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Sex Differences in Behavioral Responses to Repeat Subconcussive Events

A Thesis Presented in Partial Fulfillment for the Degree of Master of Science

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Advisor: Dr. Dorothy Kozlowski

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Chicago, IL

Table of Contents

I. List of Tables and Figures

a. Tables

b. Figures

II. Acknowledgements

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III. Abstract

Although concussions, especially those in athletes and military, have become a popular focus of neurotrauma research, subconcussions occur with higher frequency and are less well-studied. A subconcussion is loosely defined as an impact to the head that does not result in a diagnosable concussion but can result in neuronal alterations. Repeat subconcussions have been shown to produce behavioral impairments along with neuropathology that is similar to or worse than those seen in a single concussion injury. These studies have primarily included male subjects. Given the potential effects of hormones and NIH's call for sex-inclusion in biomedical research, assessing female responses to injury is essential. The current study was designed to model repeat subconcussions in the adult rat and examine sex differences in behavioral responses to injury. Using a model of closed head injury previously created in our lab, this study modified the intensity of the impact to create a subconcussive impact. All rats received a single concussion, single subconcussion, repeat subconcussion (five impacts, 24-hours apart), or sham (no impact) injury. The repeat subconcussive injury was patterned following preliminary data from our lab. Female rats received the first impact on the day of proestrus, when estrogen concentrations peak during the estrous cycle. Behavioral tests were administered two hours post impact through 31 days post-injury. All animals with a single concussion or repeat subconcussion showed deficits in locomotion, righting reflexes, and recognition memory, while animals with a single subconcussion did not. Repeat subconcussions produced deficits similar to a single concussion in righting reflex and recognition memory, but locomotor deficits were greater in rats with repeat subconcussions. When assessing sex differences in the behavioral responses to the repeat subconcussive model, female rats showed greater deficits than males in righting reflexes, locomotion, and vestibular function. Males showed greater increases in anxiety-like behaviors than the females. This study established a model of subconcussive impact where a single subconcussive impact resulted in little to no behavioral deficits but repeat subconcussive impacts resulted in deficits that are similar to or worse than a single concussion. Our data also suggest that females may experience more deleterious effects in certain outcomes following both concussive and subconcussive impacts, which supports some clinical findings. Further experiments will need to be done to examine sex differences in the neuropathology.

IV. Introduction

Traumatic Brain Injury (TBI) is one of the leading causes of deaths from injury and contributes to 42 deaths in the United States each day (Faul et al., 2010). Concussions are a form of mild TBI (mTBI) that result in a variety of symptoms such as loss of consciousness (LOS), memory-loss, confusion, lack of spatial awareness, and changes in the brain's neurochemistry (Petraglia et al., 2014). Many people with a single mTBI are able to recover, but repeat concussions have been shown to leave long-lasting neurological deficits (Gavett et al., 2011). Many studies have linked repeat concussions to neurodegenerative disorders, such as Alzheimer's disease and Chronic Traumatic Encephalopathy, especially in military personnel and athletes (Agha et al., 2004, Gavett et al., 2011, Lehman et al., 2012). The mechanistic link between repeat concussions and long-term behavioral and physiological consequences is not completely understood but has become a focus of study for many (DeFord et al., 2002). Recent studies have established that a hit to the head that does not result in clinically diagnosed concussion, known as a subconcussive event, can cause similar neuronal changes if experienced repeatedly (Bailes et al., 2013; Koerte et al., 2015).

Due to the infancy of research in repetitive subconcussions, an animal model has not been established. A goal of creating the animal model is to allow for deeper exploration of mechanisms and physiological responses that underlie important features of subconcussive events in humans. There are animal models in place for repeat concussions (Jamnia et al., 2017), but not yet for repeat subconcussions. Also, most animal models and studies examining concussions only include male rodents, which does not allow for a complete understanding of the injuries, as there may be differences in responses between males and females (Eliot and Richardson, 2016). The focus of this thesis was to validate an animal model for repeat subconcussive injury in the adult rat and examine whether sex differences in behavioral responses occur after repeat subconcussion.

