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A CLINICALLY RELEVANT CLOSED-HEAD MODEL OF SINGLE AND REPEAT CONCUSSIVE INJURIES IN THE ADULT RAT USING A CONTROLLED CORTICAL IMPACT DEVICE

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TABLE OF CONTENTS

1. LIST OF TABLES i

2. LIST OF FIGURES ii

3. ACKNOWLEDGEMENTS iii

4. ABSTRACT 1

5. REVIEW OF LITERATURE

    5.1. General Overview of TBI 3

    5.2. Clinical manifestations of repeated concussion 4

    5.3. Physiological Effects of Stress 7

        5.3.1. Hypothalamic-Pituitary-Adrenal Axis

        5.3.2. Glucocorticoid Receptors

    5.4. TBI and Hormonal Deficiencies 12

    5.5. Animal Models Addressing HPA Abnormalities 15

    5.6. Animal Models of Repeated Concussions 16

6. PILOTING CONCUSSION MODEL

    6.1. Open-Head CCI Approach 21

    6.2. Closed-Head CCI Approach 21

7. MANUSCRIPT

    7.1. INTRODUCTION 24

    7.2. METHODS

        7.2.1. Animals 27

        7.2.2. Experimental Design 29

        7.2.3. Closed-Head Controlled Cortical Impact (CCI) 30

        7.2.4. Foot Fault Test for Forelimb Coordination 31

        7.2.5. Novel Object Recognition (NOR) Task 32

        7.2.6. Open Field Test 33

        7.2.7. Corticosterone (CORT) Analysis 34

        7.2.8. Histology 34

7.2.8.1. Euthanasia

7.2.8.2. Examination of gross anatomy

7.2.9. Statistical Analysis 35

7.3. RESULTS

7.3.1. Single and repeat concussions result in hypoactivity 36

7.3.2. The number of days after injury has an effect on the percentage of total foot faults 37

7.3.3. Both single and repeat concussions result in memory deficits 38

7.3.4. Repeat concussions result in an anxiety response 41

7.3.5. Single and repeat concussions increase baseline CORT 41

7.3.6. Single and repeat concussions do not cause obvious cortical damage 42

7.4. DISCUSSION

7.4.1. Single Concussion 44

7.4.2. Repeat Concussions 48

7.5. CONCLUSION 54

8. EXTENDED DISCUSSION 56

9. WORKS CITED 60

LIST OF TABLES

Table 1 – Symptoms of Concussions 4

Manuscript

Table 1 – Groups 28
Table 2 – sTBI and sTBIac statistical measurements 36

LIST OF FIGURES

Figure 1 – The human HPA axis 9
Figure 2 – Animal models of TBI 17
Figure 3 – CHI model using a CCI device 22

Manuscript

Figure 1 – Experimental Design 29
Figure 2 – CHI model using a CCI device 30
Figure 3 – Foot Fault Test 32
Figure 4 – Novel Object Recognition Task 33
Figure 5 – Locomotor Activity in the Open Field 37
Figure 6 – Foot Fault 38
Figure 7 – Time Spent with Objects During Habituation 39
Figure 8 – Percent of Total Time Spent with Novel Object 40
Figure 9 – Anxiety-Like Behavior in the Open Field 41
Figure 10 – CORT Pre and Post Injury (ng/mL) 42
Figure 11 – Surface and Cortical Pathology 43

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ABSTRACT

Recently, cases of multiple concussions, or mild traumatic brain injury, in athletes have received increased attention. Compared to single concussion (sTBI), repeat concussions (rTBI) can produce significant long-term consequences and increased risk for neurodegenerative disease. However, mechanisms underlying this difference are poorly understood and are best elucidated using an animal model. Closed-head models of rTBI have been developed in mice and juvenile rats, but few have been developed in the adult rat. To the best of our knowledge, there is no closed-head model using a commercially available device, including the Controlled Cortical Impact (CCI) device.

We developed a clinically relevant closed-head injury model of concussion in the adult rat using a Leica CCI device. Rats were placed in a stereotax frame without ear-bars, on a foam-bed base. The head was stabilized against a Plexiglas frame to control impact while allowing head movement. A 6.5 m/s impact was delivered onto the head surface over the sensorimotor-cortex at a depth of 10.0 mm from the skin. rTBI animals received 3 injuries, 48 hours apart. A subset of sTBI animals received two subsequent exposures to anesthesia (sTBIac), 48 hours apart, to mimic the extra exposure experienced by the rTBI animals.

We initially created a model of sTBI before continuing to examine rTBI. Sham and sTBI rats were initially assessed at days 1-3 post injury using tests of memory (Novel Object Recognition), forelimb coordination (foot fault) and activity/anxiety (open field). Blood corticosterone levels were measured pre-injury and pre-sacrifice (day 4). At this time point, sTBI animals showed memory deficits without locomotor deficits or an anxiety response.

The study was then expanded by adding rTBI animals and sTBI/sTBIac animals sacrificed 8 days after the initial injury. Rats were assessed at days 5-7 after the initial injury.

Results indicate that both sTBI and rTBI animals show deficits in coordination and hypo-locomotion at day 5. Although sTBI rats showed no anxiety response rTBI rats did show anxiety (spent less time in the center of an open field). sTBI rats displayed memory deficits 3 days post-injury, but not day 7. rTBI rats continued to show memory deficits at day 7. Both had higher resting corticosterone levels post-injury compared to both their baseline levels and to sham and day 4 sTBI animals. No obvious gross pathology was observed on the cortical surface or in coronal sections. Our data presents a model of closed-head CCI in an adult rat that results in clinically relevant markers of concussion and an early delineation between single and repeat concussions.

REVIEW OF LITERATURE

The Centers for Disease Control and Prevention (CDC; 2013) defines a traumatic brain injury (TBI) as a blow to the head that disrupts normal brain function. In the United States alone, TBI affects an estimated 1.7-3.6 million people annually (Centers for Disease Control and Prevention [CDC], 2013). TBI contributes to 30.5% of all injury-related deaths in the United States (Faul and Coronado, 2015) and direct and indirect costs of TBI are estimated to be at least $60 billion (Corso et al., 2006). Currently, there are no adequate treatments for TBI.

Depending on the severity of the blow, a TBI may be classified from "mild" to "severe." A severe TBI results in cell loss and behavioral deficits such as extended unconsciousness and amnesia. A concussion (sTBI), a form of mild traumatic brain injury, results from a physical blow to the head that produces some sort of disturbance to the brain that may result in brief unconsciousness but produces other behavioral deficits that occur independently from obvious cell loss. While severe TBI can be diagnosed with the use of brain imaging technology, sTBI is difficult to diagnose and is therefore often undetected (Bazarian et al., 2006; Narayana et al., 2015). The CDC (2013) estimates that 75% of all TBIs are concussions.

The CDC (2013) classified the symptoms of concussions into four general categories: cognitive, physical, emotional, and sleep (Table 1). While some symptoms, such as dizziness or nausea, are noticed right away, others, as sleep disturbances, may persist over weeks or longer.

Table 1: Symptoms of Concussion (adapted from the CDC, 2013).

Cognitive	Physical	Emotional	Sleep
Difficulty thinking clearly	Headache, dizziness, blurred vision	Irritability	Increased sleep
Feeling slowed down	Feeling tired, having little energy	Sadness	Decreased sleep
Difficulty concentrating	Nausea or vomiting	Nervous or anxious	Insomnia
Difficulty remembering new information	Sensitivity to light or noise	More emotional	Trouble staying asleep

In general, the populations at risk for concussions include young children, teenagers, older adults, and victims of domestic abuse, but also include those in impact-prone professions such as professional sports and the military. Almost half a million annual emergency department visits for TBI are made by children from ages 0 to 14 years old (Faul and Coronado, 2015). In high school sports alone, 9% of all injuries are TBI-related (Gessel et al., 2007). There has also been a recent spike in blast-related TBIs in returning veterans and military personnel (Champion et al., 2009). The discussion of blast-related TBIs is beyond the scope of this project.

Clinical Manifestations of Repeated Concussions

The rise in head-related injury in sports and military combat has correlated with a sharp increase in neurological problems. Concussions and repeat concussions are of especial worry to

youth populations. Among young athletes, there is the highest prevalence of concussions in high school sports, including an underreporting of incidence: in one study, only 47.3% of students reported when they sustained a concussion (McCrea et al., 2004). In a survey of over 17,500 players in high school and college, 888 (5.1%) had at least one concussion, with 131 of those (14.7%) reporting a second concussion in the same season (Guskiewicz et al., 2000). This group also reported that high school populations had the highest incidence at 5.6% (Guskiewicz et al., 2000).

The immediate symptoms of concussion can include loss of consciousness, headache, dizziness, and nausea, but is difficult to diagnose. A study in 2006 found that CT scans did not detect injury post-concussion (Bazarian et al., 2006); this was supported by (Narayana et al., 2015). An *in vivo* MRI study of former NFL players found structural changes corresponding to behavioral deficits: there was significant atrophy in the right hippocampus and varied performance on verbal learning and memory tasks (Coughlin et al., 2015). Additionally, there were increases of [^{11}C]DPA-713 (*N*,*N*-diethyl-2-(4-methoxyphenol)-5,7-dimethylpyrazolo[1,5-*a*]pyrimidine-3-acetamide) binding to translocator protein (TSPO), a potential marker of brain injury and repair (Coughlin et al., 2015). However, meta-analyses of past reports have not found a biomarker that consistently predicts the manifestation and severity of post-concussion syndrome, which includes variable long-term symptoms listed previously (Begaz et al., 2006; Jeter et al., 2013)

Those with previous TBIs are at more risk for subsequent head injuries (CDC, 2013). Furthermore, while most single concussion cases recover well with adequate time, reoccurring concussions can have significant neurological consequences. Mannix and colleagues (2014) found that an increase in the number of concussions worsened verbal memory performance and

greater overall symptoms score. Studies have also linked multiple concussions to a variety of neurological problems. For example, jockeys with multiple concussions consistently preform worse on the Stroop color-word task, a measure of executive functioning and attention (Wall et al., 2006). There are similar trends in football players. These studies have found that players with 3 or more concussions have been 5 times more likely to have mild cognitive impairments, 3 times more likely to have memory problems, and 4 times more likely to have Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), and Parkinson's disease (PD) (Hoge et al., 2008; Lehman et al., 2012). Furthermore, Hazrati and colleagues found that out of four cases of Canadian Football League players, 3 had chronic traumatic encephalopathy, or CTE, (comorbid with cancer, vascular disease, and AD), and 3 had AD, ALS, and PD (Hazrati et al., 2013). CTE has been linked to repeat concussions in multiple studies (Lucke-Wold et al., 2014; McKee et al., 2009; McKee and Daneshvar, 2015).

Repeat concussions have also been linked with psychiatric disorders including posttraumatic stress disorder (PTSD), major depressive disorder (MDD), and generalized anxiety disorder (GAD) (Ma et al., 2014). Depressive disorders develop in individuals with TBI with estimates ranging from 6% to 77%, with lifetime rates of 26% to 64% (Jorge and Arciniegas, 2014a, 2014b). These rates are so variable because of the heterogeneity of depression as a disorder, meaning the symptoms can be mild (decrease in mood) to severe (suicidal ideations). Furthermore, 75% of patients who had depressive disorders were comorbid with anxiety disorders (Jorge and Arciniegas, 2014a, 2014b). Yurgil and colleagues (2014) reported that 87.2% of soldiers with a history of sTBI were twice as more likely to have PTSD post-deployment, with other studies finding similar results (Hoge et al., 2008).

Patients with depression have an increase in the endocrine stress response marked by elevated blood cortisol levels and enlarged pituitary and adrenal glands (Arborelius et al., 1999). Furthermore, there is evidence that both mood disorders (Carroll et al., 1976) and all forms of TBI (Tanriverdi et al., 2010) are also linked to dysfunctions in the physiological stress response, as controlled by the hypothalamic-pituitary-adrenal (HPA) axis.

The Physiological Effects of Stress

All organisms must maintain an equilibrium or homeostasis in order to survive. However, both internal and external events are constantly affecting this balance—here termed a stressor. Chrousos (2009) defines stress as any state where homeostasis is negatively affected which then calls for a re-establishment via complex internal mechanisms.

Stressors can be physical or emotional events or forces that vary in effect and duration. Importantly, stressors are specific to individuals, time, and place. Depending on the situation or context, an organism can react differently to the same or similar stressor. However, in all cases, the physiological stress response is governed by the same internal mechanisms. The importance of this response cannot be stressed enough; its activation increases the likelihood of an individual's survival. However, improper activation of the response can also have detrimental effects.

Hypothalamic-Pituitary-Adrenal Axis. The hypothalamic-pituitary-adrenal (HPA) axis (Figure 1) is the key system in the endocrine stress response. The hypothalamus is an area of the brain that is responsible for controlling homeostasis. One of its major outputs is to the pituitary gland, an adjacent gland that is responsible for hormone secretion. In response to a stressor, the hypothalamic paraventricular nucleus (PVN) releases a cocktail of hormones into the portal

system, which then circulates to the anterior lobe of the pituitary (Herman and Cullinan, 1997). The most important hormones released for this response are corticotropin-releasing hormone (CRH) and arginine-vasopressin (AVP) (Herman and Cullinan, 1997). These and other hormones stimulate the release of adrenocorticotropic hormone (ACTH) into the bloodstream (Herman and Cullinan, 1997). Importantly, the PVN is critical for the initiation of the stress response; lesions of PVN neurons reduce not only portal CRH levels, but also stress-induced ACTH and cortisol secretion (Makara, 1992). From here, ACTH acts on the adrenal glands, small organs atop of the kidneys. The adrenal cortex produces glucocorticoid hormones in response to ACTH, such as cortisol, the important stress hormone in humans (Herman and Cullinan, 1997). Cortisol induces a negative-feedback loop onto the hypothalamus and pituitary to regulate its production in response to the stressor at hand or future stressors (Chrousos, 2009). Interestingly, cortisol also affects other parts of the brain, such as the frontal lobe and hippocampus, which will be discussed below (Herman et al., 2005). Furthermore, overstimulation of the HPA axis can lead to insufficient/decreased responses to future stressors, indicating that the state of the axis can and does affect its responses (Stockham, 1964).

