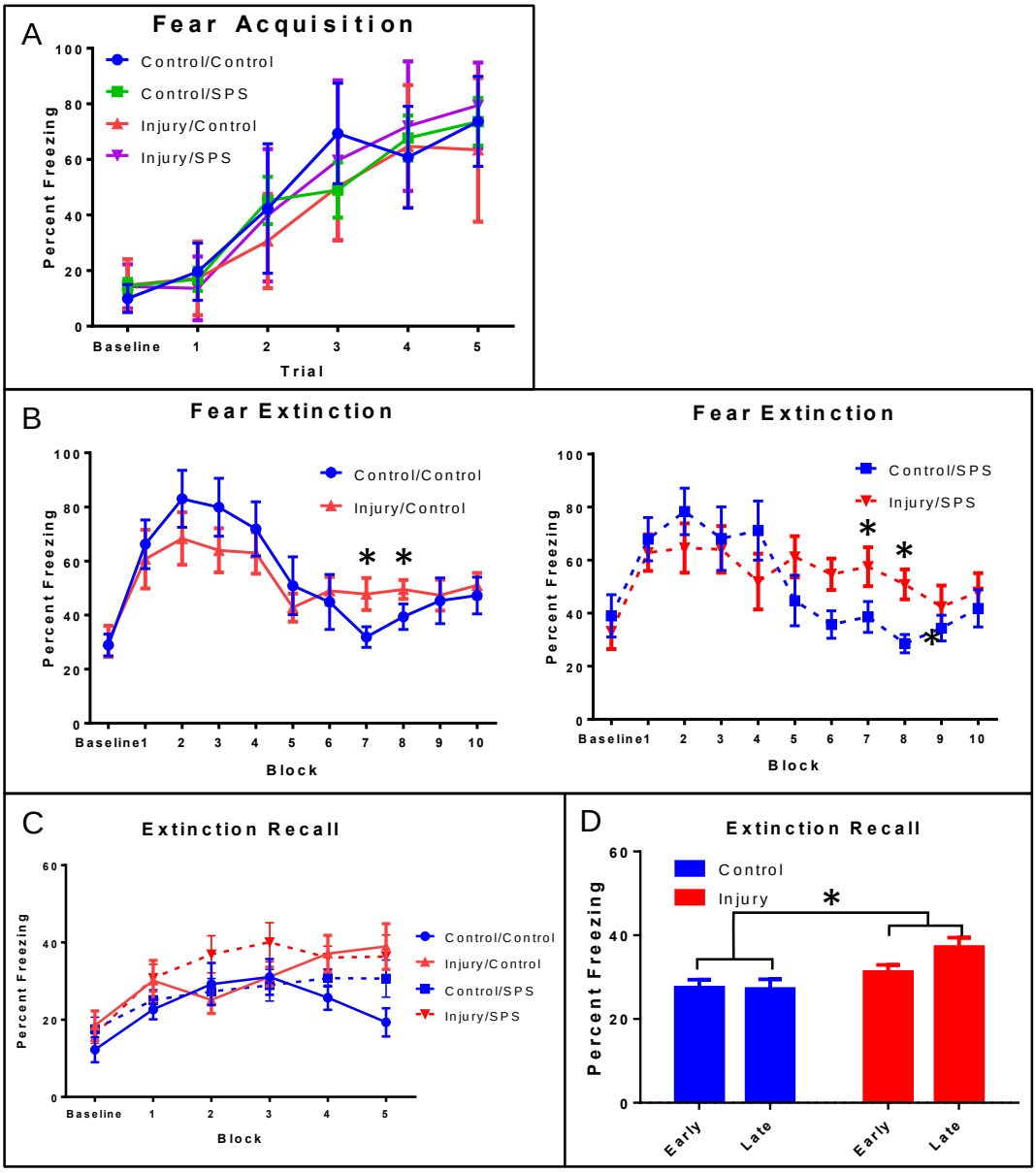

Figure II.4: Differences in freezing behavior during Experiment 2. (A) There were no differences between groups in freezing behavior during fear acquisition. (B) There was a significant interaction between group and block in freezing behavior during fear extinction indicative of impaired extinction learning in the CCI-injured animals. (C) There was a trending interaction between block, injury and SPS in freezing during extinction recall, suggesting that the combination of injury and SPS may lead to increased fear response through the extinction recall phase. (D) There was a significant interaction between time and group in freezing during extinction recall. * = p<.05

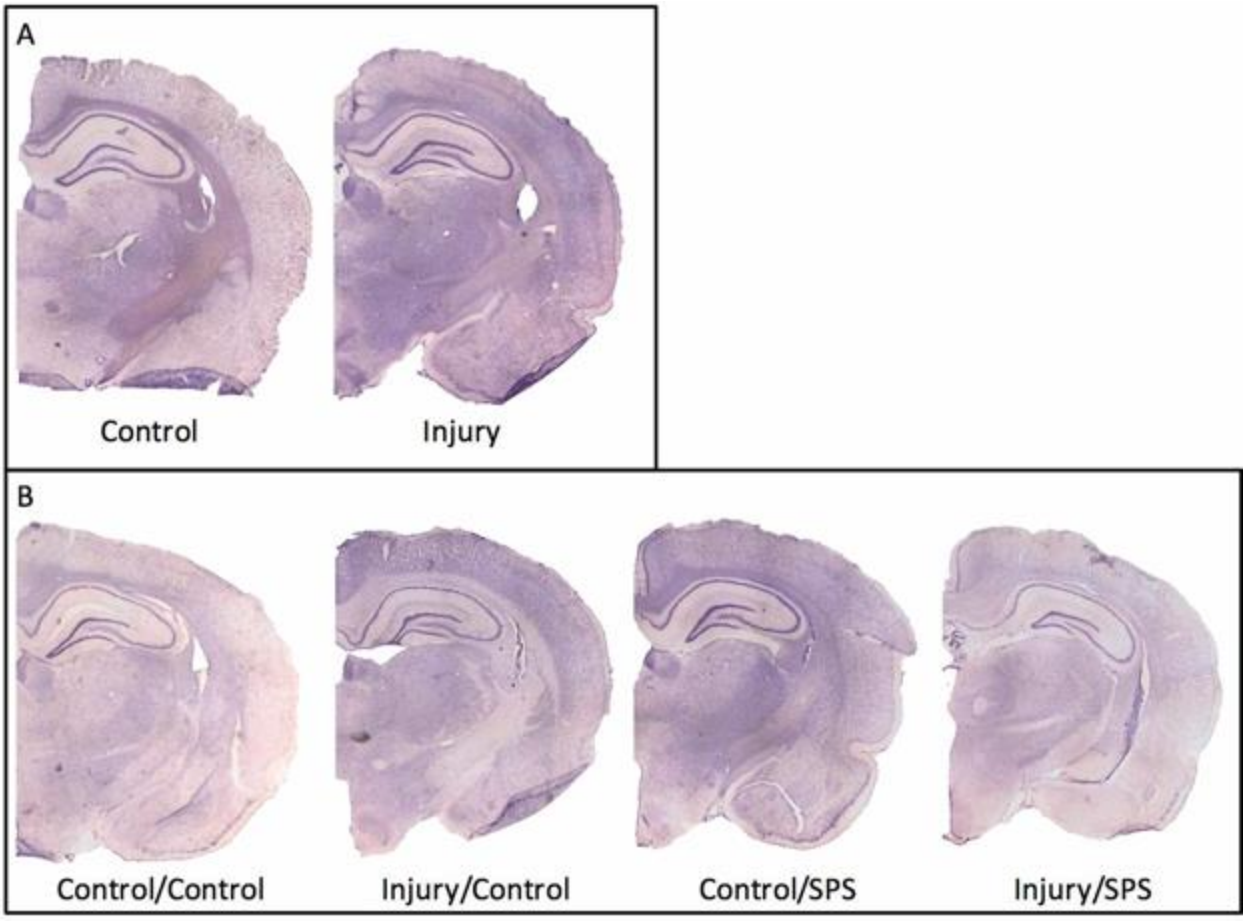

Figure II.5: Cresyl violet histology. Cresyl violet stains from (A) Experiment 1 and (B) Experiment 2 show no evidence of contusion or neuronal cell loss in injured animals.

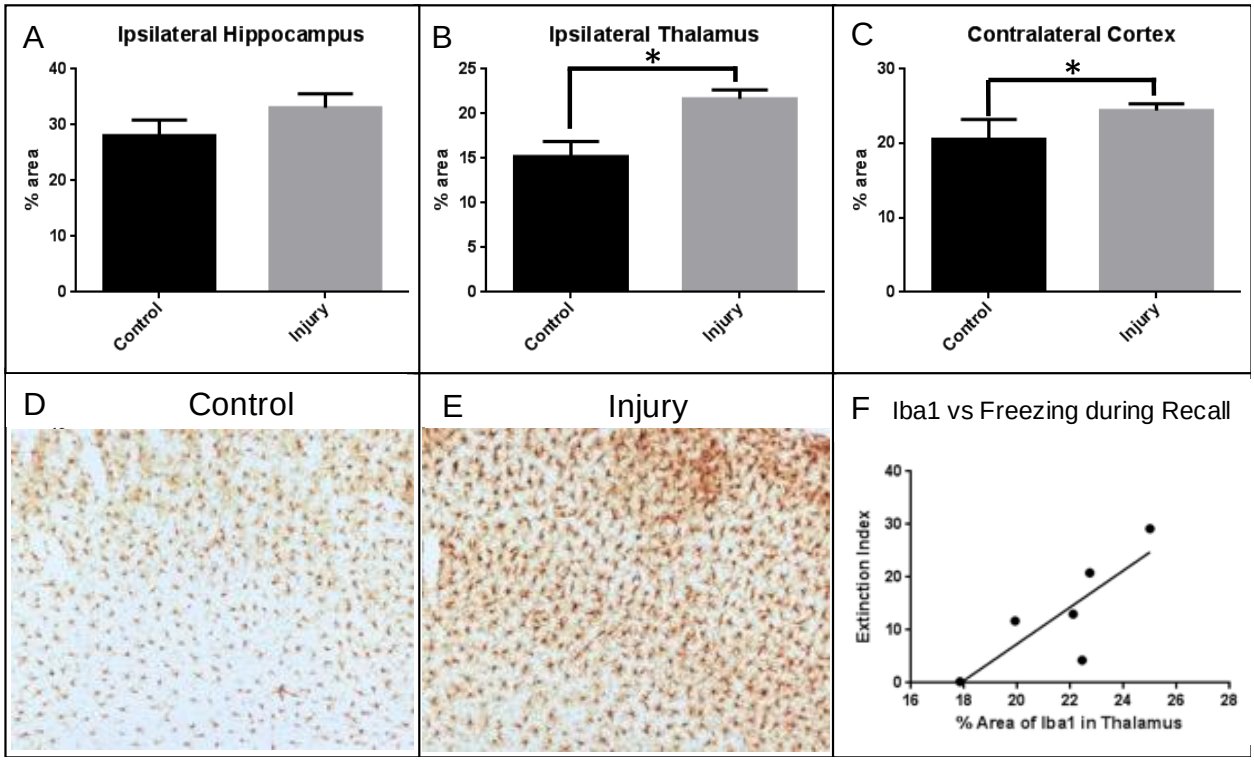

Figure II.6: Iba1 expression in (A) ipsilateral hippocampus, (B) ipsilateral thalamus and (C) ipsilateral cortex at ten days post-CCI surgery. Representative images from ipsilateral cortex in (D) control and (E) injured animals are also shown. (F) Trend-level correlation between Iba1 expression and extinction index (from extinction recall) within the injury group, suggesting increased inflammatory response is associated with greater fear response during extinction recall. * = $p<.05$

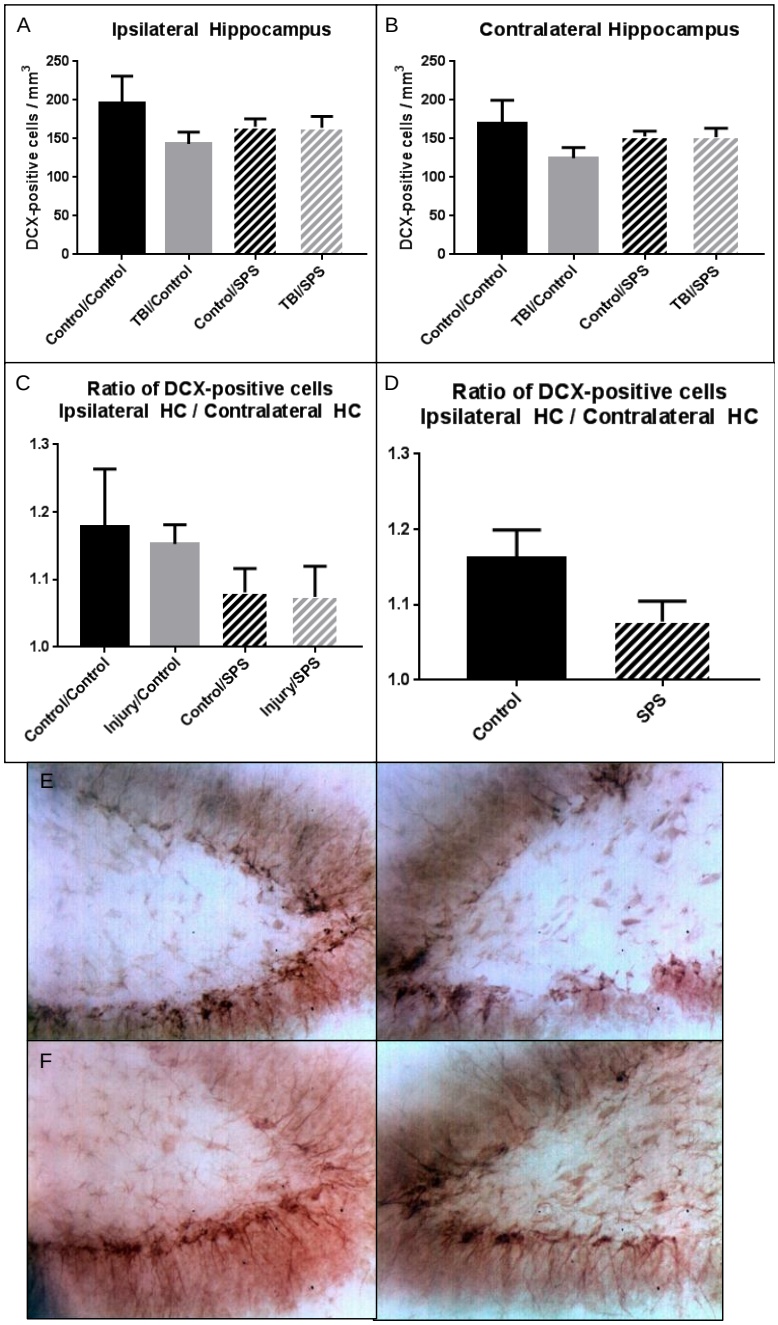

Figure II.7: Doublecortin (DCX) expression. Count of DCX-positive cells in dentate gyrus of (A) ipsilateral hippocampus (HC) and (B) contralateral HC. Ratio of the number of DCX-positive cells in ipsilateral HC versus contralateral HC split by group (C) and by SPS versus control groups (D). There was a trend-level effect of SPS on the number of DCX-positive cells in ipsilateral HC versus contralateral HC, suggesting SPS may affect neurogenesis in the recovery period following CCI and sham surgery. Representative contralateral (left) and ipsilateral (right) HC sections are shown from (E) injury/SPS group versus (F) injury/control group.