V. Literature Review

a. Traumatic Brain Injury (TBI)

Traumatic Brain injury (TBI) is defined as an external impact to the head causing an interruption to the normal function of the brain (Faul et al., 2010). It can cause lasting effects including impaired thinking, memory, movement, and cognition. Traumatic brain injury is traditionally believed to involve both primary and secondary injury phases. The primary injury phase is produced by the mechanics in the moment of impact, and secondary injury phase is classically described as the indirect result of the trauma and its subsequent pathophysiological processes (Dashnaw et al., 2012). The range of severity runs from mild to severe; from a brief change in mental cognition to an extended period of memory loss or unconsciousness (Faul et al., 2010). Concussions are a form of TBI that are typically classified as mild that can be caused by multiple events, including but not limited to sport-related injuries, combat injuries, motor vehicle injuries, violence, and falls (Faul et al., 2010).

Concussions are one of the most common brain injuries and have therefore been a focus of recent neurological studies. Because most concussion survivors are young adults with normal life-expectancy, the implications of undiagnosed post-TBI dysfunction have rarely been examined (Agha et al., 2004). Though most patients with a concussion can recover within weeks, there are some that have severe, prolonged cognitive defects that result in recovery taking months or years (Tang et al., 1997). Right now, clinical treatment options are limited due to the injury to neural structures that occurs, causing irreversible physiological damage (Shin et al., 2015).

i. Behavioral Deficits Following Concussion

Concussions can result in neuropathological changes, which in turn reflect functional deficits. These differences can include irritability, cognitive impairment, loss of memory, and emotional symptoms (McCrory et al., 2013). Although single mild brain injury has not shown significant long-term changes to cognition and behavior, multiple impacts have shown to result in significant disturbances (DeFord et al., 2002). Repeated mild concussive injuries and repeated subconcussive injuries cause cognitive damage altering complex/spatial learning, vestibular function, motor coordination and anxiety levels (Creeley et al., 2004; Dashnaw et al., 2012). Each of these changes alters performance after brain injury and overall quality of life.

Memory deficits are a common occurrence post-brain injury and can be present in multiple types of memory, such as working memory and long-term memory. Repeat concussions have been shown to cause a much higher risk for memory problems; up to 3 times more likely in NFL players with three or more concussions (Lehman et al., 2012). Two common forms of memory tested are spatial memory and recognition memory, also known as working memory; both of which have been shown to decrease post-injury (Creed et al., 2011). Spatial learning and memory is tested in rodent models with a Morris Water Maze, demonstrating lower levels of spatial learning in injured mice compared to controls (Creeley et al., 2004). The current study did not examine spatial memory, but instead measured recognition memory after repetitive subconcussive injury.

Recognition memory, also called working memory, is defined as maintaining information in limited, temporary storage while cognitively manipulating the information. It is reliant on the prefrontal cortex, which is often selectively damaged by TBI. Due to its position at the front of the brain, it is often injured in impacts to the front of the head. Traumatic brain injury patients have shown altered patterns of cerebral activation when performing working memory tasks (Christodoulou et al., 2001). This suggests that in traumatic brain injury, alternate areas of the brain are recruited for working memory once the primary areas responsible are injured, but this does not make up for loss of memory overall. The changes to recognition memory post-injury have been studied for decades and have shown that in cases of severe brain injury, recognition memory is reduced in injured patients compared to controls (Vakil, 2005). The current study examined recognition memory using the novel recognition test in adult rats. The novel-recognition test uses the natural curiosity of the rat to test working memory by measuring the amount of time it spends with a new object compared to an object that had previously introduced to the rat. Not only does it allow for a one-trial test that doesn't require learning a rule, it also provides comparisons to other studies in alternate species including primates who do similar tests for working memory (Ennaceur and Delacour, 1988).

Motor function is altered post-TBI in patients and rodent models. It is often measured by tests of motor coordination, locomotion, and righting reflexes. After single concussion, rats have shown more motor deficits than control rats in the form of decreased motor coordination and decreased amount of motor activity (Jamnia et al., 2017). Locomotion changes after concussion have shown slower movements, but not differences in path length, suggesting that impacts cause subtle motor disturbances that alter endurance (Creeley et al., 2004). Similarly, in a study done by Huang et al. (2013) a rat model of repeat mild TBI showed decreased locomotion and significantly decreased exploratory behavior. In the current study we measured motor coordination with the foot fault test and balance beam, locomotion with the balance beam and open-field test, and righting-reflexes with the air-righting reflex test.