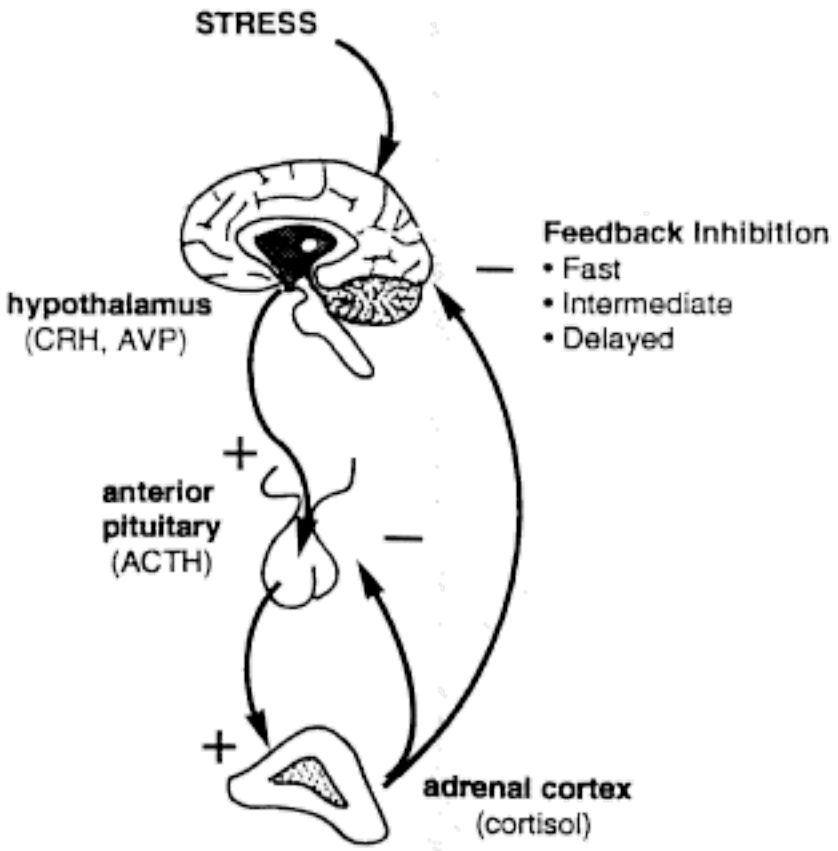

Figure 1: The human hypothalamus-pituitary-adrenal (HPA) axis. In response to a stressor, the hypothalamus releases CRH onto the anterior pituitary, which releases ACTH into the bloodstream. The adrenal cortex produces stress hormone glucocorticoids, which create a negative feedback loop back to the anterior pituitary and hypothalamus (adapted (Herman et al., 2003).

Cortisol has many systemic effects on the body. Nicknamed the "stress hormone," cortisol ultimately is released as a result of an environmental stressor—as discussed above, anything that would disrupt the body from homeostasis. The hypothalamus is highly sensitive to these stimuli and alters its production of CRH accordingly (Tsigos and Chrousos, 2002).

An exhaustive list of cortisol's downstream effects is beyond the scope of this paper. Briefly, the effects of cortisol causes the body to eventually adapt to the stressor presented.

These changes include increase alertness; improved cognition; increases in cardiovascular tone; redirection of oxygen and nutrients to the CNS; and suppression of unnecessary, energy-costing activities (Charmandari et al., 2005). Two major functions that lead to many of the downstream effects include an increase in gluconeogenesis and suppression of the immune response, as mentioned above (Arborelius et al., 1999). Through a variety of binding proteins, cortisol suppresses insulin release and breaks down glycogen to form glucose (Goldstein et al., 1993). Cortisol also reduces the inflammatory response and suppresses cytokine production (Palacios and Sugawara, 1982). Elevated cortisol levels therefore can cause hyperglycemia, possibly leading to diseases such as type-II diabetes, and lead to insufficient immune responses.

An important consideration in examining HPA function, including cortisol levels, is timing. Cortisol fluctuates throughout the day (i.e., spiking in the morning, fluctuating throughout the afternoon, and decreasing at night), and timing of the analysis of cortisol levels for research or clinical use is important. It is cortisol levels outside of this cycle that are of specific concern to the HPA axis (Dedovic et al., 2009). Krieger and Allen found that cortisol and ACTH are secreted in pulses, indicating that cortisol levels at a given instant may not be as relevant as the trends of ACTH levels over time (Krieger and Allen, 1975).

Damage to the HPA axis causes improper stress responses, but the type of response depends on the area of damage. Physiological effects of cortisol are discussed below, but two examples, hypo- and hyper-responsiveness, are briefly mentioned. For example, if the pituitary is damaged such that the production of ACTH drops, cortisol production would also be decreased, temporarily decreasing the stress response. The low levels of cortisol can then feedback to the pituitary to increase CRH production in the attempt to revert back to homeostatic levels; however, due to the physical damage, ACTH would remain at insufficient levels and blood

cortisol would remain low. This would again feed back into elevated levels of CRH, repeating the cycle. There would be a similar effect if the hypothalamus or PVN were damaged as well; however, in this case, levels of CRH would remain low and therefore depressed ACTH levels as well. The difference between secondary adrenal insufficiency, which is damage to the pituitary, and tertiary adrenal insufficiency, damage to the hypothalamus, is the difference in CRH levels. However, both of these examples would manifest a muted response to an environmental stressor. On the other hand, a hyperactive stress response would produce an overall increase in unregulated cortisol levels. Mechanisms controlling hyperactive responses are less understood and more complex, and are usually associated with a variety of disease states rather than physiological damage (Charmandari et al., 2005). The end result is ultimately increased basal cortisol levels, increased stress-induced levels, or both.

Glucocorticoid Receptors. In the brain, cortisol acts on the glucocorticoid receptors. Interestingly, glucocorticoid receptors like those for cortisol in the brain have the highest concentration not at the PVN, but at the hippocampus (Sapolsky and Pulsinelli, 1985). The hippocampus is the major site of memory consolidation in the brain. Increased alertness and attentiveness are some of the immediate effects of the normal stress response, and because the attention to detail and therefore memory can therefore aid that, it makes sense for the hippocampus to be involved. However, glucocorticoids induce metabolic vulnerability and compromise neuronal ability to survive toxic insults (Sapolsky and Pulsinelli, 1985). Therefore, prolonged elevation of cortisol can have detrimental effects on the hippocampus, as increased levels would over-activate the many glucocorticoid receptors found there. Overactivation of receptors can lead to unregulated intracellular cascades and potential cell death. Indeed, Sapolsky's work also showed that in rats, glucocorticoid levels within the physiological range

can induce neuronal death in the hippocampus (Sapolsky, 1985). Interestingly, hippocampal activation decreases CRH release and thus downstream decreases in cortisol levels (Herman et al., 2005). This is perhaps due, in part, to a negative feedback loop to decrease possible damage done, although studies are still unsure as to the actual mechanism of this action (Herman et al., 2005).

Other parts of the limbic system, including the medial prefrontal cortex and amygdala, stimulate downstream cortisol. However, the roles these areas play are not as well understood. It seems that different parts of the prefrontal cortex, for example, may play both inhibitory and excitatory roles (Herman et al., 2005). The amygdala is well-thought to activate the HPA axis, but the mechanism is related to the inflammatory response rather than CRH or ACTH (Herman et al., 2005). The amygdala also seems to be activated in response to very specific stressors, and even different regions within the amygdala may cause different levels of activation of the HPA axis (Herman et al., 2005).

TBI and Hormonal Dysfunction

Studies have linked all forms of TBI to pituitary hormone dysfunction. Studies have supported the widespread suppression of the hypothalamic-pituitary-gonadal (HPG) axis and growth axis in particular. Although these axes are not part of the direct stress response, activation of the HPA axis causes a dampening effect on the other, energy-expending hormonal axes (Charmandari et al., 2005). Bondanelli and colleagues found that over a five-year period, 51.8% of severe, mild, and moderate TBI patients had severe growth hormone deficiency (GHD) and 25.9% had hypogonadism (Bondanelli et al., 2004). In support, others found that 21% of TBI patients had primary hormonal dysfunction (Krahulik et al., 2010). Gonadotropin-releasing

hormone (GnRH) in HPG axis was primarily affected, with patients having an increased prevalence of hypogonadism and diabetes insipidus (Krahulik et al., 2010). In a study done in 2006, 41.6% of mild, moderate, and severe TBI patients (n = 52) were gonadotropin (Gn) deficient 24 hours after hospital admittance, and 20.4% had GHD (Tanriverdi et al., 2006). Interestingly, while Gn deficiency dropped to 7.7% a year later, GHD rose to 37.7% (Tanriverdi et al., 2006). Similar trends were also seen for ACTH; at 24 hours post-injury, 9.8% of patients had ACTH deficiency, while 19.2% had ACTH deficiency one year later (Tanriverdi et al., 2006). Admittedly, this increase was not significant but interesting to study further ($p = 0.175$) (Tanriverdi et al., 2006).

Other studies have estimated that 21% to 28% of TBI patients have other primary hormonal deficiencies (Agha and Thompson, 2006; Dimopoulou et al., 2004; Krahulik et al., 2010), with many of them finding HPA dysfunction. During the immediate recovery period, over 50% of surveyed patients with moderate to severe head trauma had cortisol hyporesponsiveness, resulting in a dampened stress response (Dimopoulou et al., 2004). Other pituitary or hypothalamic dysfunctions were noted, but levels of the hypothalamic-released hormone was not measured to ascertain whether there was secondary or tertiary insufficiency (Dimopoulou et al., 2004).

In a study done in 2007, Niederland and colleagues found that 61% of children (n = 27) with TBI had pituitary dysfunction independent of TBI severity. Specifically, patients had decreased levels of growth hormone (GH) secretion in response to L-DOPA or insulin stimulation. Briefly, both the dopamine precursor and the glycogen-inhibitor should increase the levels of cortisol, which acts antagonistically to bring these levels down. As GH is also regulated by a hypothalamic-pituitary axis, this implies that the HPA axis may also be affected. Indeed, the

study found that cortisol levels were significantly lower ($p < 0.05$) in TBI groups after insulin stimulation compared to uninjured patients, indicating a suppressed stress response (Niederland et al., 2007).

Cortisol responsiveness seems to be affected as well by TBI. Although decreased levels of ACTH were not found, 22.5% of patients ($n = 102$) with severe or moderate TBI showed a decrease in cortisol responsiveness to insulin (Agha et al., 2004b). In the patients that did exhibit ACTH deficiency, though, there was a corresponding decrease in basal serum cortisol levels ($p < 0.001$) (Agha et al., 2004b). In a similar study, 16% of patients ($n = 50$) exhibited peak cortisol levels lower than in normal patients, with a subnormal response to glucagon (Agha et al., 2004a). In one study, 53% of patients exhibited ACTH deficiency, with a corresponding decrease in cortisol responsiveness ($p < 0.0001$) (Cohan et al., 2005).

In 2007, Ives and colleagues examined the fascinating case study of a teenage athlete that received four concussions over the course of 4 months. The patient was diagnosed with hypopituitarism due to TBI two years after the injuries in question. At this time, he had stunted growth and a decline in strength, corresponding to decreased levels of growth hormone and thyroxine, and so was placed on hormonal supplements. What is very interesting about this study is that 5 weeks after treatment, the patient was admitted for severe ACTH deficiency, even though he had previously normal levels. Investigators concluded that the HPA axis only failed upon supplemental hormone treatment. Upon the addition of a cortisol regimen, at the time of publication, the patient appeared well (Ives et al., 2007). Fortunately, this indicates that despite the delicate structure of the pituitary, supplemental hormones are sufficient to decrease what would otherwise be life-threatening symptoms.

It seems that, overall, these and other studies have found a muted response to CRH and

ACTH in severe TBI patients (Kelly et al., 2000, Lieberman et al., 2001). Interestingly, many of the above studies mention also examining patients with concussions (sTBI) and grouping their results together. However, other studies have suggested that sTBI patients actually show a general rise in cortisol and ACTH levels (Barton et al., 1987; King et al., 1970; Sojka et al., 2006). Some studies have suggested that this rise is transient (Sojka et al., 2006), while others have shown that these may persist even up to a few days after injury (Cernak et al., 1999). Therefore, the potentially chronic nature of these rises in cortisol is debated.

Furthermore, there are still some conflicting results regarding the effects of TBI on the HPA axis. Bondanelli and colleagues (2004) found that while other axes were affected, ACTH and cortisol levels were within normal range. There is evidence that some patients have normal ACTH levels while having decreased cortisol levels (Agha et al., 2004a, Agha et al., 2004b). Furthermore, even in studies that do see a difference in these levels, their findings are conflicting. While Tanriverdi (2006) found that ACTH deficiency increased one-year post injury, Aimaretti and colleagues (2005) found that there was a decrease. Clearly, the picture is more complicated than what it seems in patients with a single concussion and even less explored in those with repeat concussions.