CHAPTER III

Neural correlates of altered fear extinction in patients with recent mild traumatic brain injury

Introduction

Mild traumatic brain injury (mTBI) is a common consequence of injury that often leads to significant, debilitating symptoms, including headache, fatigue, visual changes and problems with mood, cognition and attention (Corrigan, 2010). It is estimated that 1.5 million Americans seek treatment for TBI every year, 75% of which are classified as mild TBI (National Center for Injury Prevention and Control, 2003) that cost $16.5 billion annually in the USA (Thurman, 2001). Most mTBI symptoms fully resolve within two weeks, but approximately 6-15% of mTBI patients continue to have symptoms for over a year, or even permanently (Morissette et al., 2011). Additionally, mTBI is associated with psychiatric disorders such as major depressive disorder (MDD), bipolar disorder (BD), schizophrenia and posttraumatic stress disorder (PTSD) (Lagarde et al., 2014, Orlovska et al., 2014, Stein et al., 2015, Warren et al., 2015). The relationship between mTBI and PTSD is particularly interesting, as both disorders directly result from a traumatic experience.

The relationship between mTBI and PTSD has been well-established epidemiologically, but the exact character of this connection is still unclear. One potential explanation is that mTBI leads to deficits in fear-associated learning that

predispose these patients to PTSD development. Fear-associated learning has been used extensively in both animals and humans because altered fear learning has been hypothesized to play an important role in the development of PTSD and anxiety disorders, and experimentally it is a convenient, albeit simplistic, model of the acquisition and maintenance of fear responses, (Greco and Liberzon, 2016). By definition, patients with PTSD have improper fearful responses (hyperarousal) to cues and contexts that are actually safe (American Psychiatric Association, 2013). These real-world behaviors have been modeled using fear conditioning tasks, and dysfunction in fear-associated learning has been replicated in both animal models of PTSD and PTSD patients (Milad et al., 2009, Knox et al., 2012, Garfinkel et al., 2014), especially in the retention of extinction learning (or extinction recall). These impairments in extinction recall have also been associated with differences in brain activation, with hypoactivation commonly reported in regions such as hippocampus (HC) and ventromedial prefrontal cortex (vmPFC) that are involved in the retention of safety learning and hyperactivation in amygdala and dorsal anterior cingulate cortex (dACC), regions involved in the generation of the fear response (Milad et al., 2009, Rougemont-Bucking et al., 2011, Garfinkel et al., 2014).

Although fear-associated learning has not been examined in patients with mTBI so far, studies using animal models of TBI have demonstrated a variety of results in fear-associated learning, with some studies reporting enhanced fear acquisition (Elder et al., 2012, Meyer et al., 2012, Reger et al., 2012), others reporting impaired fear acquisition (Genovese et al., 2013, Palmer et al., 2016, Schneider et al., 2016), and others reporting no changes in fear-associated learning (Klemenhagen et al., 2013,

Sierra-Mercado et al., 2015). Additionally, several studies that examined fear extinction in rodent models of TBI have demonstrated increases in freezing behavior throughout extinction learning (Heldt et al., 2014, Davies et al., 2016, Schneider et al., 2016). A recent meta-analysis of fMRI studies in human patients with mTBI found that dACC and insula, another region intricately involved in the fear response, were commonly differentially activated in subjects with a history mTBI versus controls (Eierud et al., 2014) in paradigms assessing attention and resting-state activity, suggesting the presense of potential abnormalities in these regions. Additionally, reduced volumes of HC and amygdala have been observed following mTBI in humans (Depue et al., 2014, Zagorchev et al., 2016).

Based upon findings in animal models, previous neuroimaging findings in subjects with mTBI and our understanding of fear-associated learning deficits in PTSD, we hypothesized that mTBI leads to deficits in fear extinction and extinction recall, with underlying changes in brain activation in regions such as amygdala, HC, insula and dACC. In order to test this hypothesis, we used a validated in-scanner fear conditioning, extinction and extinction recall paradigm (Milad et al., 2009, Garfinkel et al., 2014) in human subjects who had recently sustained an mTBI.

Methods

Participants

Study participants were 41 volunteers aged 18-53 years (mean = 25.5, SD = 7.4) recruited from the Emergency Department (ED) at University Hospital in Ann Arbor, MI (University of Michigan Healthcare System). Inclusion and exclusion criteria were

adopted as specified in previous studies (Lewis et al., 2014, Wang et al., 2016). Inclusion criteria for the mTBI group were self-reported head injury, Glasgow Coma Scale (GCS) score of 13 or greater upon ED arrival, and at least one of the following: loss of consciousness (LOC) for 30 minutes or less due to the trauma, posttraumatic amnesia (PTA) or two or more post-concussive symptoms (PCS). Inclusion criteria for the control group were self-reported trauma (i.e. motor vehicle accident, fall, sports injury), GCS score of 15 upon ED arrival, no loss of consciousness, no posttraumatic amnesia and no more than one PCS. Exclusion criteria for both groups were GCS score of less than 13, LOC of more than 30 minutes, Quebec Classification of Whiplash-Associated Disorders Class III or IV (spinal fracture or dislocation, or neurologic signs including decreased/absent deep tendon reflexes or weakness), skull fracture, intracranial injury, long bone fracture, laceration with significant hemorrhage, clinically unstable patients, α-adrenoceptor or β-adrenoceptor antagonist, chronic opioid, or norepinephrine reuptake inhibitor usage, patients transferred from another hospital, patients admitted to the hospital, patients who presented more than 24 hours after injury, non–English speaking patients, pregnant patients, and those not competent to give consent. The study was approved by the Institutional Review Boards of the University of Michigan Medical School and the VA Ann Arbor Healthcare System. Participants were given full details of the study and provided written informed consent.

All participants underwent two scanning sessions on consecutive days within 14 days of recruitment in the emergency room (mean = 6.4 $\pm$ 3.1 days). On the day of the first scanning session, all participants completed self-report measures about their recent trauma (Post-Accident Pain Symptoms Questionnaire and Time and Certainty of

Recovery Questionnaire), as well as a questionnaire about previous head injuries, the State-Trait Anxiety Inventory (STAI) Y-1 and Y-2, PTSD Checklist - Stressor Specific Version (PCL-S), Acute Stress Disorder Scale (ASDS) and Major Depressive Inventory (MDI).

Fear Conditioning Paradigm

During both sessions, a fear conditioning task adapted from Milad, et al (2007), Rabinak, et al (2013) and Garfinkel, et al (2014) was performed. The paradigm is displayed graphically in Figure III.1. E-Prime was used to present stimuli and record responses (Psychology Software Tools, Pittsburgh, PA). Participants viewed stimuli through MR-compatible goggles (NordicNeuroLab, Bergen, Norway) and audio stimuli were presented through MR-compatible earbuds (Sensimetrics, Malden, MA). Subjects responded to those stimuli using an MRI-compatible button box.

On the first scanning day, each participant underwent the habituation, acquisition and extinction phases of the conditioning paradigm. During each trial of every phase of the fear conditioning paradigm, subjects were presented with a context (a picture of an office or conference room) for a period of between 2.5 and 7.5 seconds, followed by one of three conditioned stimuli (CS, a pink, yellow or blue light) for four seconds. During habituation, each CS was presented twice in each context. During fear acquisition, two conditioned stimuli (CS+E and CS+U) were followed by an unconditional stimulus (US), a burst of white noise, at a 60% reinforcement schedule. The third stimulus (CS-) was not associated with the white noise. Each CS was presented 10 times during each run, and there were two runs of fear acquisition. Rest periods between trials were jittered between 4 and 9 seconds.

During the extinction phase, subjects viewed the CS+E and CS- again in a new context, but no white noise bursts were delivered. Each CS in the extinction phase was presented 10 times per run, with two runs per subject.

On the second scanning day, subjects underwent the extinction recall phase in the scanner, where they were presented with the same context from extinction and all 3 stimuli (CS+U, CS+E and CS-) without white noise. During the extinction recall phase, there were 10 instances of each CS trial per run, and there were two runs of extinction recall per subject. Subjects were pseudorandomly assigned to one of two randomizations of the colors corresponding to each CS and the context assigned to acquisition or extinction and extinction recall.

Expectancy scores were collected during all phases of fear conditioning. After each CS presentation, subjects responded to the prompt, “Do you expect to hear the white noise?” by pressing the corresponding response button with their right hand. Button 1 (thumb) was “Definitely No”, button 2 (index finger) was “Probably No”, button 3 (middle finger) was “Maybe”, button 4 (ring finger) was “Probably Yes” and button 5 (pinky finger) was “Definitely Yes”. Subject responses were recorded for the first two seconds of the presentation of the CS. Expectancy scores were compiled for each subject over all phases, and differences between CS types were calculated using paired-samples t-tests.

Multisource Interference Task (MSIT) Paradigm

Subjects also performed the MSIT, which was adapted from Bush and Shin, 2006. Subjects were presented with a set of three numbers and instructed to respond with the identity of the number that is different than the other two numbers. In control

trials, the distractor numbers were both zeroes and the target number was in the same position as the response. In interference trials, the distractor numbers are valid responses, and the target number is in a different position from the response. The task is shown graphically in Figure III.2. Each subject performed three runs of the MSIT. Each run consisted of six alternating blocks of 16 interference or control trials, and each trial lasted 1850 milliseconds. Every block was preceded by a 12-second fixation period, and the final block of each run was followed by a 60-second fixation period.

Skin Conductance Response

Skin conductance response (SCR) was collected during the acquisition, extinction and extinction recall phases of the fear conditioning paradigm. SCR was recorded using the BIOPAC MP150 Data Acquisition System (BIOPAC Systems Inc., Goleta, CA) connected to a computer running AcqKnowledge software.

SCR data was pre-processed using AcqKnowledge 4.2 (BIOPAC Systems Inc., Goleta, CA). Data was first low-pass filtered at 25 Hz, then smoothed. After these pre-processing steps, the time of presentation of each CS was marked using AcqKnowledge. SCR for each individual trial was then scored manually by taking the difference between the maximum and minimum value during a ten second period from two seconds preceding CS presentation to eight seconds following the CS. If the maximum value preceded the minimum value temporally, the response for that trial was marked as zero. Additionally, any SCR difference below 0.01 mho was marked as zero. Subjects that had zero response throughout the run were excluded from SCR analysis on a run-by-run basis. Scorers were blinded to subject group, as well as CS trial type.

Comparisons in SCR to different CS trial types were calculated with paired sample t-tests. Group comparisons in SCR were calculated with repeated measure ANOVA.

Additionally, individual SCR differences were correlated with BOLD signal differences during fear conditioning. During acquisition, the difference in response between CS+ trials (without US) and CS- trials was calculated for each subject. During each run of extinction, the difference in SCR between CS+E and CS- trials was calculated for each subject. Finally, during each run of extinction recall, the difference in SCR between CS+U and CS+E trials was calculated. These differences were then normalized by dividing by the average SCR to all trials for each subject. Bivariate regression analyses between these SCR values and extracted beta values from ROIs in main effects contrasts were then performed to assess the relationship between SCR and BOLD signal differences.

Magnetic Resonance Imaging

Image Acquisition

MRI scanning occurred on a Philips 3.0 Tesla Achieva X-series MRI (Philips Medical Systems) at the VA Ann Arbor Healthcare System. After a T1 image (T1-overlay) was obtained, a T2*-weighted, echoplanar acquisition sequence [GRE; repetition time, 2000 ms; echo time, 25 ms; flip angle, 90°; field of view (FOV), 22 cm; 42 slices; thickness/skip, 3.0/0 mm matrix size equivalent to 64 × 64] was collected for each run of the fear conditioning protocol and the MSIT protocol. After the acquisition phase of the fear conditioning protocol was completed, a high-resolution T1 scan was obtained for anatomic normalization [FOV, 26 cm; thickness/skip, 1.0/0 mm].

Preprocessing

A standard series of processing steps was performed using statistical parametric mapping (SPM8, Wellcome Trust Center for Neuroimaging, London, UK). Scans were motion-corrected, slice-time corrected, realigned to the first scan in the experiment to correct for head motion, co-registered with the high-resolution sagittal images, anatomically normalized to the Montreal Neurological Institute (MNI) 152 template brain, resampled to 3×3×3 mm^3 voxels, and smoothed with a 5×5×5 mm^3 kernel. Motion parameters (mean displacement, mean angle) were compared across groups via independent-samples t-tests, and runs with any movement greater than 3 mm were excluded.

ROI Analysis

Small volume correction was applied using familywise error (FWE) correction within anatomical masks from the WFU Pick Atlas (ANSIR Laboratory, Winston-Salem, NC) tool in SPM8. Left and right hippocampus, left and right amygdala and left and right insula masks were created from the Automated Anatomical Labeling (AAL) images in the WFU Pick Atlas. The dACC mask was defined using previously described parameters (Cascio et al., 2015): WFU Pick Atlas was used to combine Brodmann areas 24 and 32 and the AAL anterior and middle cingulate images. Brodmann areas 8 and 9 were then subtracted from the image, and the resulting image was bounded to $-16 < x < 16$, $0 < y < 33$ and $6 < z < 52$.

Results

Participants

21 participants met inclusion criteria for the mTBI group, and 20 participants met the inclusion criteria for the control group. Sample demographics and characteristics are presented in Table III.1. The two groups did not differ by age, race or gender. There was no difference in the days between injury and the scan, or the type of injury suffered. The control group more commonly reported pain at the time of the scan (70% vs 38.1% of subjects, $t(39)=2.108$, $p<.05$), but there were no significant differences in pain intensity, estimated time of emotional or physical recovery, and certainty of recovery between the groups. There was also no significant difference between groups in the self-reported incidence of previous head injuries. Additionally, there were no differences between groups in scores on the ASDS, STAI, PCL-S and MDI. However, 19.0% of subjects in the mTBI scored above 35 on the PCL-S (a common cut-off for PTSD screening) versus 0% of subjects in the control group ($t(20)=2.828$, $p<.05$). Additionally, incidence of LOC was associated with increased ASDS scores (43.7 ± 16.5 vs 33.2 ± 10.7; $t(39)=2.045$, $p<.05$), and the number of PCS was positively correlated with scores on the ASDS ($r=0.334$, N=41, $p<.05$), STAI Y-1 ($r=0.337$, N=41, $p<.05$**),** PCL-S ($r=0.375$, N=41, $p<.05$) and MDI ($r=0.321$, N=41, $p<.05$). Only the correlation between PCS and STAI Y-1 was significant within the mTBI group ($r=.441$, N=21, $p<.05$).

Fear Acquisition

Expectancy Scores: Significant differences in expectancy scores were observed between CS trial types in all phases of fear conditioning and displayed in Figure III.3. One subject's data was removed from the analysis due to invalid responses. During fear acquisition, subjects had higher expectancy to the CS+ stimuli versus CS- stimuli during

both runs of fear acquisition (CS+: 1.86 ± 0.54, CS-: 3.46 ± 0.43, $t(38)=12.179$, $p<.001$). There were no significant effects of group in the expectancy scores.

SCR. SCR data from the all phases of the fear conditioning paradigm are shown in Figure III.4. During the second run of fear acquisition, subjects had significantly greater SCR to the CS+ stimuli (excluding trials with US) versus CS- stimuli (0.965 ±1.213 vs 0.697 ± 0.753, $t(27)=2.132$, $p<.05$). 13 subjects did not have valid SCR during the acquisition phase and were excluded from this analysis.