One of the psychological post-concussion symptoms seen in patients is anxiety. Anxiety is a psychiatric disorder that is complex at the behavioral, neural, and genetic levels (Lipkind et al., 2004). The symptoms seen in patients most commonly include a combination of anxiety, fearfulness, generalized uneasiness, and severe worrying (Moore et al., 2006). In a study done in 48 patients with mild head injury, it was shown that there was no significant change in anxiety levels between normal patients and injured patients (Schoenhuber and Gentilini, 1988). That said, a more recent study of 63 athletes from Big Ten schools that received a brain injury were shown to have increased anxiety post-injury. Although they measured anxiety using a state-trait self-reported questionnaire, they showed that anxiety levels were higher in concussion groups and was affected by support received from their family and team along with the prognosis of the injury (Covassin et al., 2012). This suggests that in humans, some anxiety could stem from an acquired psychological response of the athlete to the injury as opposed to just from the injury itself. Overall there is a history of research showing higher anxiety levels in athletes that are injured compared to their uninjured cohort, specifically in those following severe injury, preventing the athlete from returning to the sport immediately post-injury (Leddy et al., 1994). That said, when comparing injuries to the upper and lower extremities to concussive injuries a higher percentage of athletes with concussions showed mental health conditions compared to those with the other injuries (Sarac et al., 2018). The current study assessed anxiety-like symptoms using a forced swim and open field task.

ii. Physiological Damage Following Concussion

Concussions have been associated with physiological changes including low-grade neuroinflammation, increased astrocytic and microglial response, and diffuse axonal injury (Velosky et al., 2017). One key aspect about these changes in concussions are that they are very mild changes that are often difficult to see but are intensified in cases of repeat injury (Jamnia et al., 2017). When it comes to physiological damage, alterations can occur in the absence of behavioral changes (Bailes et al., 2013).

Microglia play a key role in the brain initiating inflammatory events following traumatic brain injury (Homsi et al., 2010). Microglia are the innate immune cells found in the grey matter, which activate post-injury for cellular maintenance and innate immunity. They enforce programmed elimination of neural cells, release anti-inflammatory factors, and guide stem cells to the injury site (Block et al., 2007; Dashnaw et al., 2012). The inflammatory response created is thought to contribute to motor and cognitive deficits, such as balance deficits and loss of recognition memory, that go beyond the initial damage caused by the primary injury. Therefore, microglial activation has begun to be used as a biomarker to examine the extent of brain injury. (Caplan et al., 2017).

Similar to microglia, astrocytes are glial cells that are activated after injury to the central nervous system (CNS). Astrogliosis, also known as reactive gliosis, is the increase of astrocytes in response to injury and infection. One function of this gliosis is thought to seal a blood-brain barrier that has been impacted by the injury (Barres, 2008). Astrocytes react to traumatic brain injury by altering gene expression, cell proliferation and cellular hypertrophy. In studies done in mice that were injected with an antiviral agent that ablates proliferating reactive astrocytes, the injected mice post-moderate and severe brain injury saw significant neural degeneration, inflammation, and after moderate injury saw a 42% increase in cortical tissue loss at the site of the injury compared to mice with normal astrocyte counts. This analysis suggests that astrocytes play important roles in reducing inflammation and protecting neural tissue from loss after brain injury (Myer et al., 2006).