Animal Models of TBI Addressing HPA Abnormalities. Animal models examining changes in pituitary function, especially in response to stress, are well-established. For example, Armario and colleagues (1985) found that acute stressors increase corticosterone (CORT), prolactin, luteinizing hormone, and thyroid stimulating hormone in rats. Habituation to a chronic stimulus decreased CORT response without changing the response to a novel stimulus (Armario et al., 1985).

Some studies have examined pituitary function in animal models of TBI. Overall, the

trends for sTBI models mimic those seen in the clinic. Stress-induced CORT levels increase after mild or moderate experimental injury (Griesbach et al., 2011; Kwon et al., 2011; Taylor et al., 2008). Grundy and colleagues (2001) found that hypothalamic CRH mRNA expression also increases after experimental injury. In a study done in 2013, Greco and colleagues found that rTBI results in significant acute and chronic decreases of growth hormone, implying the pituitary dysfunction might be at least just as pronounced in rTBI models. However, to the best of our knowledge, the effects of rTBI on hormonal dysfunction are still relatively unexplored.

Animal Models of Repeated Concussions

The neurobiological mechanisms of the long-term effects of repeat concussions are unknown. However, animal models are used in order to understand cellular and behavioral mechanisms of TBI to ultimately develop better treatments. Unfortunately, at this time, there is no universal model of rTBI.

Traditionally, TBI is modeled in the laboratory through a fluid percussion injury (FPI) or controlled cortical impact (CCI) (reviewed in Petraglia et al., 2014a; see Figure 2). Both require a craniotomy and result in focal lesions, motor deficits and cognitive dysfunction. FPI uses a fluid-filled tube to inject saline onto the brain, just against the dura. This produces a global movement of the brain due to the fluid pressure and therefore affects a wider area. CCI uses a piston driven by air or a magnet to drive a metal tip to impact the surface of the dura through a craniotomy. The size of the tip can vary but regardless, it results in a much more focal injury and typically results in the formation of a cavity in the brain (Petraglia et al., 2014a). Importantly, because FPI and CCI both require craniotomies, they are not preformed on an intact skull, and neither can produce the rotational effects on the head and brain seen in a typical human head

injury. Nevertheless, these models have produced the largest amount of data on the behavioral, physiological, and anatomical effects of TBI.

Figure 2. Animal Models of TBI. Fluid Percussion Injury (left) and Controlled Cortical Impact (right) (from Kozlowski et al., 2013).

Recently, other models are being used to model different forms of TBI. Blast-models are addressing TBI due to pressure compression/decompression experienced in military blasts, but are beyond the scope of this paper. Weight-drop TBI models have also been used to model forms of TBI. While weight-drop models can use a craniotomy, they are also easily altered to use in closed-head models (reviewed in Petraglia et al., 2014a). As the name implies, weight-drop models drop a predetermined weight from a specific height directly onto the surface of the skull, or onto the head directly to create the injury. Unlike FPI and CCI devices, weight-drop apparatuses are not commercially available (Petraglia et al., 2014a).

Given that, to date, there is no universal model of concussions regardless of device, Petraglia et al. (2014) suggest criteria for a clinically relevant model of rTBI. Some of their criteria include the model impacting the surface of the head over use of a craniotomy and evidence of non-overt damage such as axonal injury (Petraglia et al., 2014a). However, they also

note that this is not sufficient for a clinically relevant model. Some of the difficulties in modeling concussion include mimicking behavioral deficits seen in clinical TBI (for example, see Table 1); although there are cognitive, sensorimotor, and emotionality tests in rodents, a complete model is not plausible. Petraglia et al. (2014a) argue that even despite histological damage, models that do not display behavioral deficits may be modeling sub-concussive and not concussive injury. As a result, one of their criteria for a relevant model of rTBI or even sTBI is having behavioral deficits in an aforementioned category (Petraglia et al., 2014a).

As there is no currently accepted model of rTBI, a number of groups have attempted to model it in a variety of ways. One popular way is through the weight drop method, where rats or mice are used, with or without a helmet, with weights dropped from different heights (Namjoshi et al., 2013; Xu et al., 2014). These weights are also not uniform in mass between studies. Groups have modified CCI devices to use a rubber impactor tip against surface of the head adult mice, injuring an animal anywhere between 2 and 5 times (Mouzon et al., 2012; Hylin et al., 2013). One group created a modified CCI and weight drop apparatus that put a restrained, unanesthetized mouse on a foam bed and used a CCI device to create the impact (Petraglia et al., 2014b).

Generally, studies using these models find that rTBI results in greater histological damage and behavioral deficits than single concussions. For example, Manville and colleagues (2007) found that rTBI, but not single sTBI, causes a decrease in cerebral metabolism. rTBI also results in significant acute damage to the pituitary, including weight decrease, that seems to relate to downstream hormonal abnormalities (Greco et al., 2013). This is supported by the corresponding decrease in growth hormone (Greco et al., 2013) and findings that rTBI in female mice significantly decrease bone mass, area, density, volume, and strength, without changes in

growth factors (Yu et al., 2014).

Studies report white matter damage in both cortical and subcortical layers after rTBI (Donovan et al., 2014; Fujita et al., 2012; Hylin et al., 2013; Miyauchi et al., 2013; Mouzon et al., 2014; Namjoshi et al., 2013; Shitaka et al., 2011; Xu et al., 2014). These studies used injuries in mice with a modified CCI (Mouzon et al., 2012; Shitaka et al., 2011), weight drop (Namjoshi et al., 2013; Xu et al., 2014), or blast model (Calabrese et al., 2014). Shitaka and colleagues (2011) also noted that routine histology did not show any abnormalities in rTBI mice, which was supported by other studies (Allen et al., 2000; DeFord et al., 2002; Hylin et al., 2013; Kane et al., 2012; Mouzon et al., 2012). Some studies also reported an increase in astrocytes as measured by glial fibrillary acidic protein (GFAP) immunostaining (Allen et al., 2000; Luo et al., 2014; Kane et al., 2012; Mouzon et al., 2012; Uryu et al., 2002) and microglia as measured by Iba-1 (Huang et al., 2013; Klemenhagen et al., 2013; Mouzon et al., 2014).

In the clinic, a hallmark of repeat concussions is the diagnosis of CTE post-mortem (McKee et al., 2009). One characteristic is the aggregation of phosphorylated Tau (pTau) protein in the brain, which is responsible for the stabilization of axonal microtubules (McKee et al., 2009). Interestingly, some studies have not found an increase in pTau after rTBI (Mouzon et al., 2014; Xu et al., 2014), while others have (Ojo et al., 2013, Luo et al., 2014). There has also been a corresponding increase in amyloid beta, a protein associated with Alzheimer's Disease (Mouzon et al., 2013; Namjoshi et al., 2013; Uryu et al., 2002).

Many studies have found that rTBI causes behavioral deficits in spatial learning and memory through either the Morris Water Maze (classic or accelerated) or the Barnes Maze (DeFord et al., 2002; Huang et al., 2013; Luo et al., 2014; Mouzon et al., 2012, 2014; Uryu et al., 2002). There have been corresponding deficits also seen in the Novel Object Recognition task

(Namjoshi et al., 2013; Prins et al., 2010). The few studies that have examined emotionality through tests for depressive-like behavior, anxiety, or fear responses, have found an increase in deficits in emotional regulation in animals with rTBI (Huang et al., 2013; Klemenhagen et al., 2013; Luo et al, 2014).

Notably, eight groups used rat models, instead of mice, to examine the effects of repeat injury, and found similar behavioral and pathological results as those who used mice (Allen et al., 2000; Calabrese et al., 2014; Donovan et al., 2014; Fujita et al., 2012; Huang et al., 2013; Miyauchi et al., 2013; Wang et al., 2013; Yu et al., 2009). Of these, 3 groups used a CCI device, and all of these used a mild but still open-head paradigm (Donovan et al., 2014; Huang et al., 2013; Yu et al., 2009). As a result, the models that use a traditional CCI result in cortical cell loss and are therefore not relevant for concussion modeling.

Despite these models, to the best of our knowledge, there is no clinically relevant model of concussion in the adult rat using a commercially available device such as a CCI impactor. A clinically relevant model would not include a craniotomy but would see relevant symptoms. An easily reproducible model in the adult rat is important to compare to historical TBI literature and to clinical data.

CONCUSSION MODEL PILOT STUDIES

This project aimed to create a model of single (sTBI) and repeated concussions (rTBI) in the adult rat using a controlled cortical impact (CCI) device and to record any acute behavioral and histological differences between the two. Clinically relevant findings would include common behavioral deficits such as memory loss recorded at this early time point.

Open-Head CCI Approach

Our initial approach was to model concussion in the adult rat using a traditional CCI method, which included a crainiotomy (for example, see Adkins et al., 2015). As noted above, other groups also modeled concussion in this way. The severity of the impact is dependent on the impactor tip size, depth of the impact, and velocity of the impact. These variables were thus manipulated to produce the mildest possible injury. The velocity of the impact varied between 1.5, 1.2, and 1.0 m/s; with the depth at -0.8 mm dorsal/ventral (DV) and -0.7 mm DV from the surface of dura. Different combinations of velocity and depth were tried in adult rats. After the injury, the rats received tests of motor coordination (foot fault). Rats were euthanized 4 days following the injury, brains removed, surface of the cortex observed, and sliced coronally to be stained with Cresyl violet. While all injury parameters caused motor deficits in the foot fault test, all also caused visible damage to the cortical surface with cortical cell loss in layers I and II/III. Since obvious cell loss is not consistent with concussion, we then moved on to try using the CCI in a model.

Closed-Head CCI Approach

A closed-head model involves an impact given directly on the surface of the head or skull, as opposed to exposing the brain with a craniotomy. It is most clinically relevant for the model to impact the surface of the head. Our goal was to create the strongest impact with observable behavioral deficits without causing skull cracks or overt cortical pathology. Because foot fault is traditionally a test of asymmetrical motor deficits and we were unsure if our injury would be severe enough to observe faults, we added the Novel Object Recognition (NOR) task (as used in Prins et al., 2010) to see if we would observe acute memory deficits.

Our injury parameters were modeled after those by Prins and colleagues (2010) who used this approach in juvenile rats. Instead of a traditional CCI, the impactor hit the surface of the head directly. However, unlike the juvenile approach, we placed the rat on a foam bed instead of a wooden frame for support for two reasons: one, we did not want the impact to break the jaw; two, we wanted to mimic the acceleration-deceleration aspect of human concussions. Furthermore, we created a Plexiglas frame instead of placing the rat in ear bars. This would help stabilize the head during impact, and thus keep the area of impact more consistent (See Figure 3).

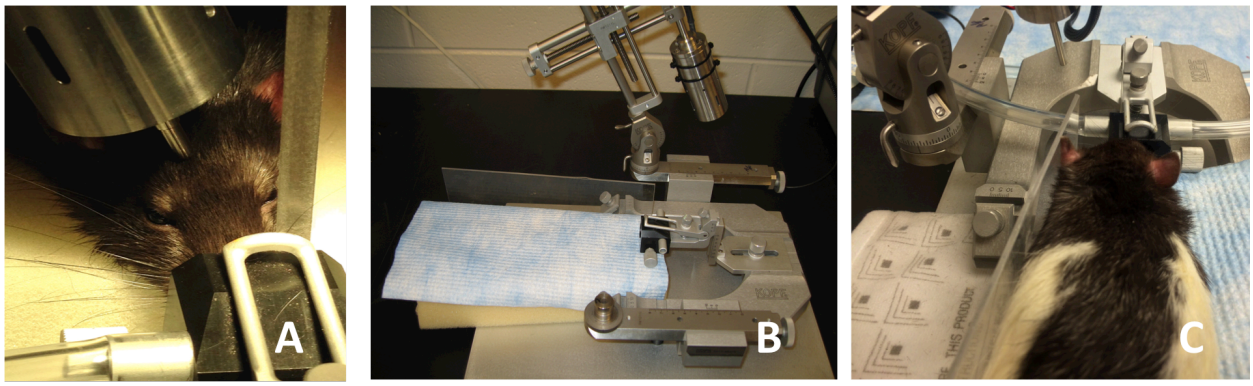

Figure 3: CHI model using a CCI device. A) Impact location. B) Plexiglas frame and foam base. C) Rat prepared for impact.

The parameters attempted were 6.5 m/s at a depth between 7.0-13.0 mm, and depths of 8.0 mm at velocities between 7.0-13.0 m/s. All depths took into account the amount of "give" or flexibility of the foam. All of these were with a 5 mm tip size. Rats were sacrificed either 24 hours post injury to examine for skull cracks or 4 days after injury. The larger velocities and depths caused skull cracks. As such, the parameters were scaled down until there were deficits in the NOR task without skull cracks or pathology observable with Cresyl violet staining. The final parameter chosen for the study was a velocity of 6.5 m/s with a depth of 10.0 mm. Based on the

lack of behavioral deficits, we believe that a milder form of the injury, 6.5 m/s at a depth of 8.0 mm, would cause a sub-concussive insult, however this was not further explored in this study.

Using these parameters, we hypothesized that at an acute time point, single and repeat concussions would cause sensorimotor and memory deficits as well as an increase in anxiety and baseline corticosterone level, without overt cortical pathology. We predicted that these deficits would be similar between the groups at this early time point. However we expected that there might begin to be some delineation between rats with a single versus repeat concussion. The results of the study are presented in the following manuscript.