BOLD fMRI. No group differences in BOLD signal were found during the fear acquisition phase of the fear conditioning paradigm.

Fear Extinction

Expectancy Scores. During the extinction phase, subjects had higher expectancy to the CS+E stimuli versus CS- stimuli in the first four trials of fear extinction (CS+E: 2.47 ± 0.98, CS-: 1.73 ± 0.76, $t(38)=4.693$, $p<.0001$). Additionally, subjects had lower expectancy to the CS+E in the last 4 trials of extinction versus the first 4 trials of extinction (CS+E First Four Trials: 2.47 ± 0.98, CS+E Last Four Trials: 1.061 ± 0.26, $t(37)=8.849$, $p<.0001$). There was no significant difference between expectancy to the last four CS+E trials vs the last four CS- trials. There were also no significant effects of group in the expectancy scores during extinction.

SCR. Subjects had higher SCR to the CS+E stimuli versus CS- stimuli during the first four trials of fear extinction (1.57 mho ± 2.32 vs 0.59 mho ±0.60, $t(18)=2.282$, $p<.05$). In the last 4 trials of extinction, subjects showed no difference between SCR to the CS+E versus CS. Additionally, subjects in the mTBI group showed a significant increase in SCR to CS+E as compared to controls, between the first and second half of

the first run of extinction (0.66 mho ± 0.99 vs -0.20 mho ± 0.46, F(1,22)=6.891, p<.05). 22 subjects did not have valid SCR during the extinction phase and were excluded from this analysis.

BOLD fMRI. No group differences in BOLD signal were found during the first run of fear extinction. However, during the second run of fear extinction (late extinction), fMRI data indicated that subjects in the mTBI group had significantly greater activation to the CS+E versus CS- in the dACC, L insula and R insula compared to controls. Location and effect sizes of significant activations are listed in Table III.2, and shown in Figure III.5. Additionally, we extracted beta values for individual trial types from each of these regions, and these values are displayed in Figure III.6. In the mTBI group, activation of dACC and bilateral insula in response to the CS+E increases from early extinction to late extinction, while it decreases in the control group. In the whole brain search, no significant group differences were found in other regions after familywise error correction.

In late extinction, normalized individual SCR differences from the main effect contrast (CS+E vs CS-) and beta values from the same contrast were significantly correlated in dACC (r(23)=.4269, p<.05). It should be noted that this correlation was also significant when SCR non-responders were included in the analysis with a response coded as zero (r(34)=.3757, p<.05). This relationship is shown in Figure III.7.

Extinction Recall

Expectancy Scores. Subjects had higher expectancy to the CS+U stimuli versus CS- stimuli during the first run of extinction recall (CS+U: 1.81 ± 0.69, CS-: 1.24 ± 0.54, t(37)=6.062, p<.0001). Higher expectancy scores were also seen to CS+E stimuli

versus CS- stimuli (CS+E: 1.62 ± 0.62, CS-: 1.24 ± 0.54, $t(37)=3.448$, $p<.01$), and CS+U stimuli versus CS+E stimuli (CS+U: 1.81 ± 0.69, CS+E: 1.62 ± 0.62, $t(37)=2.062$, $p<.05$). There were no significant differences in expectancy to the different CS types during the second run of extinction recall, and no group differences in the expectancy scores during extinction recall.

SCR. Subjects had greater SCR to the CS+U stimuli versus CS- stimuli during the first five trials of extinction recall (1.10 mho ± 1.43 vs 0.72 mho ± 0.93, $t(23)=2.176$, $p<.05$) confirming retention of fear conditioning to CS+U. Similarly, the difference in SCR between CS+U and CS+E trials also approached significance (1.10 mho ± 1.43 vs 0.70 mho ± 0.87, $t(23)=1.876$, $p=0.074$). There was no difference in SCR between CS- and CS+E trials. 17 subjects did not have valid SCR during the extinction recall phase and were excluded from analysis.

BOLD fMRI. No group differences in BOLD signal were found during the first run of fear extinction recall. In the second run of fear extinction recall (late extinction recall), fMRI data indicated that, compared to controls, subjects in the mTBI group had significantly greater activation to the CS+U versus CS- in bilateral dACC. Additionally, there was significantly greater activation in the mTBI group versus controls to CS+U versus CS+E in bilateral amygdala. Location and effect sizes of significant activations are listed in Table III.2, and shown in Figure III.8. Extracted beta values for individual trial types from these regions are displayed in Figure III.9. Compared to controls, activation of dACC and bilateral amygdala in mTBI subjects in response to the CS+U increases from early extinction recall to late extinction recall. Whole brain analysis

during both runs of extinction recall found no significant group differences after FWE correction.

In extinction recall, comparing normalized individual SCR differences and beta values yielded no significant correlations.

Multisource Interference Task

***Accuracy and Response Time*.** Behavioral results from MSIT are displayed in Figure III.10. Across both groups, subjects showed increased reaction time to interference trials versus control trials (898.9 ± 90.29 vs 606.1 ± 56.59 ms, $t(37)=29.07$, $p<.0001$). Additionally, subjects showed decreased accuracy in the interference trials versus control trials (96.49 ± 4.02 v 98.81 ± 1.80 %, $t(37)=5.128$, $p<.0001$). There were no significant differences between groups in either accuracy or response time to MSIT trials.

BOLD fMRI. Using whole brain analysis with FWE correction, significant differences were found in the task main effect (Interference vs Control) for all subjects. Location of peaks with effect sizes are listed in Table III.3, and significant activations are shown in Figure III.11. Regions found to be activated in the task included bilateral thalamus, bilateral fusiform gyrus, bilateral insula and ACC/middle cingulate gyrus. However, no significant differences were observed between the mTBI and control groups.

Discussion

The results from this study demonstrate that extinction learning is altered in patients with recent mTBI as compared to controls with recent trauma but without mTBI.

Subjects in the mTBI group showed a significant increase in SCR to the CS+E over the course of the early extinction run, as compared to the control group. Additionally, mTBI subjects showed a significantly higher SCR to CS+E versus CS- during the end of the first run of extinction. Both of these interactions showed that there was an elevated psychophysiological response to CS+E trials in mTBI patients versus controls during the extinction phase, indicative of an extinction learning deficit. Differences in BOLD signal further supported this interpretation, as CS+E vs CS- contrasts in late fear extinction uncovered greater activation in the mTBI group versus controls in insula and dACC, regions associated with the generation of the fear response (Greco and Liberzon, 2016). The beta values extracted from these regions also demonstrate that these effects are driven by sustained activation in insula and dACC to the CS+E that is only seen in the subjects with mTBI. As mentioned in the introduction, impaired extinction learning has been observed in animal models of TBI (Heldt et al., 2014, Davies et al., 2016, Schneider et al., 2016). The differences in SCR, as well as differences in BOLD signal associated with fear extinction are the first evidence of a deficit in fear-associated learning in human subjects with mTBI.

We originally hypothesized that mTBI patients would have deficits in extinction retention, as this aspect of fear-associated learning has been implicated in posttraumatic stress disorder (PTSD). Although expectancy and SCR measures confirmed that our subjects were retaining a greater fear response to the CS+U versus CS+E and CS- at the beginning of extinction recall, no significant group differences in any measure, including BOLD signal, were found during early extinction recall. However, just as in extinction, significant differences were observed during the second

run of extinction recall. During extinction recall, subjects are essentially undergoing extinction of the CS+U, as it was not formerly associated with safety like the CS+E was on the previous day. Compared to controls, subjects in the mTBI group showed greater activation to CS+U versus CS- in two areas of dACC, as well as greater activation to CS+U versus CS+E in bilateral amygdala. Analogous to the findings in late extinction, extracted beta values from extinction recall show that the mTBI patients have sustained activation in these regions to CS+U that is not observed in the control group. Again, this activation in dACC suggests that subjects with mTBI are continuing to activate the fear response, while the amygdala activation may suggest difficulties in encoding the CS-no US contingency (Greco and Liberzon, 2016). The significance of amygdala hyperactivation is harder to pinpoint due to the multiple roles of amygdala in both acquisition and extinction. Basolateral amygdala (BLA) is responsible for encoding both the acquisition and extinction memory, and centromedial amygdala projects to areas responsible for the production of the conditioned response (Maren and Quirk, 2004). However, numerous studies suggest that the central amygdala plays a critical role in fear learning (Li et al., 2013, Penzo et al., 2014, Penzo et al., 2015), and optogenetic studies suggest that different projections to the central amygdala enhance or inhibit fear learning (Tye et al., 2011). Thus, it is necessary to both examine subregions within amygdala as well as subpopulations of neurons that may increase and decrease the fear response. Conventional BOLD fMRI is unable to distinguish between these divisions of the amygdala, but future studies using multivariate pattern analysis may be able to discern where differential activation is occurring. Additionally, animal models of

mTBI are tremendously important in understanding the significance of alterations of amygdala activation during fear-associated learning following mTBI.

The limited number of previously-completed fMRI studies in subjects with a history of mTBI commonly implicate areas of the frontal lobe and centrally-located limbic structures, such as insula and ACC (Eierud et al., 2014). Additionally, measures of white matter connectivity in mTBI patients frequently find anisotropy differences in frontal tracts like the genu of the corpus callosum and anterior corona radiata, as well as cingulate bundles and the uncinate fasciculus (Eierud et al., 2014). This fairly localized damage is most likely due to the centripetal force and frontal impacts commonly involved in brain injury. The regions implicated in this study are all located in this area, and dACC, insula and amygdala are connected to each other and the hippocampus through the cingulate bundles and uncinate fasciculus, suggesting that damage or inflammation in these areas may in fact be related to differences in fear-associated learning. Further, structural neuroimaging studies have found diminished hippocampal and amygdalar volumes following mTBI (Depue et al., 2014, Zagorchev et al., 2016), and these could be associated with deficient fear-associated learning processes. Studying functional and structural connectivity, and the way that measures of connectivity relate to extinction learning deficits, may be particularly informative in understanding how the damage and inflammation caused by mTBI may manifest in behavioral abnormalities and functional deficits.

It should be noted that mTBI patients showed no difference in fear acquisition compared to controls, as evidenced in expectancy scores, SCR differences and brain activation. Fear acquisition has been shown to be intact in previous studies of patients

who have experienced trauma, both with and without PTSD (Milad et al., 2009, Garfinkel et al., 2014). Both expectancy scores and SCR results from the second run of acquisition demonstrate that subjects acquired a fearful response to the CS+ versus the CS. Additionally, expectancy scores and SCR results from early extinction and CS+U results from early extinction recall demonstrate that the acquisition memory was retained into the the extinction phase and the extinction recall phase. These findings confirm that subjects in both groups performed the task properly and learned the CS-US contingency.

Further, in our study there were no significant group differences in responses to the MSIT (both behaviorally and in BOLD signal), suggesting that attentional processes, especially conflict monitoring, remain intact in our subjects. Additionally, these results suggest that the BOLD signal differences we observed during extinction and extinction retention testing were not due to attentional deficits. This addresses the potential caveat that in the acute and subacute recovery phases from mTBI, patients often show impairments in attentional processing (Halterman et al., 2006, Malojcic et al., 2008), impairments that have been associated with hyperactivation in insula and ACC (Smits et al., 2009).

There were several limitations that require consideration. Our population was limited to subjects who had experienced a traumatic event within two weeks of scanning, so all mTBI patients were in either the acute or subacute phase of injury. In the future, subjects should be studied also at later time points in order to determine whether the differences in fear-associated learning observed in this study remain, increase or dissipate over time. We also did not include sex hormone measures in our

analysis, and levels of estrogen and progesterone have been found to be associated with fear-associated learning differences (Lebron-Milad et al., 2012, Merz et al., 2012). Although we did not see any effects of gender on activation or SCR differences, larger groups of female patients should be included in future studies so that there is a sufficient cohort to investigate the interaction of sex hormone levels with fear-associated learning in mTBI patients. This study involved a heterogeneous sample of TBI patients, with subjects who experienced a variety of different traumatic events, including motor vehicle accidents, sports injuries and falls. Although this may improve the generalizability of the results, heterogeneous injury mechanisms may limit effect sizes, as some previous studies have suggested (Jovanovic et al., 2012, Rosenbaum and Lipton, 2012, Mayer et al., 2015). Additionally, all of the subjects in this study had very mild TBIs, as all subjects had a GCS score of 15, and none were admitted to the hospital. Although our correlations between measures of injury severity (i.e. LOC, PTA and number of post-concussive symptoms) and SCR and BOLD signal differences did not identify any significant relationships during any phase of fear-associated learning, it is possible that increased mTBI severity could produce more PTSD-like effects during extinction recall, in addition to extinction deficits.

At very mild levels of injury, it is likely that slower or deficient extinction learning does not lead to deficits in extinction retention because these patients are able to eventually encode the safety trace. In more severe forms of injury, the safety trace may never be properly encoded, and retrieval of this trace will appear as a PTSD-like deficit in extinction recall. In fact, there is evidence that increasing severity of mTBI is associated with increased odds of developing PTSD (Roitman et al., 2013, Stein et al.,

2015). Further, the effect of stress may impair contextual processing and retrieval of an improperly-coded safety memory, potentiating the effect of the extinction deficit. More studies need to be performed, in populations with higher rates of PTSD, in order to determine how stress interacts with the fear-associated learning deficits that we have observed in mTBI.

As the first study of fear-associated learning in mTBI patients, the research described here demonstrates early fear extinction deficits following mTBI, confirming the fear extinction deficits observed in several animal studies. Additionally, it highlights greater differential activation during fear extinction in regions that are commonly affected by mTBI and known to be intricately involved in the fear response.

References

American Psychiatric Association (2013) *Diagnostic and statistical manual of mental disorders* (5th ed.).

Bush G, Shin LM (2006) The Multi-Source Interference Task: an fMRI task that reliably activates the cingulo-frontal-parietal cognitive/attention network. *Nat Protoc* **1**:308-313.

Cascio CN, Konrath SH, Falk EB (2015) Narcissists' social pain seen only in the brain. *Social Cognitive and Affective Neuroscience* **10**:335-341.

Corrigan JD, Anbesaw, W.S., Orman, J.A. (2010) The Epidemiology of Traumatic Brain Injury. *J Head Trauma Rehabil* **25**:72–80.

Davies DR, Olson D, Meyer DL, Scholl JL, Watt MJ, Manzerra P, Renner KJ, Forster GL (2016) Mild Traumatic Brain Injury with Social Defeat Stress Alters Anxiety, Contextual Fear Extinction, and Limbic Monoamines in Adult Rats. *Front Behav Neurosci* **10**.

Depue BE, Olson-Madden JH, Smolker HR, Rajamani M, Brenner LA, Banich MT (2014) Reduced amygdala volume is associated with deficits in inhibitory control: a voxel- and surface-based morphometric analysis of comorbid PTSD/mild TBI. *Biomed Res Int* **2014**:691505.

Eierud C, Craddock RC, Fletcher S, Aulakh M, King-Casas B, Kuehl D, LaConte SM (2014) Neuroimaging after mild traumatic brain injury: Review and meta-analysis(). *NeuroImage : Clinical* **4**:283-294.

Elder GA, Dorr NP, De Gasperi R, Gama Sosa MA, Shaughness MC, Maudlin-Jeronimo E, Hall AA, McCarron RM, Ahlers ST (2012) Blast exposure induces post-traumatic stress disorder-related traits in a rat model of mild traumatic brain injury. *J Neurotrauma* **29**:2564-2575.