Axonal injury is also a physiological response seen post-TBI in both human patients and rodent models. In a study done by Creed et al. (2011) they saw axonal degeneration in mice with a closed head concussive brain injury, which was connected with decreased action potential conduction in myelinated axons along with decreased axonal transport. Axonal injury was also connected to decreased fractional anisotropy, which is a measure of directional diffusivity, in white matter tracts. Rodent models of mild TBI have shown a significant number of damaged and sheared axons (Bailes et al., 2013), and an increased damage to the brain results in an increase in the amount of cell death observed (Jamnia et al., 2017).

b. Repeat Concussions

Milder traumatic brain injuries do not usually produce lethal prognosis, but they do account for an estimated 75-90% of traumatic brain injuries that occur, making them a worthy area of study (CDC, 2003). Due to being common in sport-related activities and military personnel, repeat concussions are being studied more frequently. In the early 1900's the first reported clinical cases of repeat concussions had been seen in boxers, and the resulting neurodegeneration seen was called "punch drunk syndrome" (Martland, 1928). This neurodegenerative disease was first linked to repeat hits in football by Dr. Bennet Omalu in a study assessing neurodegeneration in a retired football player (Omalu et al., 2005). Since 2005 there have been multiple studies further elucidating the neurodegenerative disease called Chronic Traumatic Encephalopathy (CTE) that occurs later in life after repetitive head trauma (McKee et al., 2009; Gavett et al., 2011). CTE is a progressive neurodegenerative disease that is known for widespread hyperphosphorylated tau in neurofibrillary tangles in specific patterns around sulci of the brain (McKee et al., 2013). Other neurodegenerative disorders and traumatic brain injury have been connected in multiple cases. Lehman et al., (2012), reported a neurodegenerative mortality in football players three times higher than general United States population which suggested that football players and other athletes have an increased risk of neurodegenerative diseases, such as Alzheimer's disease and Amyotrophic Lateral Sclerosis. The link to these diseases occurring post-TBI resulted in an increased interest in studying repeat concussion.

Repeat mild brain injury (rTBI) in humans can have both short- and long-term effects (DeFord et al., 2002). Repeat injury can create greater behavioral and physiological changes than the initial injury alone. In patients with mild repetitive brain injury there was increased lesion volume, increased cortical tissue damage, and increased microglial activation compared to single concussion and controls (Huang et al., 2013). Repeat mild brain injury in rats caused decreases in the cerebral metabolic rate of glucose and the group that received a second injury with a shorter time gap to the initial injury showed more significant changes in cerebral metabolic rate of glucose (Prins et al., 2013). Also, in rats age-matched to children and young adults, those that received repeat brain injury 24 hours after the initial injury showed increased axonal injury, increased astrocyte response, and increased recognition memory impairment. This further suggests additive effects of repeat brain injury (Prins et al., 2010). Mildly impacted brains were shown to be more vulnerable to repetitive injury after the initial injury. In a prospective cohort study by Guskiewicz et al., (2005) they found that with each successive injury in college football player, the risk for future concussive injuries increased. Not only that but after 3 concussions there was a 3-fold greater risk compared to players with no concussions.

Though mainly clinically studied, there are multiple animal studies emerging which model repeat concussion. Animal models have contributed greatly to the knowledge about traumatic brain injury; although the brain anatomy is not anatomically identical, it provides great insight to brain injury in humans (Dashnaw et al., 2012). In animal models there is the ability to control for outside variables and effectors that is impossible in humans. It is also possible to look at genetic mutations more easily in animal models. In addition, the ability to look at cellular responses to injury is much easier in an animal model as opposed to a clinical model. Modeling human concussions in a laboratory with rodents is challenging, but considerable effort has been made to create an animal model (Tang et al., 1997). There are multiple ways that have previously attempted to create animal models for concussion including fluid percussion methods (Dixon et al., 1987) and weight-drop methods (Tang et al., 1997; DeFord et al., 2002; Creeley et al., 2004), but each of these methods have their faults in properly modeling concussions. The fluid percussion model and weight drop models provides injury via fluid pressure and mechanical force directly on the brain parenchyma, which require craniotomies and therefore have more variables involved than a clinical concussion. One of the current models of concussion developed in our laboratory is a closed-head cortical impact, which has been able to provide clinically relevant markers of concussion including increased axonal shearing, deficits in motor coordination, and decreased locomotion, and allows for differentiation between single and repeat concussions early on after injury (Jamnia et al., 2017). In this model of concussion, the rat is placed on a foam pad to allow for free head movement and receives an impact to the surface of the head while the shoulders and body is restrained from movement. The impact is done on the surface of the head with no opening of the scalp or skull to allow for more clinical comparison. This model was modified and was used to administer traumatic brain injury to the rats in the current study.