A clinically relevant closed-head model of single and repeat concussive injury in the adult rat using a controlled cortical impact device

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INTRODUCTION

The increased incidence of traumatic brain injury (TBI) in sports and military combat has correlated with a sharp increase in long-term neurological dysfunction (Champion et al., 2009; Gessel et al., 2007; Lehman et al., 2012; Pearce et al., 2014). Of even greater concern has been the increase in individuals who experience multiple or repeat concussions during their athletic or military careers. Lehman and colleagues (2012) found that NFL players with 3 or more concussions were 5 times more likely to have mild cognitive disorders, 3 times more likely to have memory problems, and 4 times more likely to have neurodegenerative diseases such as Alzheimer's, ALS, and Parkinson's, later in life. McKee et al. (2009, 2015) has linked repeat concussions to chronic traumatic encephalopathy (CTE), an earlier-onset neurodegenerative disorder that, at the present time, can only be conclusively diagnosed post-mortem. Other studies have also reported a link between TBI and neurodegenerative disorders (Guskiewicz et al., 2000; Hazrati et al., 2013; Hoge et al., 2008; Omalu et al., 2010) as well as links to psychiatric disorders including posttraumatic stress disorder (PTSD) and major depressive disorder (Ma et al., 2014).

Traumatic brain injury is also linked to pituitary gland dysfunction both clinically and experimentally (reviewed in (Tanriverdi et al., 2010). Patients with TBI are commonly diagnosed with impairments in the hypothalamic-pituitary-gonadal (HPG) and growth hormone axes (Bondanelli et al., 2004; Krahulik et al., 2010; Tanriverdi et al., 2006). Of recent interest are the effects of TBI on the hypothalamic-pituitary-adrenal (HPA) axis, specifically modulating the stress response. Studies have shown both hypo- and hyper-responsiveness of the HPA axis in response to injury, depending on the severity of the TBI (Tanriverdi et al., 2010). Additionally, the chronic or transient nature of these changes post-injury is also debated (Agha et al., 2004b; Aimaretti et al., 2005; Barton et al., 1987; Cohan et al., 2005; Dimopoulou et al., 2004; King et al., 1970; Krahulik et al., 2010; Sojka et al., 2006; Tanriverdi et al., 2006, 2010).

Most patients experiencing a single mild TBI (sTBI) recover well with adequate time, but reoccurring concussions can result in more persistent deficits and pathological changes (Meehan et al., 2012). Furthermore, a single concussion can cause increased vulnerability to a second (Laurer et al., 2001), highlighting a significant threat for populations such as athletes. The underlying mechanisms of why repeat concussions are more detrimental than single concussions and why they are linked with neurological disorders and psychiatric symptoms are currently not clear, although studies in both patients and animal models are underway.

Animal models are better suited to more deeply explore cellular mechanisms and behavioral consequences of repeat TBI (rTBI) and how they are linked. Shultz and colleagues (2011) used a fluid percussion (FP) model of rTBI in the adult mouse that resulted in behavioral deficits, neuroinflammation, and cortical cell loss.

Other groups modified mild weight drop model parameters (as described by Khuman et al., 2011; Mannix et al., 2013) to also model repeat concussion. These studies demonstrated

transient motor coordination deficits, impaired spatial learning, and histological effects such as astrogliosis and phosphorylated Tau protein (Dapul et al., 2013; DeFord et al., 2002; Kane et al., 2012; Meehan et al., 2012). Miyauchi et al. (2013) created a similar model in the juvenile rat using a modified weight drop apparatus for a single concussion and saw significant motor/balance impairments, deficits in executive function, and depression-like behavior

Some groups (Hylin et al., 2013; Klemenhagen et al., 2013; Mouzon et al., 2012; Ojo et al., 2012; Shitaka et al., 2011; Uryu et al., 2002) have created closed-head impacts (CHI) in the adult mouse with an impactor device traditionally used for a controlled cortical impact (CCI) model of TBI. Both single and repeat injuries using these CHI models generally result in learning/memory deficits, pituitary deregulation and dysfunction, no cortical cell loss, increased aggregates of proteins such as beta amyloid precursor protein and Tau, and increased astrocyte activity (Greco et al., 2013; Hylin et al., 2013; Klemenhagen et al., 2013; Mouzon et al., 2012; Mouzon et al., 2014; Ojo et al., 2012; Kane et al., 2012; Shitaka et al., 2011; Uryu et al., 2002).

Despite the success of these mouse models of rTBI, there are few in the rat. Prins and colleagues (2010) created a CHI model using a CCI for juvenile rats, and showed similar deficits seen in mouse models, specifically an increase in astrocyte activity, axonal degeneration, and memory deficits in the Novel Object Recognition (NOR) test. To the best of our knowledge, there are currently no closed-head CCI models of concussions (mild or repeated) in the adult rat using a CCI device.

The current study describes a clinically relevant model of concussion in the adult rat using a modified CCI protocol adapted from Prins et al. (2010). Our criteria for a clinically relevant model included those presented by Petraglia and colleagues (2014a): 1) the impact would be on the surface of the skin, not require a craniotomy, and not produce skull fracture; 2)

our injury would not produce any obvious cortical pathology; 3) and the injury would cause behavior mimicking acute clinical symptoms, including sensorimotor and learning and memory deficits. Because there seems to be variable effects of concussion on the HPA axis, we took baseline corticosterone levels for a preliminary analysis. We also attempted to incorporate biomechanical aspects of human head injury by not restraining the rat in the stereotax and by placing the rat on a foam bed to allow for head movement, similar to Petraglia et al. (2014b).

METHODS

Animals

Male Hooded Long-Evans rats (Charles River Laboratory; 300-450g) were housed two to a cage in the DePaul University Research Support Facility. Rats were kept on a 12:12 hour light/dark cycle with food and water available *ad-libitum*. Animals were handled daily for approximately 1 week prior to surgery. All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Animals and were approved by the DePaul Institutional Animal Care and Use Committee.

Rats were randomly assigned into the following groups (see Table 1): sham (no injury, n = 8); repeat injuries (3 injuries spaced 48 hours apart (rTBI), n = 10); immediate (sacrificed on day 4) single injury (IsTBI, n = 9); acute (sacrificed on day 8) single injury with two subsequent anesthesia exposures spaced 48 hours apart to control for the extra exposure to anesthesia seen in the rTBI group (sTBIac, n = 9); and acute single injury with no subsequent anesthesia exposure (sTBI; n=8) sacrificed on day 8.

Table 1: Groups

Group	Euthanasia Day (post first injury)	n =
Sham	4	8
IsTBI	4	9
sTBI	8	9
sTBIac	8	8

All animals were subject to two injections of BrdU, a synthetic thymidine analog, 30 minutes after the initial injury and 2 hours before euthanasia (50 mg/kg). The results will be examined and discussed in a future study that focuses on understanding the neurogenic response to rTBI.

Experimental Design

All animals were habituated to handling 1 week prior to initial injuries through general handling. Blood samples for corticosterone analysis and baseline motor coordination score were taken 24-48 hours prior to the first injury (See Figure 1). IsTBI and sham animals received 1 injury over the right forelimb sensorimotor cortex (FL-SMC) or exposure to anesthesia, respectively, and began behavioral paradigms 24 hours after surgery. rTBI, sTBI, and sTBIac animals received 3 injuries 48 hours apart (rTBI) or 1 injury with two (sTBIac) or no (sTBI) subsequent anesthesia exposures. Behavioral paradigms for these animals began 5 days after the initial injury, which was 1 day after the final injury (rTBI) or last anesthesia exposure (sTBIac, sTBI).

Behavioral paradigms for all animals were conducted depending on the day of the final injury. For IsTBI and sham animals, testing began on the day immediately after the only injury/anesthesia exposure. rTBI and sTBIac animals, testing began on the day following the final injury/anesthesia exposure, which was 5 days after the initial injury (Figure 1). sTBI animals began testing at the same time as their anesthesia control counterparts (5 days after initial injury). The battery included the open field, foot fault, and novel object recognition tasks. Animals were euthanized for histological analysis according to group (see Table 1).

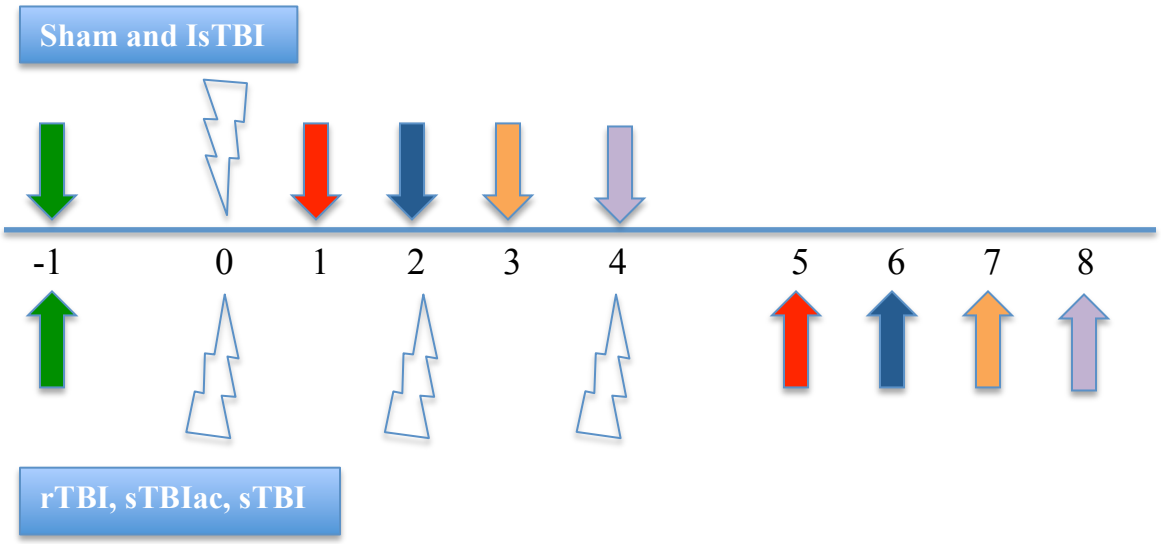

Figure 1: Experimental Design. Arrows indicate behavioral or histological tests: green = blood sample, motor baseline; red = open field, foot fault; blue = novel object habituation; orange = novel object recognition task, foot fault; purple = blood sample, euthanasia. Lightning bolts = CHI or anesthesia exposure.

Closed-Head Controlled Cortical Impact (CCI)

To inflict a closed-head traumatic brain injury we used a modified CCI approach (Leica Impact One, Leica Microsystems Inc., Buffalo Grove, IL; Prins et al., 2010; Petraglia et al., 2014b). Rats were anesthetized with 2.0-3.0 mL/min isoflurane anesthesia and placed in a Kopf

sterotaxic apparatus (Kopf, Tujunga, CA). Rats were not placed in a bit or ear bars but were kept on anesthesia through a nose cone. The rats rested on a foam bed (5 cm thick) in a Plexiglas frame. The frame consisted of a base (11.43 x 24.13 cm) and a side piece (9.525 x 22.86 cm) angled 11° from the vertical (see Figure 2B).

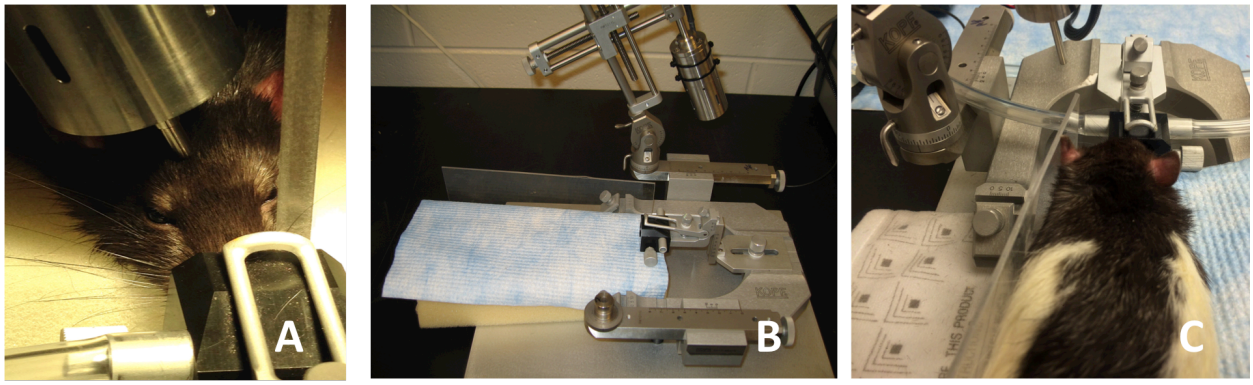

Figure 2: CHI model using a CCI device. A) Impact location. B) Plexiglas frame and foam base. C) Rat prepared for impact.

For rats in the immediate time group (sacrificed on day 4), the head was shaved and the skull was exposed just enough to center the impactor tip on bregma and move the impactor tip 0.5 mm anterior and 4.0 mm lateral to bregma, directly over the forelimb sensorimotor cortex (FL-SMC; Figure 2A). At these coordinates, the tip was placed onto the surface of the skin and the location relative to the eye and ear were measured and documented (1.1 cm between the eye and ear at an angle of 20°). In the acute (sacrificed on day 8) time point, the skull was no longer exposed and the tip was aligned over the FL-SMC using the measurements established in the immediate group. The impactor (Leica Microsystems Inc., Buffalo Grove, IL) delivered the cortical impact at an angle 20 degrees from vertical, enabling the flat tip to be perpendicular to the surface of the head; this was adjusted accordingly if necessary. To allow for the movement of the head but to also provide stability to the rat during the impact, the lateral surface of the head

was rested lightly against the Plexiglas frame (Figure 2C). All injuries were produced with a 5mm flat tip at 6.5 m/s at a depth of 10.0 mm from the surface of the skin for 300 msec. The depth accounted for the amount of give in the foam. After the impact, rats were sutured (if necessary) and topical analgesic (Xylocaine) and antibiotics (Neosporin) were applied. Body temperature was maintained at 37°C during recovery. Following the surgery, rats were returned to their home cages and monitored daily. rTBI animals had a total of 3 injuries 48 hours apart; an additional group of sTBI animals (sTBIac) were exposed to two subsequent anesthesia treatments 48 hours apart. This group was placed in an anesthetization chamber for the duration of time that the rTBI animals were anesthetized for their subsequent injuries (approximately 20 minutes).