Garfinkel SN, Abelson JL, King AP, Sripada RK, Wang X, Gaines LM, Liberzon I (2014) Impaired contextual modulation of memories in PTSD: an fMRI and psychophysiological study of extinction retention and fear renewal. *J Neurosci* **34**:13435-13443.

Genovese RF, Simmons LP, Ahlers ST, Maudlin-Jeronimo E, Dave JR, Boutte AM (2013) Effects of mild TBI from repeated blast overpressure on the expression and extinction of conditioned fear in rats. *Neuroscience* **254**:120-129.

Greco JA, Liberzon I (2016) Neuroimaging of Fear-Associated Learning. *Neuropsychopharmacology* **41**:320-334.

Halterman CI, Langan J, Drew A, Rodriguez E, Osternig LR, Chou LS, van Donkelaar P (2006) Tracking the recovery of visuospatial attention deficits in mild traumatic brain injury. *Brain* **129**:747-753.

Heldt SA, Elberger AJ, Deng Y, Guley NH, Del Mar N, Rogers J, Choi GW, Ferrell J, Rex TS, Honig MG, Reiner A (2014) A novel closed-head model of mild traumatic brain injury caused by primary overpressure blast to the cranium produces sustained emotional deficits in mice. *Front Neurol* **5**:2.

Jovanovic T, Kazama A, Bachevalier J, Davis M (2012) Impaired safety signal learning may be a biomarker of PTSD. *Neuropharmacology* **62**:695-704.

Klemenhagen KC, O'Brien SP, Brody DL (2013) Repetitive concussive traumatic brain injury interacts with post-injury foot shock stress to worsen social and depression-like behavior in mice. *PLoS One* **8**:e74510.

Knox D, George SA, Fitzpatrick CJ, Rabinak CA, Maren S, Liberzon I (2012) Single prolonged stress disrupts retention of extinguished fear in rats. *Learn Mem* **19**:43-49.

Lagarde E, Salmi L, Holm LW, et al. (2014) ASsociation of symptoms following mild traumatic brain injury with posttraumatic stress disorder vs postconcussion syndrome. *JAMA Psychiatry* **71**:1032-1040.

Lebron-Milad K, Abbs B, Milad MR, Linnman C, Rougemount-Bucking A, Zeidan MA, Holt DJ, Goldstein JM (2012) Sex differences in the neurobiology of fear conditioning and extinction: a preliminary fMRI study of shared sex differences with stress-arousal circuitry. *Biol Mood Anxiety Disord* **2**:7.

Lewis GC, Platts-Mills TF, Liberzon I, Bair E, Swor R, Peak D, Jones J, Rathlev N, Lee D, Domeier R, Hendry P, McLean SA (2014) Incidence and predictors of acute psychological distress and dissociation after motor vehicle collision: a cross-sectional study. J Trauma Dissociation 15:527-547.

Malojcic B, Mubrin Z, Coric B, Susnic M, Spilich GJ (2008) Consequences of mild traumatic brain injury on information processing assessed with attention and short-term memory tasks. *J Neurotrauma* **25**:30-37.

Maren S, Quirk GJ (2004) Neuronal signalling of fear memory. *Nat Rev Neurosci* **5**:844-852.

Mayer AR, Bellgowan PSF, Hanlon FM (2015) Functional magnetic resonance imaging of mild traumatic brain injury. *Neuroscience & Biobehavioral Reviews* **49**:8-18.

Merz CJ, Tabbert K, Schweckendiek J, Klucken T, Vaitl D, Stark R, Wolf OT (2012) Neuronal correlates of extinction learning are modulated by sex hormones. *Soc Cogn Affect Neurosci* **7**:819-830.

Meyer DL, Davies DR, Barr JL, Manzerra P, Forster GL (2012) Mild traumatic brain injury in the rat alters neuronal number in the limbic system and increases conditioned fear and anxiety-like behaviors. *Exp Neurol* **235**:574-587.

Milad MR, Pitman RK, Ellis CB, Gold AL, Shin LM, Lasko NB, Zeidan MA, Handwerger K, Orr SP, Rauch SL (2009) Neurobiological basis of failure to recall extinction memory in posttraumatic stress disorder. *Biol Psychiatry* **66**:1075-1082.

Milad MR, Wright CI, Orr SP, Pitman RK, Quirk GJ, Rauch SL (2007) Recall of fear extinction in humans activates the ventromedial prefrontal cortex and hippocampus in concert. *Biol Psychiatry* **62**:446-454.

Morissette SB, Woodward M, Kimbrel NA, Meyer EC, Kruse MI, Dolan S, Gulliver SB (2011) Deployment-related TBI, persistent postconcussive symptoms, PTSD, and depression in OEF/OIF veterans. *Rehabil Psychol* **56**:340-350.

National Center for Injury Prevention and Control (2003) *Report to Congress on Mild Traumatic Brain Injury in the United States: Steps to Prevent a Serious Public Health Problem*. Atlanta, GA: Centers for Disease Control and Prevention.

Orlovska S, Pedersen MS, Benros ME, Mortensen PB, Agerbo E, Nordentoft M (2014) Head injury as risk factor for psychiatric disorders: a nationwide register-based follow-up study of 113,906 persons with head injury. *Am J Psychiatry* **171**:463-469.

Palmer CP, Metheny HE, Elkind JA, Cohen AS (2016) Diminished amygdala activation and behavioral threat response following traumatic brain injury. *Experimental Neurology* **277**:215-226.

Rabinak CA, Angstadt M, Sripada CS, Abelson JL, Liberzon I, Milad MR, Phan KL (2013) Cannabinoid facilitation of fear extinction memory recall in humans. *Neuropharmacology* **64**:396-402.

Reger ML, Poulos AM, Buen F, Giza CC, Hovda DA, Fanselow MS (2012) Concussive brain injury enhances fear learning and excitatory processes in the amygdala. *Biol Psychiatry* **71**:335-343.

Roitman P, Gilad M, Ankri YL, Shalev AY (2013) Head injury and loss of consciousness raise the likelihood of developing and maintaining PTSD symptoms. *J Trauma Stress* **26**:727-734.

Rosenbaum SB, Lipton ML (2012) Embracing chaos: the scope and importance of clinical and pathological heterogeneity in mTBI. *Brain Imaging and Behavior* **6**:255-282.

Rougemont-Bucking A, Linnman C, Zeffiro TA, Zeidan MA, Lebron-Milad K, Rodriguez-Romaguera J, Rauch SL, Pitman RK, Milad MR (2011) Altered processing of contextual information during fear extinction in PTSD: an fMRI study. *CNS Neurosci Ther* **17**:227-236.

Schneider BL, Ghoddoussi F, Charlton JL, Kohler RJ, Galloway MP, Perrine SA, Conti AC (2016) Increased Cortical Gamma-Aminobutyric Acid Precedes Incomplete

Extinction of Conditioned Fear and Increased Hippocampal Excitatory Tone in a Mouse Model of Mild Traumatic Brain Injury. *J Neurotrauma*.

Sierra-Mercado D, McAllister LM, Lee CC, Milad MR, Eskandar EN, Whalen MJ (2015) Controlled cortical impact before or after fear conditioning does not affect fear extinction in mice. *Brain Res* **1606**:133-141.

Smits M, Dippel DW, Houston GC, Wielopolski PA, Koudstaal PJ, Hunink MG, van der Lugt A (2009) Postconcussion syndrome after minor head injury: brain activation of working memory and attention. *Hum Brain Mapp* **30**:2789-2803.

Stein MB, Kessler RC, Heeringa SG, Jain S, Campbell-Sills L, Colpe LJ, Fullerton CS, Nock MK, Sampson NA, Schoenbaum M, Sun X, Thomas ML, Ursano RJ, collaborators ObotAS (2015) Prospective Longitudinal Evaluation of the Effect of Deployment-Acquired Traumatic Brain Injury on Posttraumatic Stress and Related Disorders: Results From the Army Study to Assess Risk and Resilience in Servicemembers (Army STARRS). *American Journal of Psychiatry* **172**:1101-1111.

Thurman DJ (2001) The epidemiology and economics of head trauma. In: Head Trauma: Basic, Preclinical, and Clinical Directions (Miller L, H. R., ed) New York: John Wiley and Sons.

Wang X, Xie H, Cotton AS, Brickman KR, Lewis TJ, Wall JT, Tamburrino MB, Bauer WR, Law K, McLean SA, Liberzon I (2016) Early Changes in Cortical Emotion Processing Circuits after Mild Traumatic Brain Injury from Motor Vehicle Collision. J Neurotrauma.

Warren AM, Boals A, Elliott TR, Reynolds M, Weddle RJ, Holtz P, Trost Z, Foreman ML (2015) Mild traumatic brain injury increases risk for the development of posttraumatic stress disorder. *J Trauma Acute Care Surg* **79**:1062-1066.

Zagorchev L, Meyer C, Stehle T, Wenzel F, Young S, Peters J, Weese J, Paulsen K, Garlinghouse M, Ford J, Roth R, Flashman L, McAllister T (2016) Differences in Regional Brain Volumes Two Months and One Year after Mild Traumatic Brain Injury. *J Neurotrauma* **33**:29-34.

		Control	mTBI	
Demographics				
	Number of Subjects	20 (10 female)	21 (9 female)	
	Race	19 C, 1 AA	18 C, 3 AA	
	Age	26.4 (SD=7.1)	24.62 (SD=7.8)	
	Days Between ED Visit and Scan	6.2 (SD=3.00)	6.7 (SD=3.2)	
	Right-Handed	18 (90%)	16 (76.19%)	
	Previous mTBI (self-reported)	5 (25%)	8 (38.1%)	
Injury Characteristics				
	Self-Reported Head Injury	5 (25%)	21 (100%)	*
	Lost Consciousness	0 (0%)	6 (28.6%)	*
	Post-Traumatic Amnesia	0 (0%)	3 (14.3%)	
	Number of Post-Concussive	0.40 (SD=0.5)	4.0 (SD=1.8)	*
Type of Injury				
	Fall	4 (20%)	8 (38.1%)	
	Sports Injury	6 (30%)	5 (23.8%)	
	Hit / Hit by object	4 (20%)	5 (23.8%)	
	Motor Vehicle Accident	6 (30%)	3 (14.3%)	
Accident-Related Self-Report				
	Reported Pain at Time of Scan	14 (70%)	8 (38.1%)	*
	Pain Intensity (1-10)	3.9 (SD=2.12)	2.5 (SD=2.5)	
	Time to Recover Physically	25.7 days	22.7 days	
	Time to Recover Emotionally	27.4 days	34.5 days	
	Certainty of Recovery (1-10)	9.6 (SD=0.6)	9.1 (SD=1.7)	
Psychiatric Self-Report Measures				
	ASDS	32.7 (SD=10.2)	36.7 (SD=13.6)	
	> 55	0 (0%)	3 (14.3%)	
	STAI Y-1	30.6 (SD=10.5)	34.0 (SD=11.3)	
	STAI Y-2	33.7 (SD=10.4)	37.38 (SD=11.9)	
	PCL-S	22.0 (SD=5.8)	26.2 (SD=9.9)	
	> 35	0 (0%)	4 (19.0%)	*
	MDI	8.8 (SD=7.4)	12.5 (SD=10.9)	
	Met criteria for moderate	0 (0%)	2 (9.5%)	

Table III.1: Sample demographics and characteristics. AA, African-American; ASDS, Acute Stress Disorder Scale; C, Caucasian; MDI, Major Depressive Inventory; PCL-S, Posttraumatic Stress Disorder Checklist- Stressor Specific Version; STAI, State-Trait Anxiety Inventory. * = $p < .05$

Area	Side	Coordinates (x, y, z)	K (voxels)	Z-score	p^{FWE}
Late Extinction: CS+E > CS-, mTBI > control					
Insula*	L	-33, 8, 4	96	4.05	0.012
Insula*	R	33, 14, 4	84	3.86	0.016
dACC*		6, 23, 25	273	3.83	0.034
Late Extinction Recall: CS+U> CS-, mTBI > control					
dACC*	L	-12, 14, 34	69	4.12	0.016
dACC*	R	12, 8 , 37	52	3.21	0.031
Late Extinction Recall: CS+U> CS+E, mTBI > control					
Amygdala*	R	30, -4, -20	10	3.17	0.041
Amygdala*	L	-21, 1, -23	6	3.23	0.050

Table III.2: Brain activity during fear extinction and extinction retention. $p^{FWE} < 0.05$, * = small volume correction.

Area	Side	Coordinates (x, y, z)	K (voxels)	Z-score	p^{FWE}
Superior Parietal Lobule / Inferior Parietal Lobule	L	-27, -61, 43	1210	Inf	<0.001
Middle Frontal Gyrus / Superior Parietal Lobule	L	-30, -1, 49	273	7.33	<0.001
Inferior Occiptal Gyrus / Lingual Gyrus	R	33, -85, -5	248	7.00	<0.001
Precuneus	R	24, -58, 40	263	6.76	<0.001
Precentral Gyrus	R	27, -1, 49	88	6.61	<0.001
Inferior Frontal Operculum	L	-42, 5, 28	75	6.32	<0.001
Cerebellum	R	36, -64, -26	245	6.06	<0.001
Insula	R	36, 17, 1	21	5.20	<0.001
Thalamus	L	-12, -13, 10	26	5.06	<0.001
Insula	L	-27, 29, 1	9	5.00	<0.001

Table III.3: Brain activity during multisource interference task. Interference > control contrast for all subjects. All activation is from whole brain analysis, p^{FWE} <0.05.

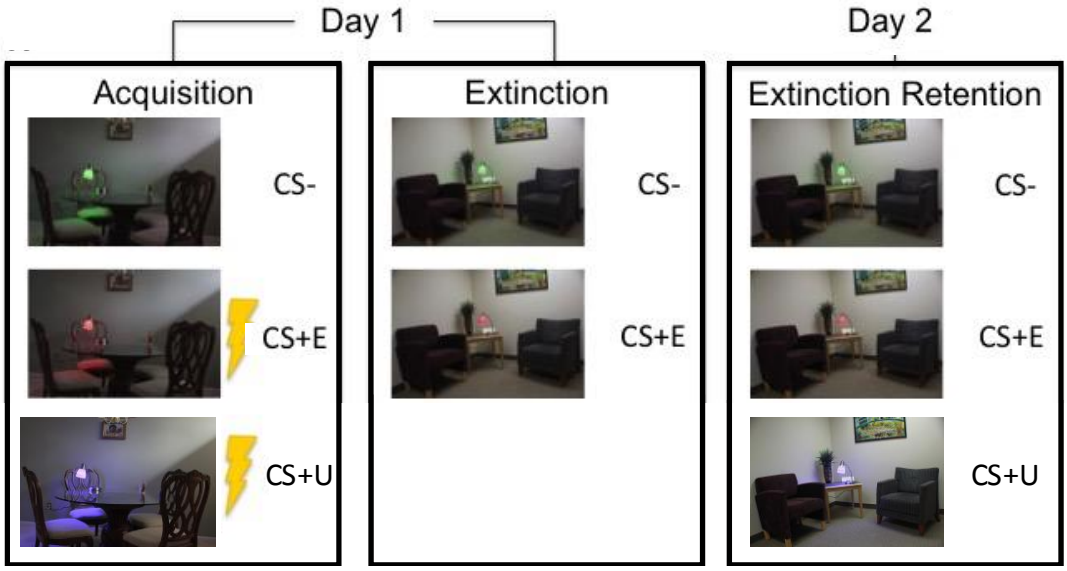

Figure III.1**:** In-scanner fear conditioning paradigm. Adapted from Milad et al, 2007 and Garfinkel et al, 2014.