c. Repeat Subconcussions

A “subconcussion” is defined as an impact to the head that does not result in overt clinical symptoms, but can manifest neuronal effects similar to those following a concussion (Bailes et at., 2013). It can cause a cascade of events in cells in the brain to cause similar behavioral and physiological neuronal changes as a concussion; similarly, repetitive subconcussions have been shown to cause damage equal to that of a single concussion (Dashnaw et al., 2012). Head impacts commonly occur multiple times during a game, or in combat, during which time symptoms may not develop to allow visible signs of neurological dysfunction (Bailes et al., 2013). A study by Koerte et al. (2015) showed that, in former professional soccer players who had a history of “heading” the ball, but did not have a history of diagnosed concussion, there was still evidence of biomarkers for neuroinflammation and neurodegeneration. This was further supported by the findings of Talvage et al. (2014) in high school football players. In this study, players who exhibited no-clinically observed concussion symptoms, but had a high number of head collision events showed decreased working memory and altered activation in the prefrontal cortex. Also, in college football players where none experienced a clinically evident concussion, there were significant white matter changes that persisted to 6-months post-TBI (Bazarian et al., 2014). In a recent study done assessing Chronic Traumatic Encephalopathy (CTE) after closed-head impacts that did not show signs of a clinical concussion, it was found that even subconcussive injuries can accrue to cause a neurodegenerative disease such as CTE (Tagge et al., 2018). Currently there is no method of predicting the effects of injury based on biomechanics of head impacts or previous injuries, so each case is evaluated individually (Broglio et al., 2011).

d. Sex Differences

There is no question that sex-related variables are of valid concern in all scientific study. In 2010 the National Institute of Health (NIH) announced a plan to advance women's health and research on women. This plan has three goals: (1) increase sex differences research in basic science, (2) incorporate findings of that research into development of new technology, and (3) establish methods of prevention, diagnostics, and therapeutics for girls and women (Pinn et al., 2010). In 2014 the NIH augmented a new policy that requires the balance of male and female animals in preclinical studies unless a previously defined exception allows for it (Clayton and Collins, 2014). Studies including sex differences are becoming more prevalent but there is still a lot of ground to cover.

Recent studies have been looking at the difference between males and females in response to traumatic brain injury (Eliot and Richardson, 2016; Velosky et al., 2017). There is an assumption that a sex difference in neurophysiology means that there is a sex difference in behavior. Rather than assuming, more work should use the connection between the sex and behavior as a hypothesis for testing (McCarthy, 2016).

i. Sexual Dimorphism

Differences in males and females that affects an individual's health and response to injury and disease begin to appear early in life and continue across the lifespan. Sexual dimorphism begins early in development as a result of genetic and hormonal events, and can manifest as physiological differences that may account for disease differences. These differences include females having a higher percentage body fat, higher cerebral blood flow, lower body weight, and less blood volume (Becker et al., 2005). Beyond contrasting overall anatomy, there are differences between male and female brains and their functioning. In one of the first well recognized animal studies that observed canaries and zebra finches, it was discovered that there were dramatic sex differences in the brains of males and females, specifically, in the three vocal control portions of the brain that correlated to the differences in singing behaviors (Nottebohm and Arnold, 1976). The most robust behavioral sex differences, in memory, motor functioning, and cognition in humans can be directly related to findings in animals that involved either hormonal or genetic manipulation (McCarthy, 2016).