Foot Fault Test for Forelimb Coordination

Motor coordination was examined using the foot fault test before injury (for baseline) and 1 and 3 days after final injury. Previous studies have shown that rats with a significant unilateral injury over the FL-SMC exhibit deficits in forelimb coordination contralateral to the injury (Adkins et al., 2015; Hernandez and Schallert, 1988); however the test can also be used to examine overall motor coordination. Rats were placed on a grid made of test tube racks (33.02 x 25.40 x 7.62 cm, with openings of 2.54 cm) for 50 steps (Figure 3). A step was defined as the movement of both forepaws sequentially. The number of left and right forelimb "faults" was counted, which was defined by a forelimb falling through the opening of the rack. Our injury model was not severe enough to produce forelimb asymmetries. Therefore, data presented are of percent total foot faults ((left faults + right faults/ number of steps)*100).

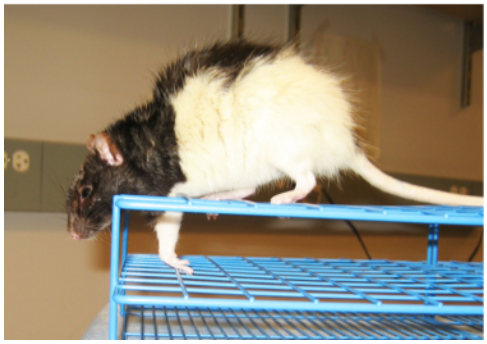

Figure 3: Rat demonstrating forelimb fault in the foot fault test.

Novel Object Recognition Test

The novel object recognition (NOR) task is a well-established measure of hippocampal memory in rodents (Ennaceur and Delacour, 1988; Mathiasen and DiCamillo, 2010). Previous studies have shown that juvenile rats undergoing both mild and repeat concussions show deficits in the task and thereby in memory (Prins et al., 2010; Reger et al., 2009).

Briefly, the original task as described by Ennaceur and Delacour (1988) uses spontaneous behavior of rodents to explore objects placed in their environment, and the tendency to spend more time with a previously unexamined object than a familiar one. This task is especially useful because it requires no food deprivation, reinforcement, or other stimuli other than object exposure. The NOR task takes advantage of an enclosed space to track locomotor behavior and object interaction.

There are 3 stages to the task: arena habituation; 'familiar' object habituation; and the 'novel' object test. 24 hours after a single injury or anesthesia (sham, IsTBI) or after the final injury (in the groups sacrificed at day 8: rTBI, sTBI, sTBIac) rats were first given ten minutes to get acclimated to and explore the arena, an open field (San Diego Instruments, San Diego, CA). During this habituation, the open field test was conducted (see below). After a set latency period, in this case 24 hours, the animals were reintroduced to the open field with two of the same

"familiar" objects (Kong Classic dog toy), and were given 5 minutes to explore and interact with the objects (Figure 4B). After the same latency period as before, one of the objects was replaced with a 'novel' object (generic Target dog toy), and rats were allowed to again explore and interact for 5 minutes (Figure 4C). Each trial was videotaped. The duration of time spent (in seconds) with each object was assessed in both the object habituation and test trials. Time spent with object was defined as direct interaction, such as touching or sniffing, or the snout facing the object within a few centimeters. Data was converted to percent total time spent with the novel object: [(time with novel/(time with novel + familiar)*100].

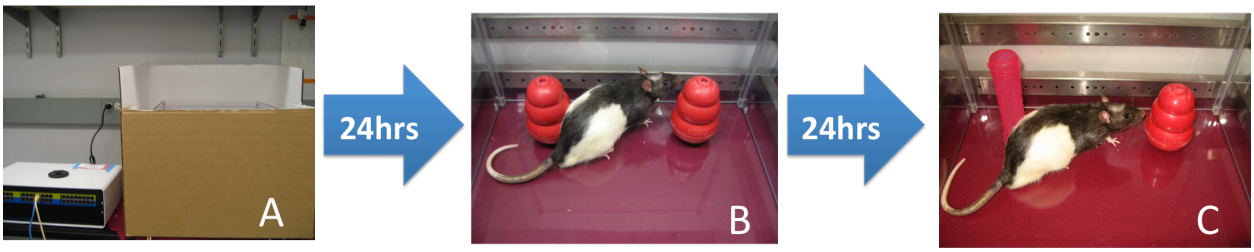

Figure 4: Novel Object Recognition Task: A) Open field apparatus for habituation. B) 24 hours later, rats were placed into the open field with two of the same objects. C) 24 hours afterwards, rats were again placed in the open field with one familiar and one novel object.

Open Field Test

The open field test can be used as a test of both locomotor activity and anxiety (Walsh and Cummins, 1976). Since the open field arena was used for the novel object test, habituation to the arena for the novel object test was used to assess locomotor activity and anxiety in an open field (Figure 4A). On day 1 post first injury (IsTBI, sham), or day 5 post first injury (rTBI, sTBI, and sTBIac groups), rats were placed into the open field for 10 minutes (San Diego Instruments, San Diego, CA; 40.64 cm x 40.64 cm x 38.1 cm). Using PAS-Open Field software (San Diego

Instruments, San Diego), locomotor activity was monitored using 16 x 16 beams on the XY plane, measuring which beams are broken per predesigned interval (15 seconds). The open field was furthermore broken into a "center" (middle 40% of the arena) and "surround" (Markel et al., 1989; Meziane et al., 2007). Rodents displaying an anxious phenotype will spend less time in the center than their control counterparts (Katz et al., 1981). Center and surround areas were calculated based on the 16 x 16 beam break structure of the open field and the number of unique beam breaks in each area was tallied.

Corticosterone (CORT) Analysis

To examine resting corticosterone levels, blood samples were collected 1 day prior to injury (baseline) and two hours before euthanasia. Samples were collected using heparinized capillary tubes from the proximal end of the tail (approximately 150 μL). All blood samples were collected between 9 am and 11 am. Samples were spun at 1800 RPM for 10 minutes or until separation. Serum and pellet were separated and stored at -80°C until analysis. All samples were analyzed using methods previously described using a MP Biomedicals, LLC (Orangeburg, NY) radioimmunoassay kit (Dimitrov et al., 2007). Data is presented as ng/mL.

Histology

Euthanasia. Animals were sacrificed 4 or 8 days following the first injury. Rats were deeply anesthetized with Equithesin (149 mg/100 g chloral hydrate, 31 mg/100 g sodium pentobarbital i.p.) prior to euthanasia. Brains were extracted after cardiac perfusion using PBS with 0.005% heparin and 4% paraformaldehyde in phosphate buffered saline (PBS). After extraction, brains were post-fixed, cryoprotected, and kept at 4°C until sectioning. Brains were

flash-frozen and sliced serially in 40 μm coronal sections in sets of 7 using a cryostat. In 1 set of tissue, cell bodies were stained using Cresyl violet to examine underlying cytoarchitecture.

Examination of gross anatomy. Sections were mounted on gelatin-coated slides and stained with Cresyl violet. The sections were examined qualitatively on a Leica microscope (Leica Microsystems Inc., Buffalo Grove, IL) for the presence of contusions, obvious areas of cell loss, and cortical layer disorganization.

Statistical Analysis

Results were analyzed with the online statistical software VassarStats (Vassar College, Poughkeepsie, NY). The foot fault test was analyzed with a two-way repeated measures ANOVA. The NOR and open field tests were analyzed with a one-way ANOVA. Comparisons were preformed across IsTBI and sham groups for the immediate time points, and all groups in acute time points. Tukey's *post-hoc* test was applied when relevant to reveal the significant main effects or interactions. Data is presented as mean ± SEM.

To examine whether the extra exposure to anesthesia received by the rTBI group played a role in our measures, we first compared sTBI with no further anesthesia exposure and a single TBI group with two subsequent exposures (sTBIac) to mimic the extra anesthesia received by the rTBI group. In all of the analyses, we first conducted a one-way ANOVA between these two groups (sTBI and sTBIac groups) to see if the extra anesthesia produced effects. In all measures presented below, there were no significant differences found; therefore these two groups were pooled and labeled sTBI (see Table 2). All further reference to sTBI includes sTBI (n = 9) and sTBIac (n = 8).

Table 2: Statistical values indicating no difference between sTBI and sTBIac anesthesia control groups for each measure.

Task	F Value	p value
Open field (locomotion)	$F_{1, 15} = 2.21$	$p = 0.15$
Foot fault	$F_{12\ 30} = 3.31$	$p = 0.06$
NOR (habituation, total time)	$F_{1, 15} = 0.28$	$p = 0.60$
NOR (novel vs familiar)	$F_{1, 15} = 0.46$	$p = 0.51$
Open field (anxiety)	$F_{1, 15} = 0.03$	$p = 0.86$
CORT	$F_{1, 15} = 0.19$	$p = 0.67$

RESULTS

Single and repeat concussions result in hypoactivity

A one-way ANOVA revealed a significant main effect of injury type on locomotor activity, measured by the number of beam breaks in the open field ($F_{3, 41} = 13.19$, $p < 0.001$; Figure 5). A Tukey HSD *post-hoc* test demonstrated that both the rTBI and sTBI (tested on day 5) groups had significantly fewer beam breaks than both shams ($p < 0.05$) and IsTBI animals (tested on day 1) ($p < 0.01$). There were no significant activity differences between shams and IsTBI groups nor between rTBI and sTBI groups. Therefore, 1 day after their injury, animals with a single concussion have locomotor activity similar to shams; however, by day 5, rats with a single concussion are hypoactive. Compared to single concussion, rats with repeat concussions were not more hypoactive at this early time point.

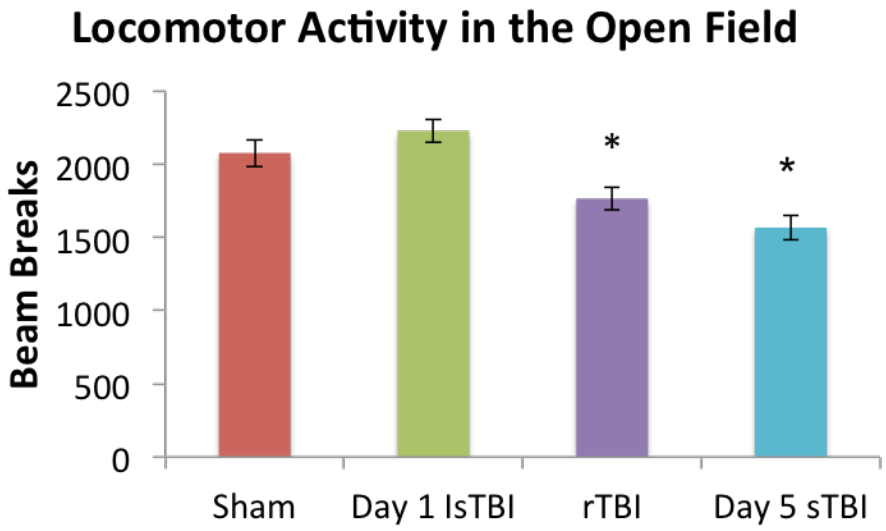

Figure 5: rTBI and sTBI animals are significantly hypoactive 5 days after the initial injury (*$p < 0.05$ compared to sham).

The number of days after injury has an effect on the percentage of total foot faults

Foot fault is typically a test of asymmetries; although the impact in our model was unilateral, our injury did result in foot fault asymmetries. Therefore, we analyzed the total number of faults. To examine changes in locomotor function over time, rather than at a single time point, we focused on the data of the shams, pooled sTBI, and rTBI groups, sacrificed on day 8.

There was a significant effect of injury type ($F_{2,24} = 4.49$, $p < 0.01$), day of testing ($F_{2,68} = 12.65$, $p < 0.001$), and an interaction between injury type and day of testing ($F_{4,110} = 2.77$, $p < 0.05$; Figure 6). Further *post hoc* analysis revealed no differences among groups for baseline faults ($p = 0.11$) or day 7 faults ($p = 0.227$), but did show a significant increase of faults on day 5 ($F_{2,34} = 6.71$, $p = 0.0035$). Animals with a single injury had significantly more faults 5 days after the initial injury compared to shams ($p < 0.01$) and to their baseline score ($p < 0.01$). However, by day 7, these deficits recovered ($p < 0.05$) such that the number of faults on day 7 were not

significantly different from the baseline data. Repeat injuries did show a non-significant increase in faults compared to shams that was maintained, rather than resolved, at day 7. The number of faults at day 7 was more significant than at baseline within the rTBI group ($p < 0.05$). Otherwise, single and repeat injured animals were not significantly different from each other in the foot fault task.