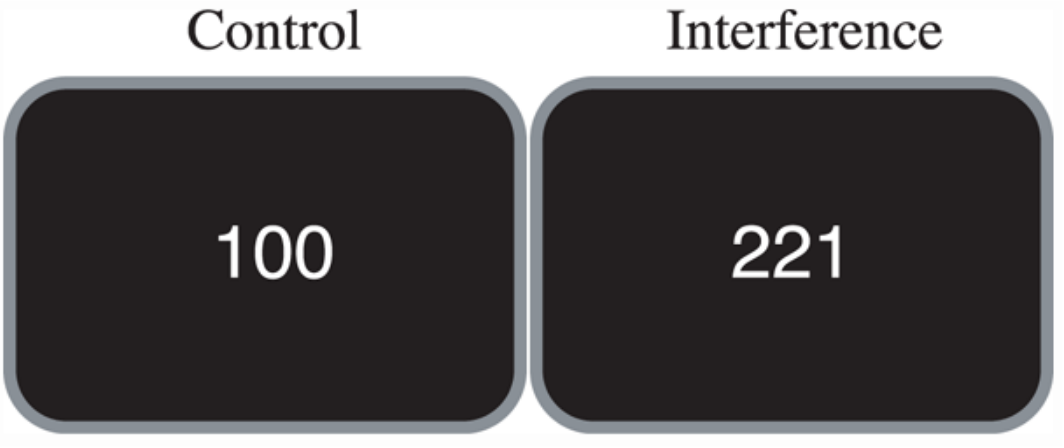

Figure III.2: Sample multisource interference task (MSIT) trials. Adapted from Bush and Shin, 2006.

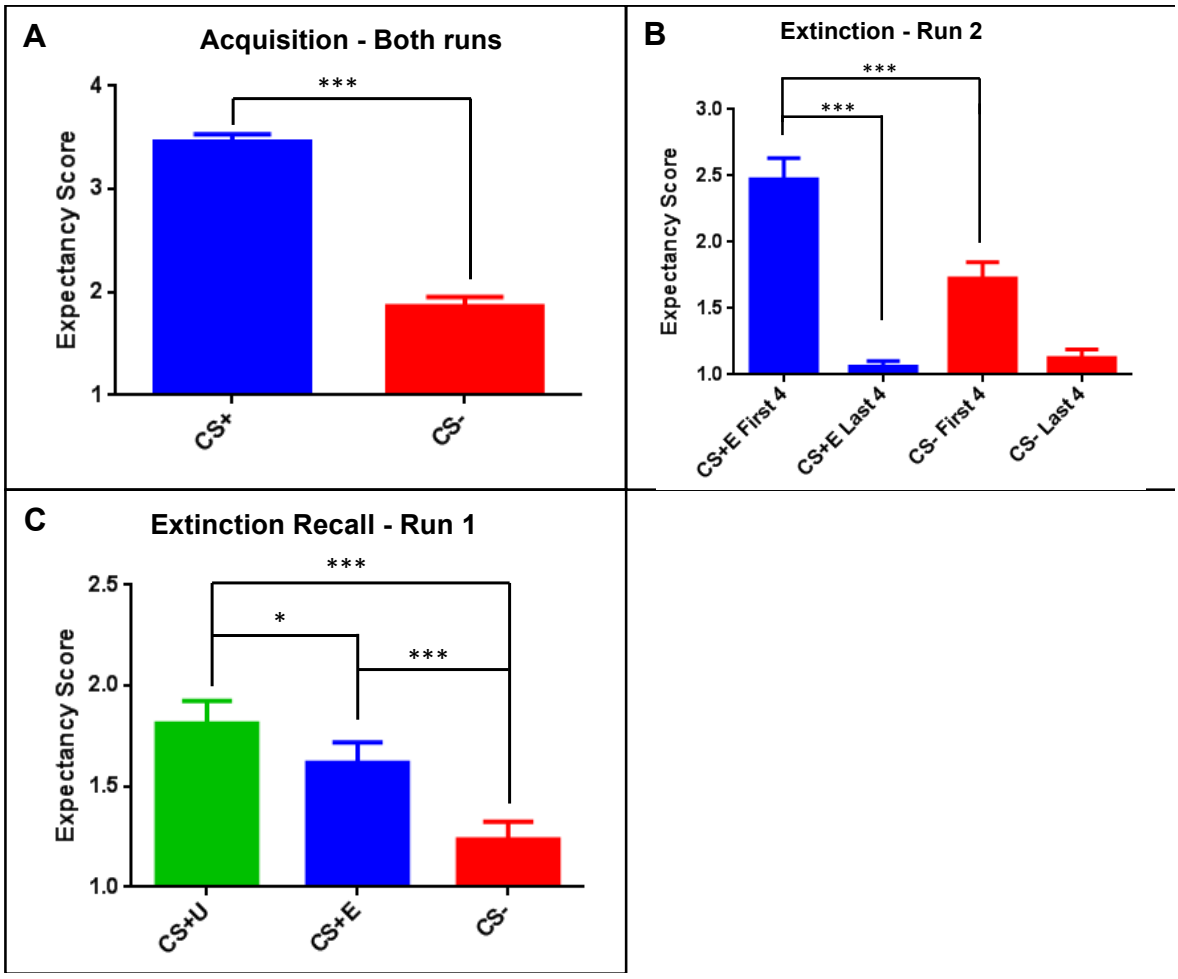

Figure III.3: Expectancy scores from the fear conditioning task across both groups. Scores were coded on a scale of 1 to 5. (A) Expectancy scores from fear acquisition demonstrate that subjects learned the CS-US contingency. (B) Expectancy scores from fear extinction demonstrate that subjects maintained the CS-US contingency into early extinction and properly extinguished response to the CS+E by the end of extinction. (C) Expectancy scores from the first run of extinction recall demonstrate that subjects maintained the acquisition memory to CS+U. * = p<.05, ***=p<.001

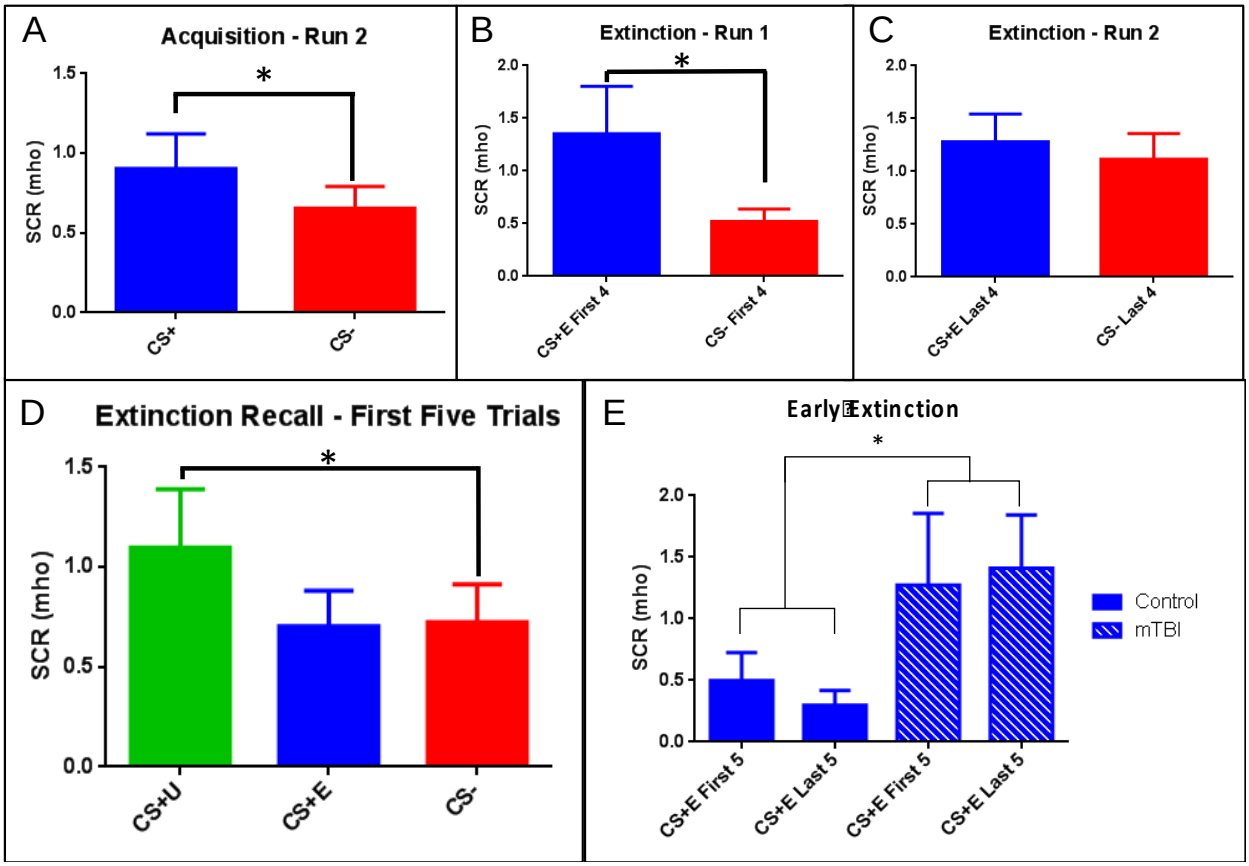

Figure III.4: Skin conductance response (SCR) measures from fear conditioning. **(**A) SCR differences in late fear acquisition demonstrate that subjects across groups acquire the fear response to the CS+. (B) SCR differences in early fear extinction demonstrate that subjects across groups maintain the fear response to the CS+ into the beginning of the extinction phase. (C) SCR differences in late fear extinction demonstrate that subjects properly extinguish the fear response to CS+E. (D) SCR differences in early extinction recall show that . (E) Group differences in SCR during the first run of fear extinction. * = p <.05

Late Extinction: CS+E > CS-, mTBI > Control

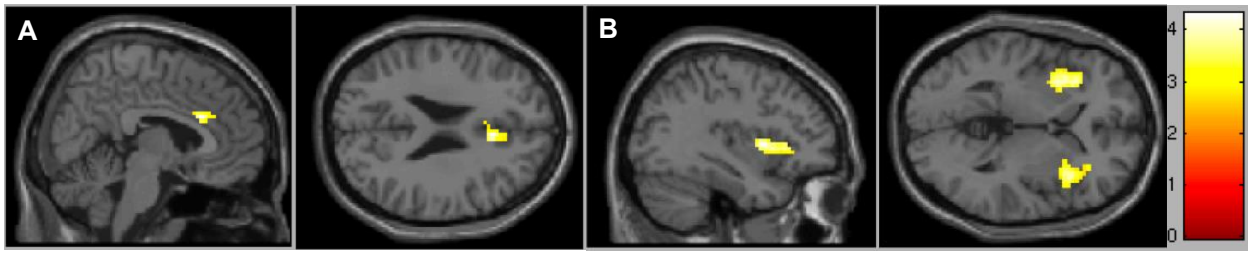

Figure III.5: Group differences in BOLD signal during the second run of extinction in (A) dorsal anterior cingulate cortex (dACC) and (B) bilateral insula. All images are from the CS+E> CS-, mTBI > control contrast, $p<.05$ with familywise error after small volume correction.

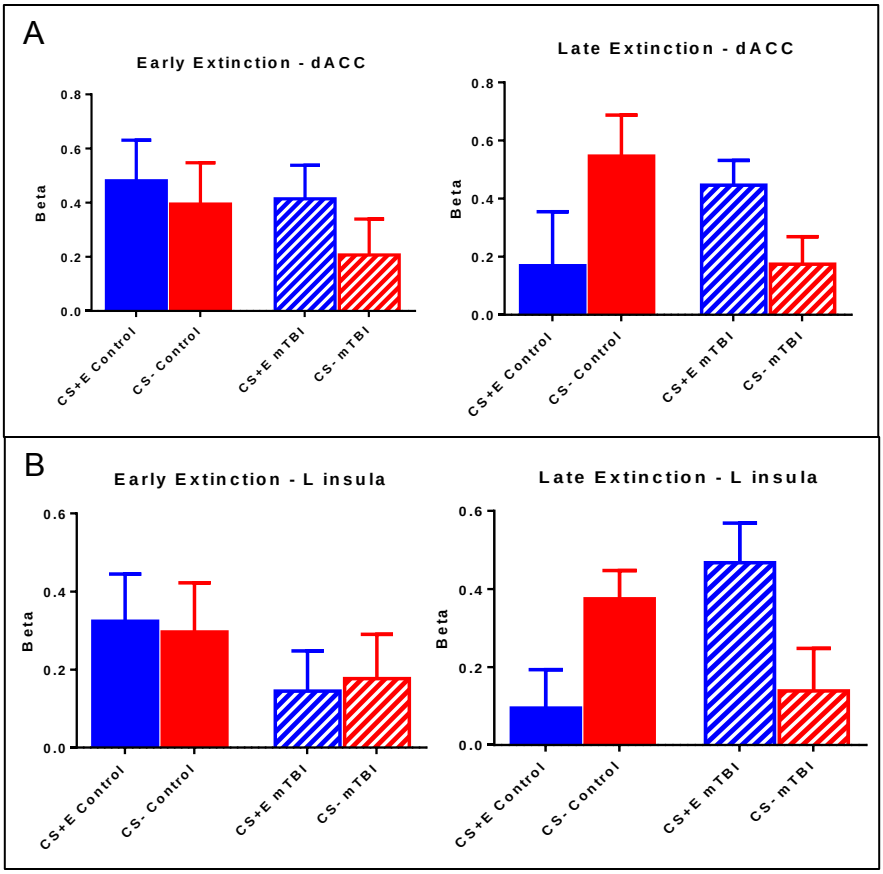

Figure III.6: Beta values from extinction runs 1 and 2, extracted using a 5 mm sphere centered around peaks from late extinction CS+E > CS-, mTBI > control contrast in (A) dorsal anterior cingulate cortex (dACC, 6, 23, 25) and (B) left insula (-33,8,4).

SCR versus BOLD signal in dACC to CS+E>CS-, Late Extinction

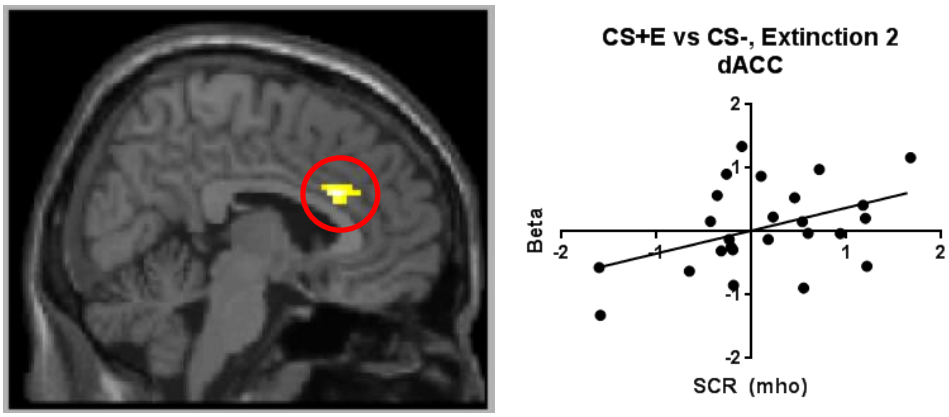

Figure III.7: Skin conductance response (SCR) versus differential blood oxygen level dependent (BOLD) response in dorsal anterior cingulate cortex (dACC) to CS+E versus CS- during late fear extinction. Increased fear response was associated with increased dACC activation to CS+E trials versus CS- trials.