ii. Hormones

Hormones are commonly investigated as a cause of sex differences due to the different hormones and hormone levels in males and females. Testosterone is the dominant sex hormone in males, while estrogen and progesterone are dominant in women. (Becker et al., 2005). Female mammals go through cycles of internal hormonal concentrations to aid in reproduction, while there is no concrete evidence that males have hormonal cycles. In a study done using patients with mild TBI a connection was found between menstrual cycle phase and outcomes of TBI. In patients whose TBI occurred in the luteal phase, when progesterone and estrogen spike, there were more symptoms reported and lower overall health scores compared to those in the follicular phase and synthetic progestin group (Wunderle at al., 2013). Similar to the menstrual cycle in humans, female rats see a hormone fluctuation that occurs during the 4 to 5 day long estrous cycle which has 4 separate portions: proestrus, estrus, metestrus, and diestrus (Figure 1). It was found that the stage of estrous cycle affected mice in anxiety-like behaviors, balance, and reflex responses (Meziane et al., 2007). A female's reproductive status and ovarian cycle must be considered to study sex differences in TBI, similar to previous studies looking at sex differences in disease incidence, manifestation, and prognosis (Becker et al., 2005). Female rodents have shown protection from TBI in the proestrus phase, when estrogen levels are high (Engler-Chiurassi et al., 2015). Similarly, in studies done by Shanksky et al., (2004) there was a distinctive difference between males and females in the cognitive responses to stress that suggested a connection to the sex hormone's capability of modulating factors in stress-induced prefrontal cortex dysfunction.

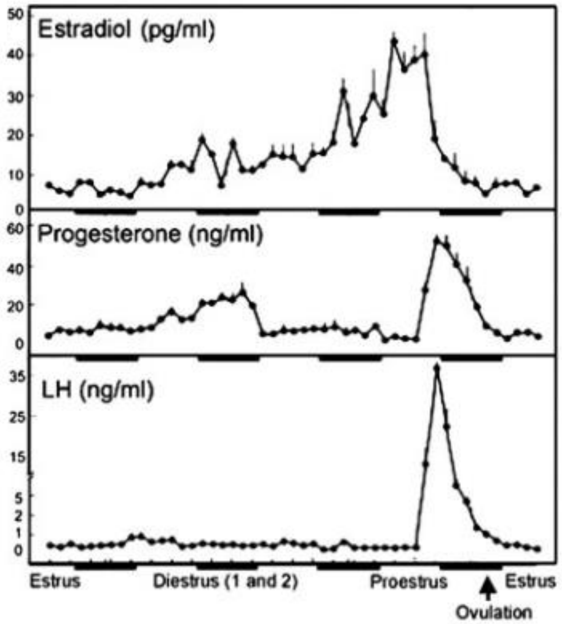


Figure 1: Levels of hormones at each portion of the estrous cycle of a female rat, taken from Smith et al. (1975).

iii. Estrogen and Progesterone

Estrogens are steroid hormones that are synthesized from cholesterol through multiple chemical reactions in the ovaries after the ovaries are signaled by the anterior pituitary to increase estrogen concentrations. Although mainly found in females, there is a smaller concentration of estrogen found in males as well. These sex hormones affect more than just reproductive hormones and can travel to alternate effector targets in the body including the central nervous system. Estrogen is important for many reasons including regulating skeletal homeostasis, metabolism of carbohydrates and lipids, and cardiovascular health (Engler-Chiurazzi et al., 2016). The estrogens travel through the blood to bind receptors in target tissues. There are three main estrogen receptors: Estrogen Receptor-α (ERα), Estrogen Receptor-β (ERβ), and G protein-coupled estrogen receptor 1 (GPER1). ERα and ERβ alter transcription and translation of DNA directly and indirectly by binding to estrogen response elements at gene promotors after estrogen binds. GPER1 is found on the membrane and is used for more rapid actions that do not alter gene expression, such as activation of protein-kinase cascades. There are three main types of circulating estrogens: estrone (E1), 17β-estradiol (E2), and estriol (E3), all which play roles as neurobiological ligands (Vrtacnik et al., 2014).

Progesterone is a steroid hormone with a similar synthesis process to estrogen, but is a precursor to estrogen. It exists in male and female brains and similar to estrogen, influences more than just reproduction. It has known neuroprotective effects including protecting the blood-brain barrier, decreasing inflammation through inhibition of inflammatory cytokines, and decreasing cellular apoptosis (Skolnick et al., 2014). When administered post-severe TBI, it was shown to decrease cell proliferation in the hippocampus, reduce number of immature neurons, and decrease cell death in dentate gyrus, where neurogenesis occurs. (Barha et al., 2011). Although progesterone has been used in potential treatments for patients with severe TBI, a study done by Skolnick et al. (2014) showed no clinical benefit of administration of progesterone to patients with severe TBI in two phase 3 clinical trials.