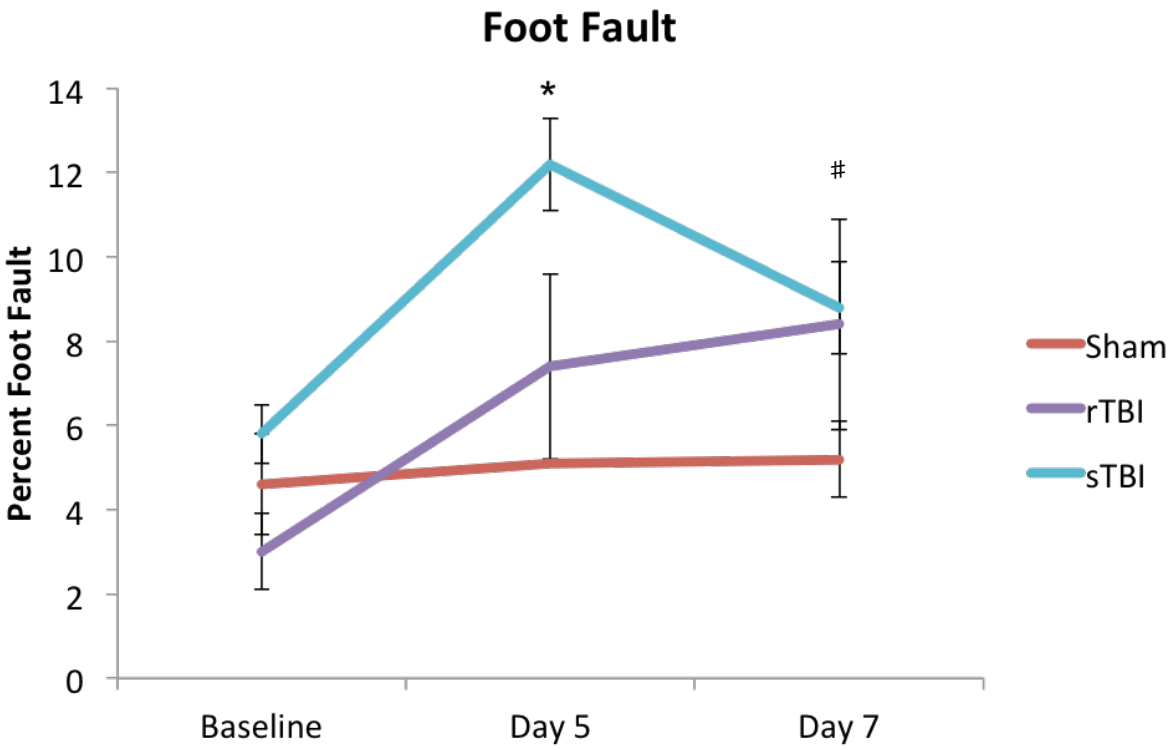

Figure 6: sTBI animals have significantly more foot faults 5 days after the initial injury that decreases on day 7 (*$p < 0.05$ compared to sham). Although there was an increase in foot faults in the rTBI group, it did not reach significance. However, the number of faults in the rTBI group at day 7 was significantly more than their baseline faults ($^{\#}p < 0.05$).

Both single and repeat concussions result in memory deficits

Habituation to the Objects. Because concussions produced a decrease in locomotor activity in the open field, we examined the "habituation" phase of the NOR task to see if injury

had an effect on overall exploratory activity and interaction with the objects. This trial occurred on day 2 for sham/IsTBI and day 6 (after first injury) for rTBI/pooled sTBI, and the objects used were the same for all animals.

A one-way ANOVA of all groups showed a main effect of group on the total time spent exploring the objects ($F_{3, 40} = 5.82$, $p < 0.002$). However, although *post-hoc* analysis revealed that animals with a single concussion spent significantly less time with the objects overall on day 6 than on day 2 ($p < 0.01$; Figure 7), there were no differences in time spent with objects among IsTBI, sham, or rTBI groups. Thus, in consideration of this variability in time spent with objects between animals, data was analyzed as "percent of total time spent with the novel object."

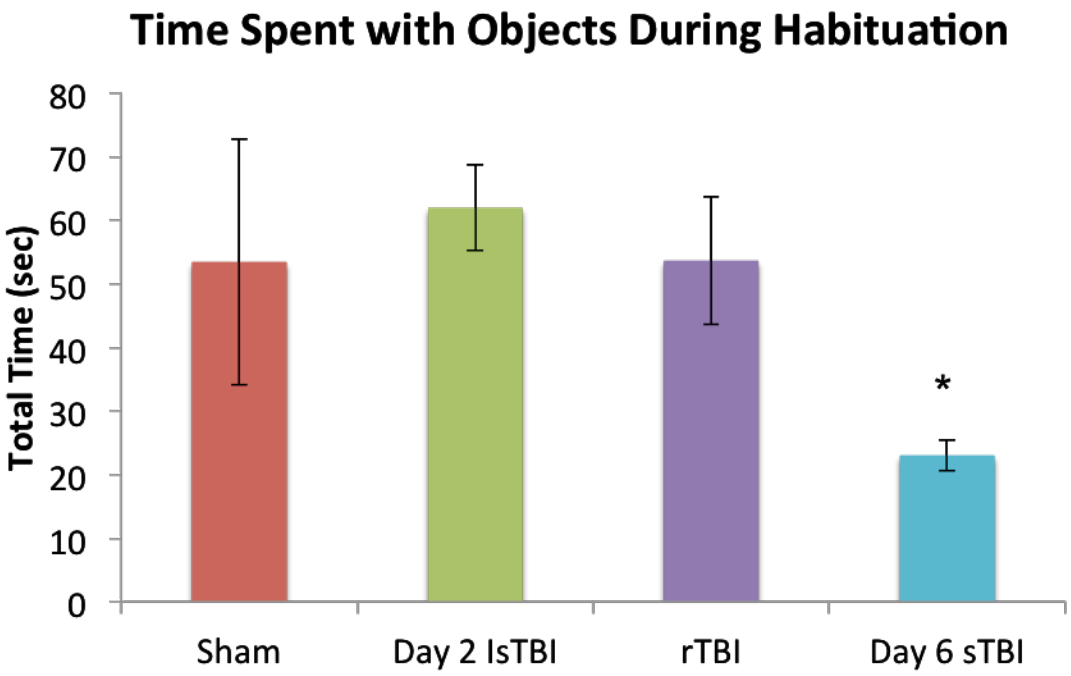

Figure 7: sTBI animals spend the least amount of time with the objects during habituation (*significantly different from sham, $p < 0.05$).

Memory Task. Both the sTBI and rTBI groups did the NOR task on day 7 after their initial injury, while the IsTBI did the task on day 3. One-way ANOVA demonstrated an effect of

injury type on time spent with the novel object ($F_{3,39} = 12.8$, $p < 0.001$). As expected, uninjured shams spent more time with the novel object ($p < 0.02$; Figure 8). Animals with a single concussion, however, 3 days after their injury, spent equal time between the two objects ($F_{1,8} = 0.6$; $p = 0.46$; data not shown) and a significantly less overall percentage of their time with the novel object than shams ($p < 0.01$). Animals with a single concussion recovered by day 7 and spent more time with the novel object ($F_{1,16} = 12.66$; $p = 0.003$) as did shams. However, at day 7, animals with repeat concussions did not spend most of their time with the novel object and were significantly different from both shams and sTBI groups ($p < 0.05$).

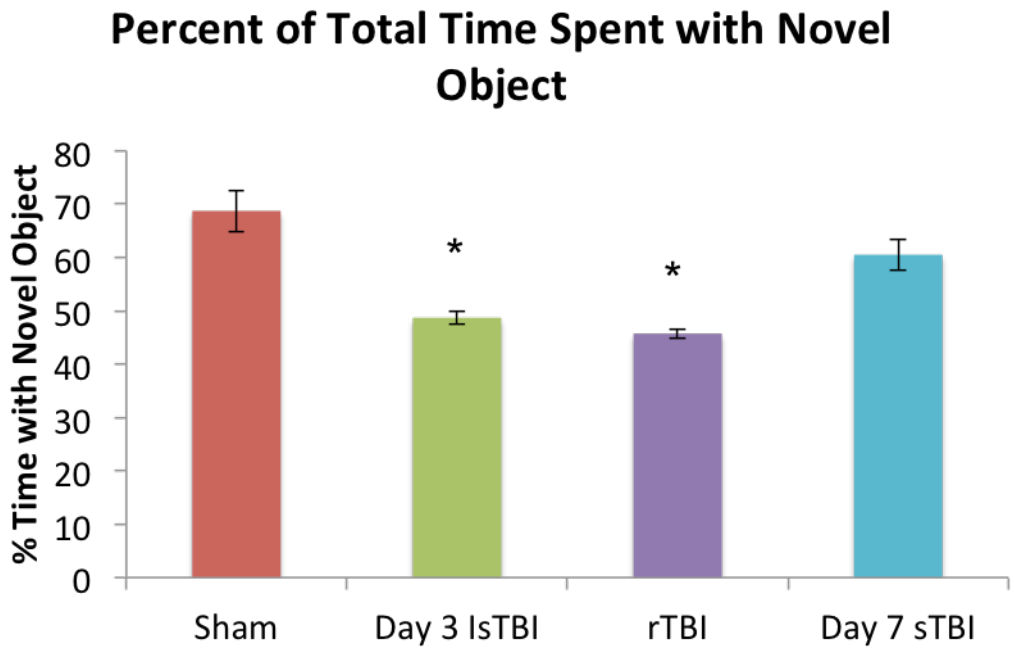

Figure 8: Sham animals spend more time with the novel than familiar object. Animals with a single concussion spend equal time with objects but recover the ability to recognize the novel object by day 7. Animals with rTBI continue to show deficits in the NOR task (* $p < 0.05$, from sham).

Repeat concussions results in an anxiety response

As previously discussed, decreased time spent in the center of the open field is reflective of an anxiety response. A single concussion did not decrease time spent in the center of the open field 1 or 5 days after the injury (Figure 9). However, the animals with a repeat concussion showed significantly less time spent in the center of the open field compared to both sham and IsTBI groups ($F_{3, 41} = 5.36$; $p < 0.01$). Although there was a trend for rats with a single concussion sTBI rats to spend less time in the center by day 5, it did not significantly differ from the other groups.

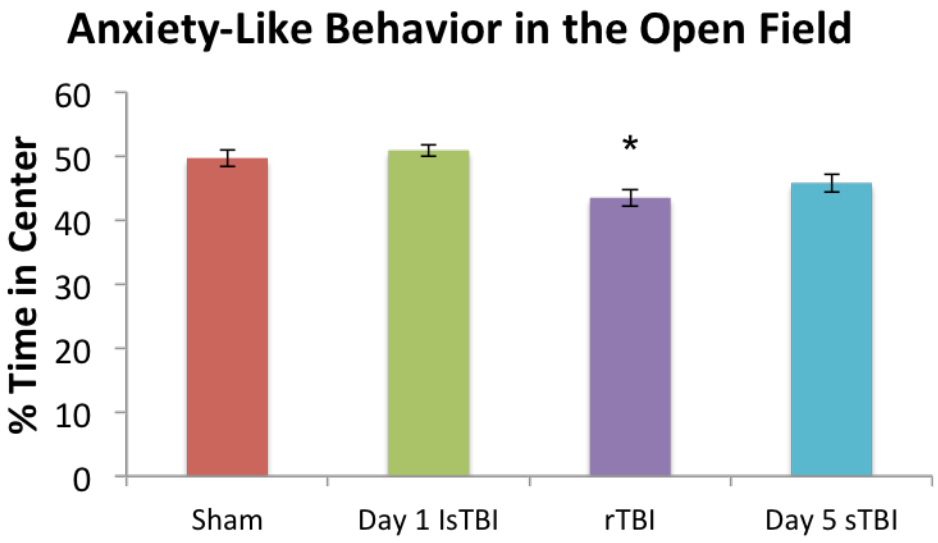

Figure 9: rTBI animals spend significantly less time in the center of the open field (*$p < 0.05$ from sham and IsTBI).

Single and repeat concussions result in increased baseline corticosterone levels

There was a significant effect of day ($F_{1,40} = 17.8$, $p < 0.0001$) and a group by day interaction in CORT levels ($F_{3, 87} = 4.8$; $p = 0.006$). There were no differences in CORT levels between injury types at baseline ($p = 0.85$). 4 days after an injury, animals with a single

concussion had a non-significant increase in CORT levels compared both to their pre-injury levels and to sham animals ($p = 0.19$). 8 days after an injury, however, animals with a single concussion did have significantly higher baseline CORT levels compared to both sham ($p < 0.01$) and IsTBI groups ($p < 0.05$). Similarly, animals with repeated concussions also had higher baseline CORT levels compared to sham ($p < 0.01$) and IsTBI groups ($p < 0.05$). However, rTBI and sTBI animals did not differ from each other at day 8 (Figure 10).