Late Extinction Recall: mTBI > Control

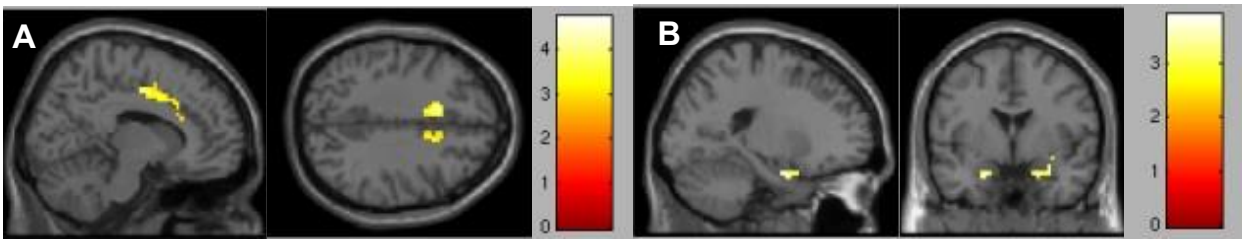

Figure III.8: Group differences in BOLD signal during the second run of extinction recall in (A) left and right dorsal anterior cingulate cortex, CS+U versus CS-, mTBI vs control contrast, and (B) left and right amygdala, CS+U versus CS+E, mTBI vs control contrast. $p<.05$ with familywise error after small volume correction.

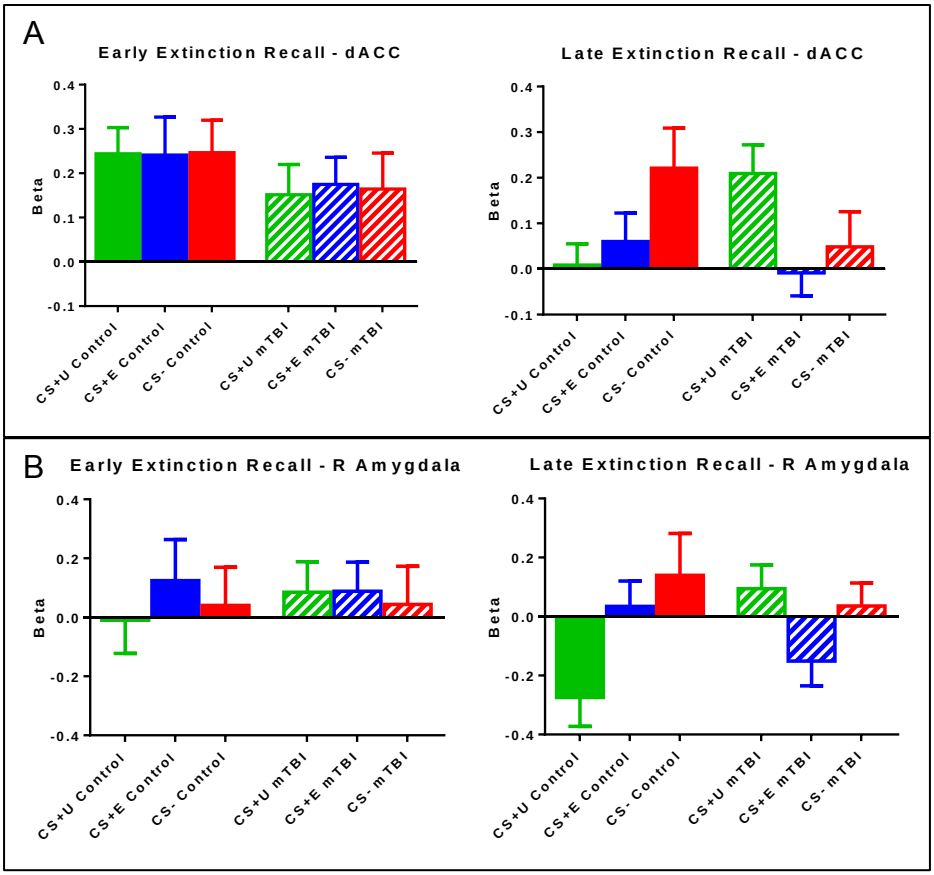

Figure III.9: Beta values from extinction recall runs 1 and 2. From 5 mm sphere centered around peaks from late extinction recall; (A) CS+U > CS-, mTBI > control contrast in dorsal anterior cingulate cortex (dACC, 12, 8, 37) and (B) CS+U > CS+E, mTBI > control contrast in right amygdala (anatomical mask).

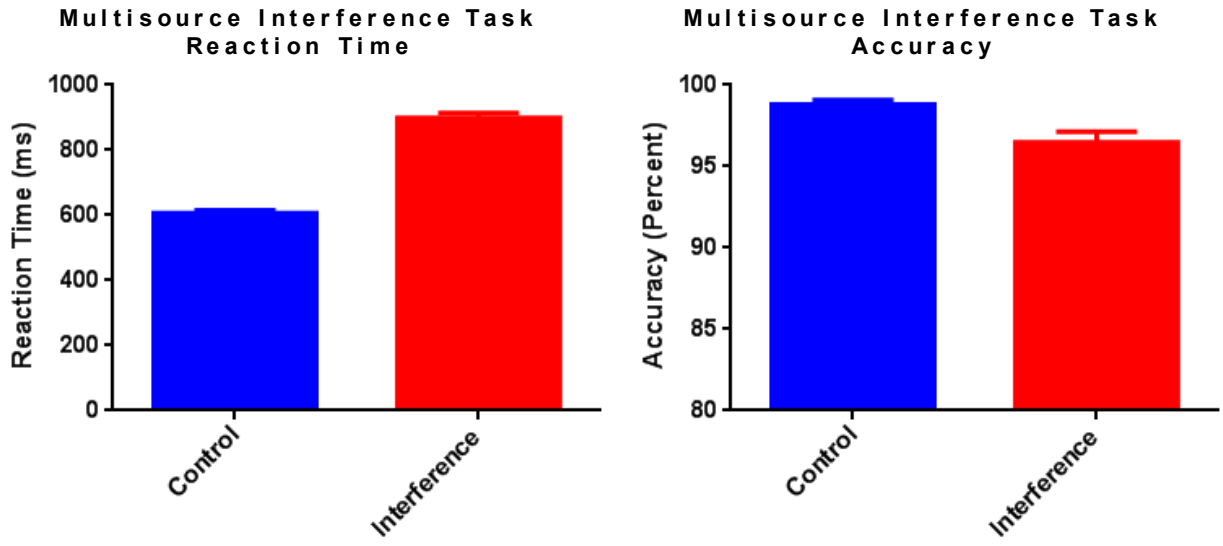

Figure III.10: Behavioral results from the multisource interference task across groups. A) Subjects showed increased reaction time to interference trials versus control. B) Subjects showed decreased accuracy in the interference trials versus control trials. Results indicate that there were no impairments in attention in either group during the task.

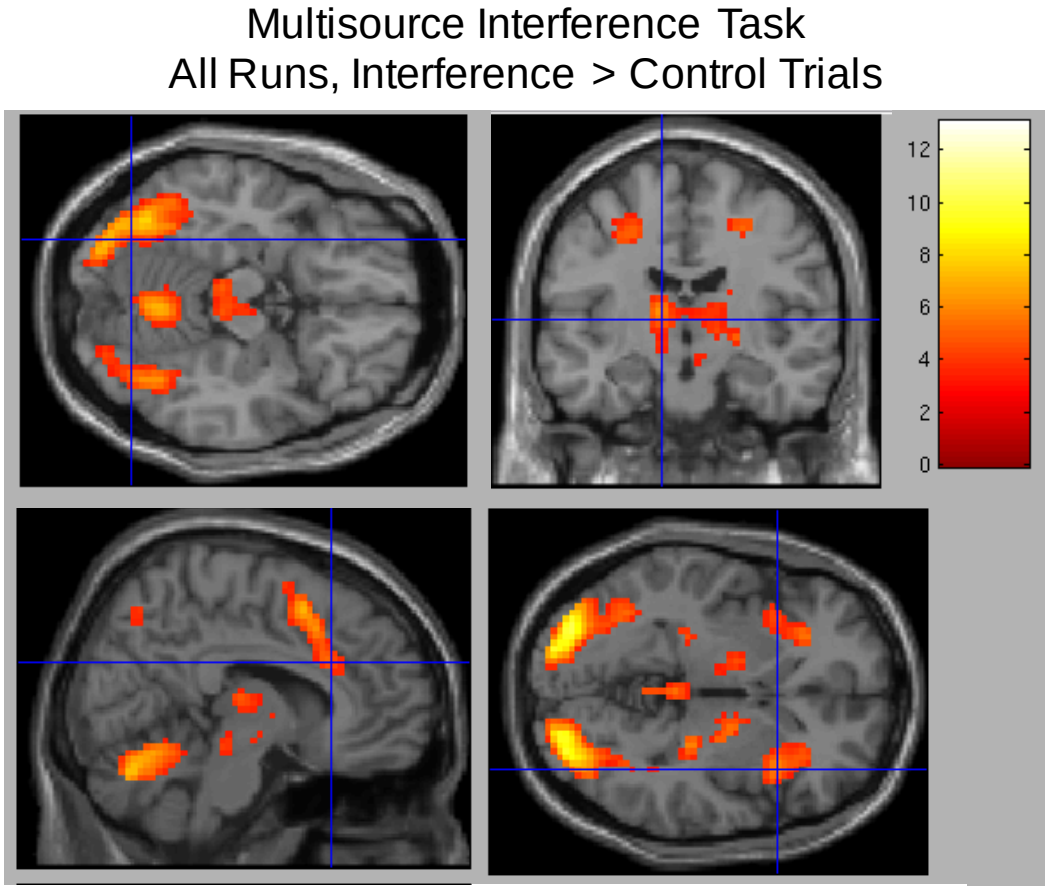

Figure III.11: BOLD signal differences in the multisource interference task from all subjects, interference > control trials. Clockwise starting from top left, bilateral fusiform gyrus, bilateral thalamus, bilateral insula and dorsal anterior cingulate cortex / middle cingulate cortex. Whole brain analysis, $p<.05$ after family-wise error correction.

CHAPTER IV

Discussion

As stated throughout this dissertation, mTBI is a significant public health challenge, and the epidemiological relationship between mTBI and PTSD is particularly important and poorly-understood. Understanding if and how mTBI leads to increased prevalence of posttraumatic stress symptoms opens the door for new treatments that may prevent the development of PTSD following brain injury.

The work performed here specifically investigated the hypothesis that mTBI leads to deficits in fear-associated learning that may predispose patients to PTSD development. First, we sought to establish that there were differences in the behavioral response to conditioned stimuli during fear acquisition, extinction and/or extinction retention (recall). We initially expected that the deficits in mTBI would mirror those seen in both animal models of PTSD and PTSD patients i.e. impaired extinction retention (Milad et al., 2009, Knox et al., 2012a, Garfinkel et al., 2014). However, both behavioral results in rats that underwent controlled cortical impact (CCI) and behavioral results in patients with recent mTBI instead pointed to an alteration in extinction learning.

Fear acquisition was fully intact in all experimental groups, across rat and human modalities. Rats across all groups conditioned fully, according to freezing behavior during the acquisition phase and at the beginning of the extinction phase. Additionally,

expectancy scores and SCR data confirmed that human subjects conditioned properly, and mTBI subjects did not differ from controls in either of these measures. Further, there were no group differences in brain activation during fear acquisition. Some studies of freezing behavior in animal models of TBI have suggested that brain injury enhances fear acquisition (Elder et al., 2012, Meyer et al., 2012, Reger et al., 2012) while others have suggested that it impairs acquisition (Palmer et al., 2016), but no differences in fear conditioning were observed in any of our experiments. As mentioned in previous chapters, it is possible that differences in both injury and fear-associated learning protocols are responsible for inconsistent results. Most of the studies that found differences in fear acquisition collapsed measures of freezing behavior across time periods (in contextual conditioning) or cue presentations, which limits the ability to distinguish deficits in acquisition from deficits in extinction.

In our experiments, there was remarkable consistency in the results we observed during the extinction phase in both animals and patients with recent mTBI. In both animal experiments, there was a significant interaction between time and injury that suggested that injured animals were not encoding the safety trace as efficiently as the uninjured animals. This effect was seen across the SPS manipulation and in animals at both 8 days and 16 days post-surgery. In humans, we noted increased SCR to CS+E vs CS- in the mTBI group versus controls in the middle of extinction learning, as well as increased SCR to CS+E during the second half of the first run of extinction versus the first half. This effect was not observed in controls.

As stated earlier, several previous studies of fear-associated learning in animal models of TBI have found deficits in extinction learning (Heldt et al., 2014, Davies et al.,

2016, Schneider et al., 2016). Similar findings were observed here, but several studies have reported negative results in extinction that should be addressed. Meyer, et al (2012) did not report differential extinction effects following weight-drop injury, but their extinction procedures occurred in the same context as conditioning. Retaining the same visual, olfactory and auditory contextual inputs significantly impacts extinction processing, as there is complete overlap between the contextual information that is being encoded in the safety trace and the contextual information that was previously encoded in the fear trace during acquisition. Widely varying injury mechanisms, as well as parameters within the same protocols may also explain some of the variability in findings. However, since mTBI in humans has so many different injury mechanisms and varying severities of impact, it is important that modelling mTBI in animals also examines different mechanisms. With all of these models, cross-validation of behavioral results and neural differences with findings in human mTBI patients is tremendously important in establishing models of TBI in rodents.

Additionally, we wanted to test the hypothesis that changes in neurobiology and brain activation in animal models of mTBI and patients with recent mTBI, particularly in amygdala, HC, dACC and insula, would underlie dysfunctions in fear-associated learning. In animals, the CCI model does not cause specific damage to ACC, insula or amygdala (due to location of insula and ACC, and the depth of the amygdala below the cortical surface). Therefore, we focused our histopathological measures on hippocampus and general cortex. The relatively mild severity of impact did not lead to visible neuronal cell loss or contusions (as examined with cresyl violet staining), similar to the lack of notable findings in conventional CT and MRI imaging of human patients

with mTBI (Shenton et al., 2012). We did, however, observe an increased inflammatory response (signified by increased microglial activation) in ipsilateral thalamus and cortex at 10 days post-injury in CCI-injured animals versus controls, and the area of microgliosis in ipsilateral thalamus was associated (at trend levels) with increased freezing during extinction recall in injured animals. The inflammation in thalamus could be a general marker of the brain-wide inflammatory response, or it could directly contribute to deficient extinction retention. Further behavioral studies are necessary to determine whether this trend-level interaction is legitimate, and subsequent examination of the many modulatory neurotransmitter systems (including GABAergic, serotonergic and adrenergic systems) in thalamic nuclei is required to understand the nature of this relationship.

There was not a significant difference in activation of microglia in ipsilateral hippocampus at 10 or 18 days post-injury, but we also did not see differences in numbers of neuronal progenitors. There were no significant differences in BOLD signal observed within the hippocampus, suggesting that contextual modulation processes thought to be compromised in patients with PTSD might not be affected immediately in mTBI. Despite negative results, it is highly important to continue investigating changes in hippocampal activation and concentrations of hormones (particularly mineralocorticoids and glucocorticoids), neurotransmitters (including GABA and glutamate) and their receptors, as there is substantial evidence in previous studies of mTBI that suggest that brain injury specifically affects hippocampal function (Girgis et al., 2016). It is also possible that the timing of our animal experiments did not allow us to capture changes in hippocampal neurogenesis following injury (Yu et al., 2008), and it is

also possible that increased severity of mTBI in humans may lead to functional changes in extinction learning in hippocampus.

Results from neuroimaging confirmed the extinction deficits that we observed in behavioral data and demonstrated that several of hypothesized regions of interest were differentially activated during fear-associated learning. Greater dACC and bilateral insula activation to CS+E versus CS- trials during the second run of extinction in mTBI patients versus controls suggests that these regions involved in the generation of the fear response are hyperactive to a stimulus that is being extinguished. Additionally, greater activation in dACC to CS+U versus CS- suggests that increased activation of the fear response also occurs when the CS+U should be extinguished during late extinction recall. Greater activation in bilateral amygdala to CS+U versus CS+E in mTBI patients versus controls in late extinction recall may also signify an exaggerated fear response, or it may correspond to extended encoding of the CS+U – no US contingency in the safety trace.

Our investigations into how PTSD interacts with mTBI in animal models were less informative. In the second animal experiment, increased freezing behavior was observed in injured animals during fear extinction, but there was no effect of SPS. This was expected, as previous studies in our laboratory have not found differences during the extinction phase in SPS animals (Knox et al., 2012b, Knox et al., 2012c). However, we did not replicate an effect of SPS on the extinction recall phase of fear learning, in animals with or without CCI. It is possible that the added stress of surgery (with or without CCI) masked the effect typically seen in SPS animals. Further experiments may elucidate the effect of timing on this interaction. A seven-day recovery period following

injury, although important for the wellbeing of the experimental animals, means that the next step of the experimental protocol occurs late in the subacute phase of injury recovery. Adding the 8-day SPS procedure then pushes behavioral testing into chronic phase of recovery. This was witnessed in the immunohistochemistry experiments, as differences in microglial activation were only seen at 10 days post-injury, and only in ipsilateral thalamus and cortex. Since the CCI procedure was well-tolerated by all animals, it should be possible to shorten the recovery period and examine whether behavioral differences are intensified in the acute and subacute phases of injury.

In the human neuroimaging experiments described here, we did not perform follow-up assessments looking into development of PTSD, as we had a small sample size (41 total subjects), and were limited by timing and the inability to recruit large numbers of patients. Despite the increased risk of PTSD in mTBI patients, the rate of PTSD in civilians with mTBI is relatively low, with one recent study of trauma patients finding that 18% of mTBI patients met criteria for PTSD diagnosis at 3 months post-injury (versus 9% in matched trauma-exposed controls) (Warren et al., 2015), and another study found only 8.8% of mTBI patients met criteria for PTSD at 3 months post-injury (versus 2.2% of trauma-exposed controls) (Lagarde et al., 2014). Our sample size necessarily precluded us from the ability to comprise a large enough group of mTBI patients who later developed PTSD. Further, a majority of our subjects experienced fairly minor trauma, with a large proportion experiencing sports injuries or falls rather than events with higher rates of PTSD development like motor vehicle accidents or assault. This is reflected in scores on the posttraumatic stress disorder checklist (PCL-S) and acute stress disorder scale (ASDS), as only 9.8 and 7.3% of subjects met

common screening criteria for PTSD and ASD, respectively. We analyzed correlations between SCR and BOLD signal differences and injury characteristics (number of postconcussive symptoms (PCS), loss of consciousness (LOC)), but we did not detect significant relationships. This is likely due to power concerns in our sample, as we only enrolled 3 subjects with posttraumatic amnesia and 6 subjects with LOC. As incidence of LOC during mTBI is associated with subsequent increased rates of PTSD (Roitman et al., 2013), and increased mTBI severity is also associated with increased PTSD rates (Stein et al., 2015), further studies with a larger and a slightly more severely injured sample are critical.

The findings in animals exposed to CCI and human subjects with recent mTBI potentially suggest that a different mechanism is responsible for the fear-associated learning deficits seen in mTBI versus PTSD. The impairments in extinction recall that are consistently observed in PTSD patients and models of PTSD were not seen here. Deficits in extinction learning have been reported inconsistently in PTSD patients or animal models of PTSD, but this phase of fear-associated learning was clearly the most affected by TBI in our model and in our subjects. Further, the greater differential activation in amygdala and dACC that we observed in mTBI patients and the increased freezing behavior we observed in injured rats during extinction retention do not appear to be problems in the recall of the safety trace; rather, they both occurred late in extinction retention testing and are also likely indicative of an impairment in extinction learning. The data in animals does suggest that the safety trace is encoded by the end of the extinction phase, and this is also suggested by the lack of deficits or differential activation in human subjects at the beginning of extinction retention testing. It is

possible that slightly increased severity of injury could lead to greater deficits in extinction learning that do cause the safety trace to be improperly coded. Extinction retention testing may then uncover a deficit in extinction recall, although the mechanism of this deficit would be different than what is hypothesized in PTSD. Studies of extinction retention and fear renewal suggest that contextual processing is impaired in PTSD, rather than just an inability to recall the safety trace (Garfinkel et al., 2014). Stress from more severe traumatic events than those experienced by subjects in our sample may lead to contextual processing deficits that combine with mTBI-induced extinction learning deficits to cause impairments in extinction recall, even if the mTBI injury is no more severe than the mTBIs seen in our sample. In order to distinguish between these various possible mechanisms, studying (slightly) more severe injury and traumatic events is necessary, and properly characterizing the sample (based on injury severity and posttraumatic stress symptoms) is extremely important.

The role of chronicity may also be studied in human patients. In fact, one possible way to recruit more patients with more “moderate” mTBIs is to expand the time between trauma and the scanning session. This would answer questions about the timing and length of fear-associated learning deficits in mTBI. There is some debate as to whether the same trauma can be responsible for both mTBI and PTSD (Vasterling, 2012), and examining fear-associated learning at different time periods after the traumatic event will shed light on the period of time that extinction deficits occur following mTBI. The obvious caveat with such a study is the need for increased power. Although increasing the time period between mTBI and study participation would make it easier to enroll subjects who may have more symptoms that preclude their immediate

enrollment, increased subject numbers would be necessary to account for the different time points post-injury.

As suggested earlier, one of the most exciting aspects of these experiments is the cross-validation of behavioral results in an animal model with behavioral results from humans. To our knowledge, no studies have been completed that investigated fear-associated learning in patients with mTBI. Establishing a well-tolerated and representative model of TBI (+/- a similarly valid PTSD model) will allow for future studies that dive deeper into the neurotransmitter, hormonal and inflammatory changes that may underlie the deficit in fear extinction we observed in these experiments.

Investigating changes in the levels of stress hormones and hormone receptors may elucidate potential mechanisms involved in impaired fear learning in TBI, as well as the interaction between TBI and PTSD. Interestingly, limited studies suggest that TBI and PTSD have opposite effects on glucocorticoid receptor (GR) expression within the hippocampus, as several studies using the SPS model have demonstrated enhanced GR expression in hippocampus and prefrontal cortex (Liberzon et al., 1999, Wang et al., 2009, Knox et al., 2012c), while studies in rodent models of TBI have found either unchanged or diminished GR expression in hippocampus (Gao et al., 2012, Griesbach et al., 2012). This does suggest a different mechanism of impaired fear-associated learning in the two disorders, but further research, as well as correlations with freezing behavior should be conducted. Additionally, investigating changes in GR in the combined model of CCI and SPS would be interesting given the opposing effects seen in previous research.

The contributions of glutamate to fear-associated learning have been well-described, while contributions of GABA are less clear. However, changes in receptor and neurotransmitter concentration in both of these systems following TBI may play a role in the changes observed in fear-associated learning. Glutamate receptors (both NMDA and AMPA) within amygdala are necessary for fear acquisition, and NMDA receptors appear to be involved in extinction as well (Lee and Kim, 1998, Walker and Davis, 2002). Additionally, studies of inhibitory GABAergic neurons within hippocampus, amygdala and PFC have suggested a variety of different contributions, including disruption of fear acquisition in hippocampus and amygdala (Brioni et al., 1989, Harris and Westbrook, 1999, 2001, Heldt and Ressler, 2007), disruption of fear extinction (Akirav et al., 2006, Hart et al., 2009) and disruption of extinction retention (Corcoran et al., 2005). The preponderance of evidence suggests that GABA inhibits the consolidation of both the danger and safety traces following acquisition and extinction (Makkar et al., 2010). Findings in glutamatergic neurons following LFPI have been inconsistent, with Schwarzbach et al. (2006) and Aungst et al. (2014) finding that glutamatergic excitatory transmission within hippocampus is weakened, while Reger et al. (2012) found increased NMDA receptor concentration in basolateral amygdala. Additionally, both decreases and increases in inhibitory transmission have been observed in hippocampus following TBI (Witgen et al., 2005, Mtchedlishvili et al., 2010). Generally, evidence suggests that the balance between excitatory and inhibitory activity within hippocampus goes awry following TBI (Atkins, 2011). This variance may underlie some of the inconsistent findings in fear-associated learning in rodent models of TBI. Examining the relationship between behavior during fear extinction and neurobiological

and electrophysiological measures of GABAergic and glutamatergic transmission will shed light on the contribution of each system to the deficits that we observed in our experiments.

Assessing the effect of CCI and SPS on the ability of neurons to communicate within and between brain regions with electrophysiology may provide further insight into network-level changes in brain injury. Deficits in long-term potentiation within hippocampus following TBI in rodents have been observed in numerous studies (Schwarzbach et al., 2006, Cohen et al., 2007, Atkins, 2011, Aungst et al., 2014), and, given the crucial role of hippocampus in encoding contextual information during extinction, this may explain why a decreased rate of extinction learning was observed in animals who received CCI. Examining excitability and long-term potentiation in hippocampus following fear-associated learning experiments will allow for correlations of behavioral measures of freezing with electrophysiology and help determine whether deficits in hippocampus underlie impaired extinction learning following TBI. Additionally, reduced excitability within amygdalar circuits (and in outputs from basolateral amygdala) has been shown following lateral fluid percussion injury (LFPI) (Palmer et al., 2016). This study found decreased freezing behavior during a short acquisition testing period, but did not examine extinction (Palmer et al., 2016). Given the role of amygdala in both acquisition and extinction learning, it is feasible that damage, inflammation or other changes in amygdala following TBI cause problems with encoding the CS-no US contingency during extinction. Interpreting findings in amygdala, particularly in humans, is complicated by its role in both acquisition and extinction, and that separate projections to the amygdala appear to enhance and inhibit fear learning (Tye et al.,

2011). These complexities require electrophysiological and possibly optogenetic techniques that can specifically target subpopulations of neurons within amygdala and determine which pathways are responsible for the differences we observe in fear-associated learning following TBI.

Large-scale histological measures of neuronal cell death and contusion volume did not capture differences between sham and CCI surgery, but more sensitive measures of apoptosis could capture subtler differences in cell death. Markers of apoptosis could also be used to determine whether the extent of cell death following CCI relates to fear-associated learning deficits. Timing is quite critical in these experiments, as one previous study using H&E, DNA electrophoresis and terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) showed that apoptotic changes peak at 1 day post-CCI (with significantly greater velocity and impact depth than used in our experiments) and match controls by day 14 (Newcomb et al., 1999). Increased amounts of dystrophic neurons have been observed in amygdala, thalamus and hippocampus following CCI and weight-drop injuries (Colicos et al., 1996, Meyer et al., 2012). Correlating measures of apoptotic cell death in these regions of interest with measures of freezing behavior following CCI/SPS could help determine which regions are responsible for impairments in extinction learning and suggest a possible mechanism. The effect of SPS on cell death following CCI could also be determined, but it will probably be necessary to begin the SPS protocol more quickly after CCI surgery to see any effects.

Although the forces generated during an mTBI cause damage to individual brain regions (and we observed numerous regional differences in extinction learning), they

tend to be particularly harmful to connections between regions (Bigler and Maxwell, 2012). Differences in mean diffusivity (MD) and fractional anisotropy (FA) in cingulum bundles (Wu et al., 2010, Wilde et al., 2012) have been reported in DTI studies of mTBI, and abnormalities in resting-state connectivity (within default mode network, DMN (Spreng et al., 2008, Qin and Northoff, 2011)) have also been reported after mTBI (Tang et al., 2011, Johnson et al., 2012, Zhou et al., 2012). However, the source and the link of these findings to pathophysiology remain unclear. Our experimental design included neuroimaging modalities that provide several measures of network connectivity. Before the fear conditioning paradigm on both scanning days, subjects underwent 10-minute scans at rest in order to measure resting state connectivity. Additionally, we included a 6-minute diffusion tensor imaging (DTI) scan to obtain measures of white matter integrity like FA and MD. We analyzed the resting-state scans with seed-based connectivity measures, taking candidate seed regions within the default mode network (vmPFC and posterior cingulate cortex (Sripada et al., 2012)), as well as hippocampus and amygdala, to determine whether there were any differences in functional connectivity between mTBI subjects and controls. We did not see any significant differences in these connectivity measures, either due to limited power (20 control and 21 mTBI subjects), the quite mild severity of injuries that were sustained by our subjects, and/or the heterogeneity of injury mechanisms present in our sample. However, further analyses that do not rely upon pre-selected seeds (including independent component analysis) will be completed in order to investigate whether any changes in functional connectivity were present in our sample, and whether these changes correlate to differences in SCR or task-based activation. Additionally, analysis

of the DTI data for our subjects will be completed, and relationships between FA and MD in white matter tracts (i.e. cingulum bundles, genu of the corpus callosum) and measures of task-based activation, functional connectivity and SCR will be analyzed. Again, recruiting a sample with higher injury severity or more homogenous injury mechanisms may help improve effect sizes in measures of brain connectivity, with the same caveat of reduced generalizability.

Finally, as the ultimate goal of most clinical and translational research is to improve patient outcomes, investigating the effect of treatment on fear-associated learning in mTBI patients is an exciting future direction. The endocannabinoid system (ECS) is a particularly interesting target for treatment in PTSD, as several studies using agonists and knockouts of the cannabinoid type 1 (CB1) receptor have suggested that the ECS is required in extinction learning (Ruehle et al., 2012). Specifically, CB1 knockout mice have shown impairments in within-session extinction, the same type of impairment that we observed in our animal and human studies of mTBI (Plendl and Wotjak, 2010). Additionally, there is evidence that Delta9-tetrahydrocannibinol (THC) administration enhances fear extinction recall in humans (Rabinak et al., 2013, Rabinak et al., 2014). Given the role of endocannabinoids throughout extinction learning, agonists such as THC may improve the rate of extinction learning in mTBI patients.