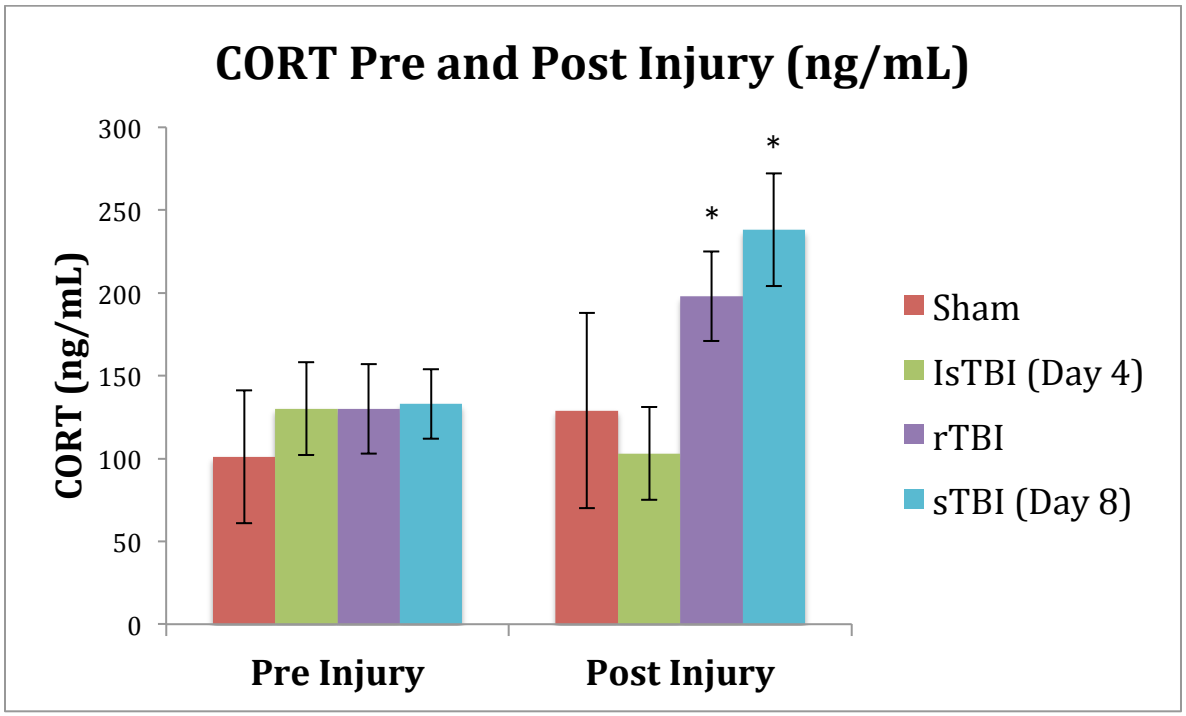

Figure 10: Both rTBI and sTBI animals have higher CORT levels day 8 after the initial injury compared to sham and day 4 single hit animals (*$p < 0.05$).

There was no obvious cortical damage in sTBI or rTBI animals.

After euthanasia, brains were extracted and examined for any surface damage. Coronal sections of brains were mounted and stained with Cresyl violet and examined for obvious cell loss or cortical layer disorganization. Neither single nor repeat concussions resulted in any

surface damage, nor caused any cortical layer disorganization, contusions, or other obvious neuronal cell loss (Figure 11).

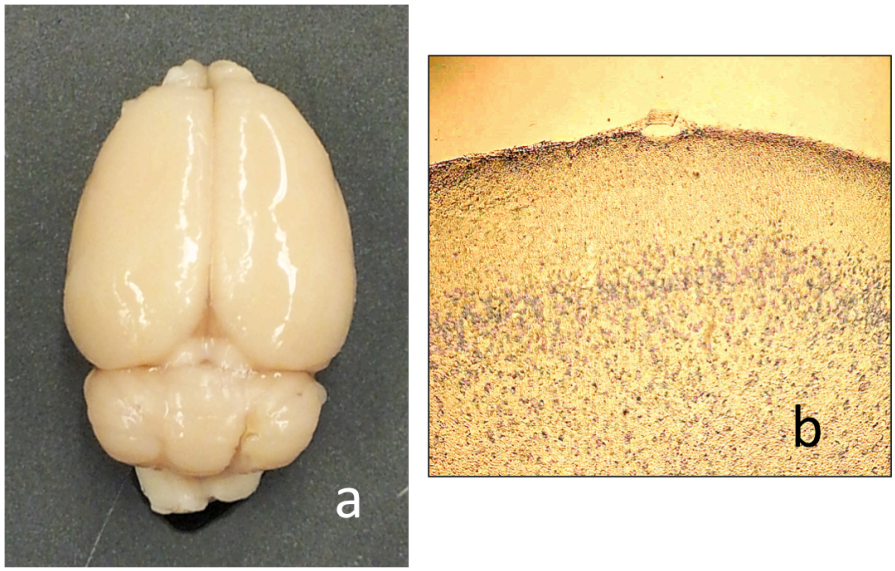

Figure 11: The concussion model does not produce surface damage or cortical disorganization following both single and repeat concussions.

DISCUSSION

Our closed-head concussion model produces deficits in motor coordination and locomotor activity (foot fault, open field), deficits in non-spatial learning and memory (NOR task), and anxiety-like behavior (open field) in animals with both single and repeat injuries, with repeat injuries producing longer lasting disruption in memory and longer lasting anxiety. These behavioral results, along with the lack of observable pathology, demonstrate the first viable model for both single and repeat closed-head injury in the adult rat.

Animal models have been used to examine various severities of TBI in order to understand its complex consequences. However, until recently, many groups have focused more on moderate and severe TBI (Petraglia et al., 2014a) because the multiple challenges in modeling milder forms of TBI, like concussions, are multifold. For example, mimicking the physical blow

to the head and its resulting biomechanics and, at the same time, an adequate number of clinically relevant symptoms, is not a simple task. In adult rats, this is especially difficult given the relative skull thickness, which is not an issue in juvenile rats and mice. The current traditional models have inherent limitations for modeling CHI. Fluid percussion and CCI involve craniotomies and focal lesions, neither of which are clinically present in patients with concussions (Petraglia et al., 2014a). Although weight drop models do produce an actual physical blow to the surface of the head, they are not commercially available and are often more variable and less reproducible than other models (Xiong et al., 2013). Here, we present a novel CHI model in the adult rat using a commercially available CCI device that allows free movement of the head. Models representing this acceleration-deceleration injury (also called coupe-contra coupe) are crucial to understanding the diffuse pathology and nature of human concussions, but, to the best of our knowledge, are less represented in the literature (Petraglia et al., 2014a). Our CHI model was also able to produce both a single concussion and a model of repeat TBI, which will be helpful in expanding the understanding of the pathology and consequences of repeat concussions.

Single Concussion

Although concussion symptoms can be quite variable and unique from one individual to another, one of the most universal symptoms of concussion is memory loss. In our model, a single concussion causes acute memory deficits in the Novel Object Recognition Task 24 hours after habituation (3 days after the initial injury) that recover at a later time point (7 days after the initial injury). Similarly, Prins (2010) reported that 1 hour after habituation, injured juvenile rats were still able to discriminate between the novel and familiar object; however, this ability was

lost 24 hours after habituation, 3 days after the initial injury. Another study used a 4 hour interval at days 2, 7, and 14 post-injury, finding deficits at each time point (Namjoshi et al., 2013). Unlike this study, our study did not find deficits 24 hours after habituation on day 7 post-injury. Namjoshi (2013) used a weight-drop model on mice, impacting the skull directly instead of the surface of the head. A direct skull impact may have resulted in a more severe injury that would account for the deficits in their model, but not in ours.

NOR is a test of non-spatial and declarative memory. Many groups instead use tests of spatial working memory, such as the Morris Water Maze and Barnes Maze, in their concussion models. However, even at an acute time point, results vary for single concussions. Luo (2014) found that a single concussion in mice causes deficits in the radial arm water maze but only at 2 and 6 months after the injury. Other groups also did not observe memory deficits at 10/11 days after the initial injury (DeFord et al., 2014; Hylin et al., 2013). However, without deficits at an acute time point, it is difficult to say whether these were true concussions or subconcussive injuries. Clinically, a single subconcussive injury typically does not result in acute memory deficits, but if multiple subconcussive injuries are present, it has been thought to play a role in later memory deficits, similar to what is seen following repeated concussions (Dashnaw et al., 2012).

Other symptoms seen following a single concussion involve motor function, such as a lack of coordination, dizziness, and lethargy. Indeed, clinically, athletes showed motor abnormalities including tonic posturing, clonic movements, and gait abnormalities immediately following a concussion (McCrory and Berkovic, 2000). In the current study, animals with a single concussion were slightly hyperactive compared to shams 1 day after the injury, although this difference was not significant. However, 5 days after the injury, animals with a single

concussion were significantly hypoactive. Interestingly, another group found that a single weight-drop concussion in the mouse caused hyperactivity in a novel cage 5 days after injury, but not 30 (Kane et al., 2012). This difference may be due to how locomotion was assessed; although this group used a 16 x 16 beam break as well, the mice were placed in a transparent, clean cage. On the other hand, these differences may speak to injury severity and modeling. Although, theoretically, a weight-drop model and CCI model of concussions that are both clinically relevant should have comparable pathology and behavior, there may be some differences, perhaps in regards to weight-drop affecting a larger surface area on the skull and a more severe injury in the mouse whose skull is thinner.

In addition to examining locomotor activity, other studies have also focused on more specific motor abnormalities such as motor learning and coordination (rotarod test). In our study, in addition to changes in overall locomotor activity, animals with a single concussion also display a significant number of total faults in the foot fault task, which demonstrates deficits in motor coordination. However, these deficits are fairly transient, as they are seen 5 days after injury but are ameliorated by day 7. To the best of our knowledge, only one other group examined foot fault after a single concussive insult. Hylin (2013) showed that mice after a single concussion did not have significant impairments or asymmetries in foot fault compared to shams from days 1 through 6. However, the foot faults were presented as an asymmetry score, not as a measure of total faults, which most likely accounts for the difference between this finding and ours, which was that a single concussion produced deficits in total foot fault 5 days post-injury.

Nevertheless, foot fault is traditionally a test of forelimb asymmetry, sensitive to deficits caused by unilateral CCIs that create contusions (Adkins et al., 2015). Although the injury induced in the current study was unilateral, there were no asymmetrical foot faults. This

demonstrates that despite the fact that the area of impact was in one hemisphere, the injury was more global and affected both hemispheres. This may be due to use of a larger tip size for impact or diffusion of the impact over the skull. Hylin et al. (2013) did demonstrate that mice with concussions had deficits in the balance beam test, another measure of motor coordination, from days 1 until 6 after the final injury. In another measure of motor coordination and learning, the rotarod, Mouzon (2012) found that a single concussion causes decreased performance 1, 3, and 5 days after injury that recovered by 7 days post-injury. Chen et al. (2014) also found significant rotarod deficits days 1-3 post-injury, but did not examine further time points. Together these studies support our finding of a transient decrease in motor coordination following a single concussion. It also suggests that following concussion, motor deficits should be examined with tests that more closely examine motor coordination in a bilateral, not unilateral method.

There have been varying results in regards to the relationship between concussion and anxiety. In our study, a single concussion did not produce an anxiety response in the open field at an acute time point (24 hour post-injury). Furthermore, there was no change in baseline corticosterone levels 4 days after the injury, but there was an increase by day 8. It is well accepted that there is a link between mood disorders and the HPA axis (Carroll and Mendels, 1976), and that TBI affects that axis in patients (Tanriverdi et al., 2010) and animal models (Taylor et al., 2008). However, the mechanisms behind the link between TBI, deficits in the HPA axis, and resulting mood disorders are still not fully understood. Factors to consider include how TBI affects different levels of the axis and different endocrine systems. Although we did not see an increase in anxiety 24 hours post-injury, other groups have found that concussion can produce an anxiety response after a more chronic (2-6 months) time point (Luo et al., 2014; Mouzon et al., 2014) and not at an acute (days 12-14) time point (Hylin et al., 2013). We did demonstrate,

however, that a single concussion produced an increase in baseline corticosterone levels by day 8 post-injury. Although the direct relationship between corticosterone levels and anxiety is not fully understood, it is possible that after chronic elevation of corticosterone an anxiety response might be shown (Charmandari et al., 2005). Although previous studies show that concussion can produce anxiety, these groups did not look at levels of CORT. Future studies will need to be conducted to examine this relationship more closely.

Other studies that examine corticosterone levels after TBI have done so in response to a stressor. For example, Griesbach and colleagues (2011) found that 7 and 14 days post injury, animals with a mild FPI had significant increases in both CORT and ACTH levels after a restraint stress. At days 7, 21, and 24, however, Taylor et al. (2008) found that a mild open-head CCI caused a decrease in CORT in response to a restraint or forced swim stressor. Interestingly, on days 34, 54, and 70, animals with a mild CCI had significantly higher CORT levels after the stressor. Although both studies attempted a milder TBI, the CCI was a focal injury that caused lesions, which may account for the differences in CORT responsiveness compared to our study and those of Griesbach (2011). Examining corticosterone levels in response to a stressor in our model will add to our understanding of the effect of CHI on the HPA axis.

Repeat Concussions

Both clinically and experimentally, multiple concussions result in more cognitive deficits and physical impairments than single concussions (Guskiewicz et al., 2005). In our study, we found that compared to a single concussion, rats with repeat concussions showed a prolonged memory deficit in the NOR task, and anxiety response in the open field. They showed similar deficits however in motor tasks and baseline CORT levels.

Rats with repeat concussions spent significantly less time in the center of the open field than animals with a single concussion or shams 5 days after the initial injury. Spending decreased time in the center of the open field is indicative of an anxiety response (Katz et al., 1980). Other studies that have examined emotionality through tests for depressive-like behavior, anxiety, or fear responses, also have found an increase in symptoms in animals with repeat compared to single concussions (Huang et al., 2013; Klemenhagen et al., 2013; Luo et al, 2014; Mouzon et al., 2014). However, these groups looked at these behaviors at chronic time points and not within the first week, as in our study. As such, and given the clinical population of individuals with repeat concussions that manifest psychiatric conditions years later, it is surprising that an anxiety response is seen so early in the current study. It is possible that other models of repeat concussions may also show an anxiety response within the first week after the injuries if measured at this time point.