One commonly used and effective therapy in PTSD that may also affect fear-associated learning in mTBI is prolonged exposure (PE) therapy, which involves repeated exposure to the traumatic memory (RETM). One neuroimaging study using functional connectivity measures showed that RETM is associated with strengthened connectivity of the amygdala with regions such as hippocampus and insula, as well as

mPFC with insula, and hippocampus with dACC (Cisler et al., 2014). Additionally, exposure therapy in patients with specific phobia has been associated with increased activation in PFC and decreased activation in amygdala, insula, and cingulate cortex during the presentation of phobia-related images (Hauner et al., 2012). We observed hyperactivation in several of these regions during extinction in mTBI patients. PE could be investigated as a preventative therapy in populations of mTBI patients with high percentages of PTSD development, including veterans, assault victims, and motor vehicle accident survivors.

Clearly, there are many important follow-up studies to be performed in human mTBI patients and rodent models that will help determine the underlying neurobiological mechanisms of impaired TBI. However, the research performed in this dissertation represents a step forward in the field, as it provides evidence of differential extinction processing in human mTBI patients. The parallel findings in humans and rats can provide a standard for behavioral and neural findings in other models of brain injury.

References

Akirav I, Raizel H, Maroun M (2006) Enhancement of conditioned fear extinction by infusion of the GABA(A) agonist muscimol into the rat prefrontal cortex and amygdala. *Eur J Neurosci* **23**:758-764.

Atkins CM (2011) Decoding Hippocampal Signaling Deficits after Traumatic Brain Injury. *Transl Stroke Res* **2**:546-555.

Aungst SL, Kabadi SV, Thompson SM, Stoica BA, Faden AI (2014) Repeated mild traumatic brain injury causes chronic neuroinflammation, changes in hippocampal synaptic plasticity, and associated cognitive deficits. *J Cereb Blood Flow Metab* **34**:1223-1232.

Bigler ED, Maxwell WL (2012) Neuropathology of mild traumatic brain injury: relationship to neuroimaging findings. *Brain Imaging Behav* **6**:108-136.

Brioni JD, Nagahara AH, McGaugh JL (1989) Involvement of the amygdala GABAergic system in the modulation of memory storage. *Brain Res* **487**:105-112.

Cisler JM, Steele JS, Lenow JK, Smitherman S, Everett B, Messias E, Kilts CD (2014) Functional reorganization of neural networks during repeated exposure to the traumatic memory in posttraumatic stress disorder: an exploratory fMRI study. *J Psychiatr Res* **48**:47-55.

Cohen AS, Pfister BJ, Schwarzbach E, Grady MS, Goforth PB, Satin LS (2007) Injury-induced alterations in CNS electrophysiology. *Prog Brain Res* **161**:143-169.

Colicos MA, Dixon CE, Dash PK (1996) Delayed, selective neuronal death following experimental cortical impact injury in rats: possible role in memory deficits. *Brain Res* **739**:111-119.

Corcoran KA, Desmond TJ, Frey KA, Maren S (2005) Hippocampal inactivation disrupts the acquisition and contextual encoding of fear extinction. *J Neurosci* **25**:8978-8987.

Davies DR, Olson D, Meyer DL, Scholl JL, Watt MJ, Manzerra P, Renner KJ, Forster GL (2016) Mild Traumatic Brain Injury with Social Defeat Stress Alters Anxiety, Contextual Fear Extinction, and Limbic Monoamines in Adult Rats. *Front Behav Neurosci* **10**.

Elder GA, Dorr NP, De Gasperi R, Gama Sosa MA, Shaughness MC, Maudlin-Jeronimo E, Hall AA, McCarron RM, Ahlers ST (2012) Blast exposure induces post-traumatic stress disorder-related traits in a rat model of mild traumatic brain injury. *J Neurotrauma* **29**:2564-2575.

Gao W, Xu H, Liang M, Huang JH, He X (2012) Association between reduced expression of hippocampal glucocorticoid receptors and cognitive dysfunction in a rat

model of traumatic brain injury due to lateral head acceleration. *Neurosci Lett* **533**: 50-54.

Garfinkel SN, Abelson JL, King AP, Sripada RK, Wang X, Gaines LM, Liberzon I (2014) Impaired contextual modulation of memories in PTSD: an fMRI and psychophysiological study of extinction retention and fear renewal. *J Neurosci* **34**:13435-13443.

Girgis F, Pace J, Sweet J, Miller JP (2016) Hippocampal Neurophysiologic Changes after Mild Traumatic Brain Injury and Potential Neuromodulation Treatment Approaches. *Front Syst Neurosci* **10**.

Greco JA, Liberzon I (2016) Neuroimaging of Fear-Associated Learning. *Neuropsychopharmacology* **41**:320-334.

Griesbach GS, Tio DL, Vincelli J, McArthur DL, Taylor AN (2012) Differential effects of voluntary and forced exercise on stress responses after traumatic brain injury. *J Neurotrauma* **29**:1426-1433.

Harris JA, Westbrook RF (1999) The benzodiazepine midazolam does not impair Pavlovian fear conditioning but regulates when and where fear is expressed. *J Exp Psychol Anim Behav Process* **25**:236-246.

Harris JA, Westbrook RF (2001) Contextual control over the expression of fear in rats conditioned under a benzodiazepine. *Psychopharmacology (Berl)* **156**:92-97.

Hart G, Harris JA, Westbrook RF (2009) Systemic or intra-amygdala injection of a benzodiazepine (midazolam) impairs extinction but spares re-extinction of conditioned fear responses. *Learn Mem* **16**:53-61.

Hauner KK, Mineka S, Voss JL, Paller KA (2012) Exposure therapy triggers lasting reorganization of neural fear processing. *Proc Natl Acad Sci U S A* **109**:9203-9208.

Heldt SA, Elberger AJ, Deng Y, Guley NH, Del Mar N, Rogers J, Choi GW, Ferrell J, Rex TS, Honig MG, Reiner A (2014) A novel closed-head model of mild traumatic brain injury caused by primary overpressure blast to the cranium produces sustained emotional deficits in mice. *Front Neurol* **5**:2.

Heldt SA, Ressler KJ (2007) Training-induced changes in the expression of GABA(A)-associated genes in the amygdala after the acquisition and extinction of Pavlovian fear. *Eur J Neurosci* **26**:3631-3644.

Johnson B, Zhang K, Gay M, Horovitz S, Hallett M, Sebastianelli W, Slobounov S (2012) Alteration of brain default network in subacute phase of injury in concussed individuals: resting-state fMRI study. *Neuroimage* **59**:511-518.

Knox D, George SA, Fitzpatrick CJ, Rabinak CA, Maren S, Liberzon I (2012a) Single prolonged stress disrupts retention of extinguished fear in rats. *Learning & Memory* **19**:43-49.

Knox D, George SA, Fitzpatrick CJ, Rabinak CA, Maren S, Liberzon I (2012b) Single prolonged stress disrupts retention of extinguished fear in rats. *Learn Mem* **19**:43-49.

Knox D, Nault T, Henderson C, Liberzon I (2012c) Glucocorticoid receptors and extinction retention deficits in the single prolonged stress model. *Neuroscience* **223**:163-173.

Lagarde E, Salmi L, Holm LW, et al. (2014) ASsociation of symptoms following mild traumatic brain injury with posttraumatic stress disorder vs postconcussion syndrome. *JAMA Psychiatry* **71**:1032-1040.

Lee H, Kim JJ (1998) Amygdalar NMDA receptors are critical for new fear learning in previously fear-conditioned rats. *J Neurosci* **18**:8444-8454.

Liberzon I, Abelson JL, Flagel SB, Raz J, Young EA (1999) Neuroendocrine and psychophysiologic responses in PTSD: a symptom provocation study. *Neuropsychopharmacology* **21**:40-50.

Makkar SR, Zhang SQ, Cranney J (2010) Behavioral and Neural Analysis of GABA in the Acquisition, Consolidation, Reconsolidation, and Extinction of Fear Memory. *Neuropsychopharmacology* **35**:1625-1652.

Meyer DL, Davies DR, Barr JL, Manzerra P, Forster GL (2012) Mild traumatic brain injury in the rat alters neuronal number in the limbic system and increases conditioned fear and anxiety-like behaviors. *Exp Neurol* **235**:574-587.

Milad MR, Pitman RK, Ellis CB, Gold AL, Shin LM, Lasko NB, Zeidan MA, Handwerger K, Orr SP, Rauch SL (2009) Neurobiological basis of failure to recall extinction memory in posttraumatic stress disorder. *Biol Psychiatry* **66**:1075-1082.

Mtchedlishvili Z, Lepsveridze E, Xu H, Kharlamov EA, Lu B, Kelly KM (2010) Increase of GABAA receptor-mediated tonic inhibition in dentate granule cells after traumatic brain injury. *Neurobiol Dis* **38**:464-475.

Newcomb JK, Zhao X, Pike BR, Hayes RL (1999) Temporal Profile of Apoptotic-like Changes in Neurons and Astrocytes Following Controlled Cortical Impact Injury in the Rat. *Experimental Neurology* **158**:76-88.

Palmer CP, Metheny HE, Elkind JA, Cohen AS (2016) Diminished amygdala activation and behavioral threat response following traumatic brain injury. *Experimental Neurology* **277**:215-226.

Plendl W, Wotjak CT (2010) Dissociation of within- and between-session extinction of conditioned fear. *J Neurosci* **30**:4990-4998.

Qin P, Northoff G (2011) How is our self related to midline regions and the default-mode network? *Neuroimage* **57**:1221-1233.

Rabinak CA, Angstadt M, Lyons M, Mori S, Milad MR, Liberzon I, Phan KL (2014) Cannabinoid modulation of prefrontal-limbic activation during fear extinction learning and recall in humans. *Neurobiol Learn Mem* **113**:125-134.

Rabinak CA, Angstadt M, Sripada CS, Abelson JL, Liberzon I, Milad MR, Phan KL (2013) Cannabinoid facilitation of fear extinction memory recall in humans. *Neuropharmacology* **64**:396-402.

Reger ML, Poulos AM, Buen F, Giza CC, Hovda DA, Fanselow MS (2012) Concussive brain injury enhances fear learning and excitatory processes in the amygdala. *Biol Psychiatry* **71**:335-343.

Roitman P, Gilad M, Ankri YL, Shalev AY (2013) Head injury and loss of consciousness raise the likelihood of developing and maintaining PTSD symptoms. *J Trauma Stress* **26**:727-734.

Ruehle S, Rey AA, Remmers F, Lutz B (2012) The endocannabinoid system in anxiety, fear memory and. *J Psychopharmacol* **26**:23-39.

Schneider BL, Ghoddoussi F, Charlton JL, Kohler RJ, Galloway MP, Perrine SA, Conti AC (2016) Increased Cortical Gamma-Aminobutyric Acid Precedes Incomplete Extinction of Conditioned Fear and Increased Hippocampal Excitatory Tone in a Mouse Model of Mild Traumatic Brain Injury. *J Neurotrauma*.

Schwarzbach E, Bonislawski D, Xiong G, Cohen A (2006) Mechanisms Underlying the Inability to Induce Area CA1 LTP in the Mouse After Traumatic Brain Injury. *Hippocampus* **16**:541-550.

Shenton ME, Hamoda HM, Schneiderman JS, Bouix S, Pasternak O, Rathi Y, Vu MA, Purohit MP, Helmer K, Koerte I, Lin AP, Westin CF, Kikinis R, Kubicki M, Stern RA, Zafonte R (2012) A review of magnetic resonance imaging and diffusion tensor imaging findings in mild traumatic brain injury. *Brain Imaging Behav* **6**:137-192.

Sierra-Mercado D, McAllister LM, Lee CC, Milad MR, Eskandar EN, Whalen MJ (2015) Controlled cortical impact before or after fear conditioning does not affect fear extinction in mice. *Brain Res* **1606**:133-141.

Spreng RN, Mar RA, Kim ASN (2008) The Common Neural Basis of Autobiographical Memory, Prospection, Navigation, Theory of Mind, and the Default Mode: A Quantitative Meta-analysis. *Journal of Cognitive Neuroscience* **21**:489-510.

Sripada RK, King AP, Welsh RC, Garfinkel SN, Wang X, Sripada CS, Liberzon I (2012) Neural dysregulation in posttraumatic stress disorder: evidence for disrupted equilibrium between salience and default mode brain networks. *Psychosom Med* **74**:904-911.

Stein MB, Kessler RC, Heeringa SG, Jain S, Campbell-Sills L, Colpe LJ, Fullerton CS, Nock MK, Sampson NA, Schoenbaum M, Sun X, Thomas ML, Ursano RJ, collaborators

ObotAS (2015) Prospective Longitudinal Evaluation of the Effect of Deployment-Acquired Traumatic Brain Injury on Posttraumatic Stress and Related Disorders: Results From the Army Study to Assess Risk and Resilience in Servicemembers (Army STARRS). *Am J Psychiatry* **172**:1101-1111.

Tang L, Ge Y, Sodickson DK, Miles L, Zhou Y, Reaume J, Grossman RI (2011) Thalamic resting-state functional networks: disruption in patients with mild traumatic brain injury. *Radiology* **260**:831-840.

Tye KM, Prakash R, Kim S-Y, Fenno LE, Grosenick L, Zarabi H, Thompson KR, Gradinaru V, Ramakrishnan C, Deisseroth K (2011) Amygdala circuitry mediating reversible and bidirectional control of anxiety. *Nature* **471**:358-362.

Vasterling JJ, Bryant, R.A., Keane, T.M. (2012) Understanding the Interface of Traumatic Stress and Mild Traumatic Brain Injury. In: PTSD and Mild Traumatic Brain Injury (Vasterling, J. J., Bryant, R.A., Keane, T.M., ed) New York: The Guilford Press.

Walker DL, Davis M (2002) The role of amygdala glutamate receptors in fear learning, fear-potentiated startle, and extinction. *Pharmacology Biochemistry and Behavior* **71**:379-392.

Wang HT, Han F, Shi YX (2009) Activity of the 5-HT1A receptor is involved in the alteration of glucocorticoid receptor in hippocampus and corticotropin-releasing factor in hypothalamus in SPS rats. *Int J Mol Med* **24**:227-231.

Warren AM, Boals A, Elliott TR, Reynolds M, Weddle RJ, Holtz P, Trost Z, Foreman ML (2015) Mild traumatic brain injury increases risk for the development of posttraumatic stress disorder. *J Trauma Acute Care Surg* **79**:1062-1066.

Wilde EA, McCauley SR, Barnes A, Wu TC, Chu Z, Hunter JV, Bigler ED (2012) Serial measurement of memory and diffusion tensor imaging changes within the first week following uncomplicated mild traumatic brain injury. *Brain Imaging Behav* **6**:319-328.

Witgen BM, Lifshitz J, Smith ML, Schwarzbach E, Liang SL, Grady MS, Cohen AS (2005) Regional hippocampal alteration associated with cognitive deficit following experimental brain injury: A systems, network and cellular evaluation. *Neuroscience* **133**:1-15.

Wu TC, Wilde EA, Bigler ED, Yallampalli R, McCauley SR, Troyanskaya M, Chu Z, Li X, Hanten G, Hunter JV, Levin HS (2010) Evaluating the relationship between memory functioning and cingulum bundles in acute mild traumatic brain injury using diffusion tensor imaging. *J Neurotrauma* **27**:303-307.

Yu T-S, Zhang G, Liebl DJ, Kernie SG (2008) Traumatic brain injury-induced hippocampal neurogenesis requires activation of early nestin-expressing progenitors. *J Neurosci* **28**:12901-12912.

Zhou Y, Milham MP, Lui YW, Miles L, Reaume J, Sodickson DK, Grossman RI, Ge Y (2012) Default-mode network disruption in mild traumatic brain injury. Radiology 265:882-892.