As mentioned, studies have linked dysfunctions in the stress axis with these types of anxiety behaviors. In the current study we found an increase in baseline corticosterone levels in rats with both multiple and single concussions at an early time point, but no significant difference between groups. It is curious that there was an anxiety response in the open field in animals with multiple concussions but not animals with single concussions, as both groups had elevated basal corticosterone levels at day 8 post-injury. It is possible that the elevation of CORT in rTBI rats began earlier than in sTBI rats. As mentioned above, the link between corticosterone and anxiety is not fully understood (Charmandari et al., 2005); if the animals with multiple concussions had longer exposure to elevated CORT levels than animals with a single concussion, that may account for the differences in the anxiety response.

The changes in corticosterone may be linked to damage done to the pituitary gland. rTBI has been shown to result in significant damage to the pituitary 24 hours after a repeated injury that is related to downstream hormonal abnormalities (Greco et al., 2013). This is supported by the corresponding decrease in growth hormone 1 month after repeated injuries, implying that the damage may require extended time to act on downstream systems (Greco et al., 2013). While this is not directly related to the stress axis, it does imply unspecific pituitary damage. This is also supported by findings that rTBI in female mice significantly decrease bone mass, area, density, volume, and strength, without changes in growth factors 2 weeks after the initial injury, decreases which are related to dysfunctions in pituitary-released hormones (Yu et al., 2013). Again, although the HPA axis was not examined directly, studies have linked activation of the HPA axis to dampening the effects of other energy-expending axes, including those that affect growth as mentioned above (Charmandari et al., 2005). Thus, if other endocrine axes are affected, it seems likely that the HPA axis would also be affected by injury.

Other studies have not examined the HPA axis in brain injury through either baseline or stress-induced CORT levels after closed-head concussions in a rodent model. As discussed above, that research is mostly in open-head models and have found that mild or moderate injuries increase stress-induced CORT levels even at acute time points (Griesbach et al., 2011; Taylor et al., 2008). Based on other groups who did use a concussion model, and found hormonal decreases in other axes (Greco et al., 2013; Yu et al., 2013), it is unsurprising that there was a response at this time point. However, due to the heterogeneity of the clinical data from a single injury (Tanriverdi et al., 2010), it is interesting to see a significant increase in CORT from our repeated injury group.

In the current study, we examined only baseline CORT levels, rather than their change in response to a stressor. As clinical studies have found deregulation in the hypothalamic-pituitary axes as a whole (Agha and Thompson, 2006; Dimopoulou et al., 2004; Krahulik et al., 2010), there may be important differences seen following concussion when the HPA axis is activated by a stressor rather than just in its baseline state. Traditional CCI and FPI models do cause an increase in stress-induced levels of CORT (discussed above; Griesbach et al., 2011; Taylor et al., 2008). Indeed, we predict that concussion may induce more dramatic effects when the HPA axis and CORT levels are examined in response to a stressor. We also are confident that CORT levels of single and repeat concussion groups will diverge at a more chronic time point, with repeat concussions causing a greater increase of stress-dependent CORT levels than what is seen following a single concussion. Although we did not examine CORT levels in response to a stressor in this study, we are currently examining this response in our model at a chronic time point.

In addition to an anxiety response, repeat concussions produced by our CHI model produced longer lasting memory deficits. Patients with multiple concussions can have chronic memory deficits and a higher incidence of cognitive disorders, such as Alzheimer's Disease (Lehman et al., 2012). Indeed, we found that rats with multiple concussions spent significantly less time with the novel object than shams or animals with a single concussion 7 days after their initial injury, indicating a persistent memory deficit. Although a single concussion did produce deficits in the novel object task at day 3, by day 7 they no longer showed memory deficits. Similar to our data, Prins (2010) found that juvenile rats with multiple and single concussions preform worse in the NOR task than shams.

Other groups who have studied spatial memory have found that animals with repeated concussions also do worse in these tasks and take longer to recover from these types of memory deficits (DeFord et al., 2002; Huang et al., 2013; Luo et al., 2014; Mouzon et al., 2014; Uryu et al., 2002). For example, mice with repeated injuries preform worse in the Morris Water Maze compared to both sham and animals with a single injury the first week after injury (Shitaka et al., 2011). These deficits can persist for up to 12 months following injury in animals with repeated injury but not those with a single injury (Mouzon et al., 2014). As these were done in spatial memory tasks, it will be interesting to see if our rTBI group has deficits in declarative memory through the NOR task 30 days after the initial injury. This is currently being examined.

It is interesting to note that although rats with a repeat concussion spent a significantly smaller portion of their time with the novel object compared to shams, they did spend significantly more time with the familiar object within the group. In light of the open field data, that demonstrates that these rats also exhibit anxiety, this then may represent more of an anxiety response and not a deficit in memory. Ennaceur et al. (2006) have reported that the introduction of a novel object in an enclosed environment causes an anxiety response in rats. Nevertheless, during the habituation phase of the NOR task, animals with repeat concussions spend as much time exploring the familiar objects as did sham and IsTBI groups; therefore, it is not an overall decrease in exploratory behavior. On the other hand, rTBI animals had decreased locomotor activity in the open field 5 days after their initial injury, and decreased exploratory activity has been linked to depressive-like behavior (Sousa et al., 2004). The delineation between an anxiety response and a memory deficit will need to be further explored in future studies.

Repeat concussions resulted in motor deficits, however they were not significantly greater than those seen with a single concussion. Similar to animals with a single concussion,

animals with repeat concussions were significantly hypoactive 5 days after their initial injury. Huang (2013) also found hypoactivity in animals with repeat concussions, but at a chronic time point (PID 30). Motor coordination was also decreased following repeat concussions, but not significantly different from rats with a single concussion or sham. Nevertheless, when compared just to baseline performance, animals with multiple injuries had significant deficits in motor coordination seven days after their initial injury, but there was not more dysfunction than that following a single concussion.

Other groups have also found motor deficits following repeat concussion at acute time points, using either rotarod or beam walking tasks and similar to our findings, did not show differences in performance between a single or multiple concussions (Huang et al., 2013; Hylin et al., 2013; Mouzon et al., 2012; Namjoshi et al., 2013). Some groups have continued to not see differences in motor coordination between these groups between 2 and 14 days after the initial injury (Allen et al., 2000) and up to 4 weeks after injury (Luo et al., 2014; Uryu et al., 2002). Perhaps the transient nature of these deficits means that more nuanced tests are required at earlier time points, even within hours of the injury.

The lack of a difference in motor coordination between repeat concussion animals and other groups may relate to the fact that they did show decreased locomotion. It was qualitatively observed across several experimenters that during the foot fault task, rats with repeat concussions had increased freezing behavior and increased time spent completing the task. Unfortunately, this was not measured quantitatively, but did suggest a change in the foot fault task for future studies that can capture this change in overall locomotion. Alternatively, it may be beneficial to measure motor coordination in this model in other ways such as the rotarod test or beam walking. Other groups have previously used the vermicelli handling test and single pellet test to show deficits in

forelimb coordination in response to brain injury (Adkins et al., 2015; Maldonado et al., 2008); although it is unknown whether the current concussion model would result in such fine forelimb use deficits.

In our model of CHI, neither single concussion nor repeat concussions produced skull fractures, surface damage, or gross cellular changes. This is comparable to what is seen in the clinic. Individuals with concussions do not typically present with skull cracks, or evidence of cortical pathology on MRI or CT (reviewed in (Bazarian et al., 2006). Other studies of animal models of rTBI have examined more specific markers of pathology, such as axonal shearing, phosphorylated Tau, and beta amyloid to uncover differences between single and repeat concussions. These groups generally find that repeat concussions cause greater expression of markers of pathology than single concussions (Kane et al., 2012; Mouzon et al., 2013), usually in chronic time points. Some groups however, have seen that repeat concussions produce significantly more axonal damage at acute time points (Fujita et al., 2012; Hylin et al., 2013; Prins et al., 2010). We are currently examining the tissue from the current study for these markers of pathology and predict we will find evidence of greater pathology following repeat concussions.

CONCLUSIONS

We have presented a model of single and repeat CHI using a CCI device that results in clinically relevant symptoms including deficits in memory, motor function, and emotion, with no obvious signs of skull fracture or cortical pathology. Our model uses a commercially available injury device, and materials that are easily accessible and is reproducible. It has been shown to model a single concussion as well as a repeat concussion and can be very useful to begin to

elucidate the mechanisms underlying the link between repeat concussions and long-term deficits and neurodegeneration.

EXTENDED DISCUSSION

The current project set out to create a model of closed-head injury (concussion) and to compare single and repeated injuries. We found that a controlled cortical impact (CCI) device could be used to produce an injury directly on the head that caused concussion-like symptoms such as change in locomotion, anxiety-like behavior, and memory deficits. At an acute time point (8 days), animals with multiple concussions have sustained memory deficits and an anxiety response not seen in animals with a single concussion. Furthermore, our collaborators are examining brain tissue from these animals. Preliminary data has found that animals with repeat concussions have cortical thinning and increased amounts of phosphorylated Tau compared to animals with a single concussion.

We are currently using this model to examine differences in behavior and histology between single and repeated concussions at a more chronic time point. Although there were few differences between single and repeat injuries in the current study at the acute time point, based on the literature, we predict a divergence by post-injury day 30 in behavioral tasks. As animals with a single injury recovered both motor coordination and memory by day 7 after their injury, their progress would likely stay similar to sham levels throughout the 30 days. Animals with multiple injuries, on the other hand, may continue to show these deficits and perhaps develop others. If animals with repeat concussions do recover these behavioral functions over time, it is expected that they would take longer than animals that have had only a single injury.

We are also expanding our behavioral paradigms to include other symptoms, such as depressive-like behavior in the Porsolt forced swim test. As previously mentioned, there is a strong link between TBI and mood disorders (Jorge & Arciniegas, 2014a, 2014b), which has not been explored in-depth in models of concussion. Depression and anxiety are also strongly co-

morbid (Jorge & Arciniegas, 2014a, 2014b). Thus, we predict that animals with multiple concussions will have increased depressive-like behavior compared to uninjured animals. The forced swim test, at a chronic time point, may find depressive-like behavior in animals with a single concussion. Although rats with a single concussion did not exhibit anxiety behavior in the open field at post-injury day 7, it is possible that these symptoms may manifest later. It has been shown that symptoms of emotionality after concussions manifests up to 3 months after the initial injury in humans and 1 month in mice (reviewed in Broshek et al., 2015).

To support our behavioral data, we will examine markers of cell proliferation and degeneration, accumulation of amyloid beta and phosphorylated Tau, and stereological volume data at both the chronic and this acute time point. It will be interesting to see if there is an increase in neuronal degeneration in concussions over time as there is in more severe form of TBI. Additionally, if there is cell proliferation in response to the injuries, where is it located and what becomes of the proliferating cells. Preliminary results for pTau expression show that at 8 days after the initial concussion, animals with repeated concussions have significantly more pTau in the cortex and hippocampus than animals with a single concussion in the cortex and hippocampus. Phosphorylated Tau is linked to neurodegenerative diseases such as CTE and may therefore account for some symptomatology related to memory and mood.

Insights into the molecular mechanisms of the difference between repeat and single concussions are applicable to neuroscience and biology on a larger scale. Understanding the aggregation of pTau could help us understand the aggregation of other proteins, for instance, and how the aggregation of proteins leads to pathology. Some data have suggested that aggregates may relate to a larger systemic immune response. The recent discovery of lymphatic vessels in the brain (Louveau et al., 2015) opens the possibility of whole-body effects of TBI.

Research in traumatic brain injury may seem geared towards a specific population, but TBI is a serious public health concern, affecting every race, gender, and profession. The cases of athletes with multiple concussions have received plenty of media attention, and recently, there has been growing concern with veterans who were exposed to blast waves without ever having a physical impact. However, focusing on these populations ignores other cases, such as accidents, work-related incidents, and, of seriously underrepresented concern, domestic violence. Although models of repeat concussions were developed in response to findings in professional athletes and combat veterans, their findings can apply to all of these other populations as well. Moving forward, now that our model is established, it can be used to understand repeat concussions from a mechanistic level so that possible treatments can be developed.

Modeling is integral to research in all areas of biology, but especially the etiology and treatment of human disease. Questions that cannot be answered through clinical research must, for ethical and methodological reasons, be examined in an animal. While some questions can initially start to be answered through the use of *in vitro* methods, an animal model is necessary to understand systemic, chronic, and behavioral effects, along with their interactions. For example, mice have been used to elucidate mechanisms behind cancer metastasis, use and regulation of specific genes, and identifications of other disease-related mutations (Rosenthal and Brown, 2007). Animal models allow researchers to test drugs before clinical trials, including those for mental health problems such as depression. Models in non-human primates have led to the development of Deep Brain Stimulation for the treatment of neurological disease, work towards an HIV/AIDS vaccine, and new treatments using stem cells and gene transfer (Capitanio and Emborg, 2008). Pavlov famously used dogs to understand classical conditioning (Dickinson and

Mackintosh, 1978). Pasteur tested the first rabies vaccine in rabbits before using it in humans (Hendriksen, 1996).

Animal modeling has been integral to understanding and treating human disease. Having an accepted model of single and repeat concussions will help elucidate the sequale after injury, with which we can move towards prevention and treatment measurements. Our study was designed to be reproducible by other labs without large difficulty or arduous set up. It is our hope that our model continues to demonstrate clinical relevance for these purposes.

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