Estrogen has been identified as an important hormone for correct neurological functioning. Estrogen has anti-inflammatory actions and increases plasticity in the central nervous system, both forms of neuroprotection, which can impact cognitive function (Engler-Chiurazzi et al., 2016). More specifically 17β-estradiol has been shown to influence not only neurodegeneration, but also memory formation, cognition, mood, and motor coordination (Fiochetti et al., 2012). The connection to estrogen and neuroprotection has been suspected and studied for over a decade, but it was not until recently that the mechanisms were understood.

iv. Estrogen Mechanisms

There are two ways that 17β-estradiol affects cellular apoptosis pathways (Figure 2). One pathway involves a neuron's exposure to oxidative stress which induces the release of cytochrome c (cyt c), which regulates activation of apoptosis-protease activating factor-1, which follows a pathway to apoptosis. 17β-estradiol binds estrogen receptors that induce transcription of neuroglobin (Ngb) that facilitates a connection with cyt c to prevent it from initiating the pathway to apoptosis (Fiochetti et al., 2012). The other pathway includes estrogen inhibiting signaling pathways that go through mitochondrial signaling of apoptosis, such as p53 and MAPK, that lead to Bax and p53-upregulated modulator of apoptosis (PUMA) (Numakawa et al., 2011). Each of these pathways show how estrogen can effect damage and death of neurons induced from traumatic brain injury.

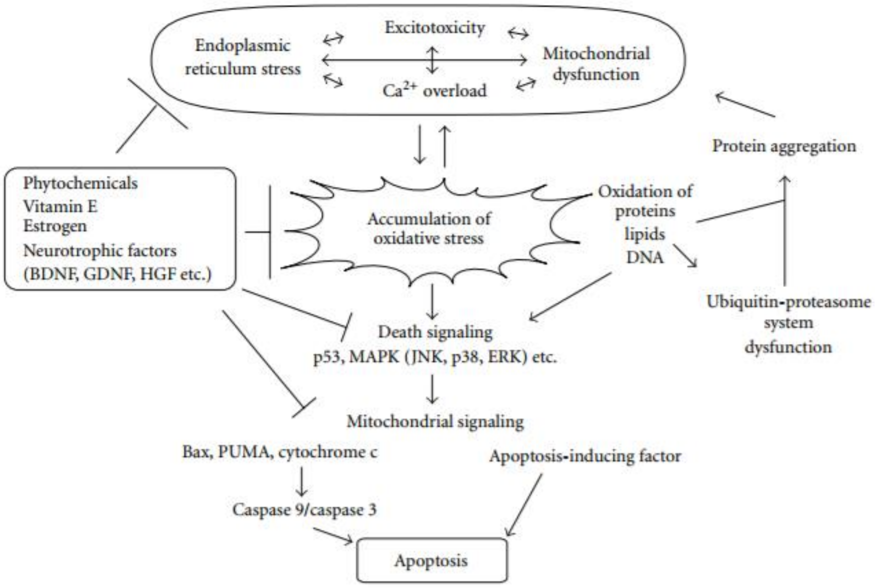


Figure 2: Pathways of E2 preventing apoptosis (Taken from Numakawa et al., 2011)

v. Hypopituitarism Following TBI

As seen above there is significant potential for estrogen as a neuroprotective agent, but females are still seeing symptoms worse than males post traumatic brain injury (Bazarian et al., 2010). There have been studies suggesting that hypopituitarism following brain injury could be the cause of the increased severity and longevity of symptomology in females. Hypopituitarism was found in 40% of patients with head injury at differing degrees and one of the most common deficiencies was in gonadotrophins (Kelly et al., 2000). Since that study there have been more cases where hypopituitarism has been found post-brain injury. Similar data from Wagner et al., revealed 43% of female patients showed low estradiol levels and at least 77% showed a reduction in three hormone levels, which persisted until post-injury day 9 when the observation period was concluded (2010). In this same study they assessed patients across an age range of 14 years to 80 years, and the reductions in gonadotrophins were not significantly different between age groups, suggesting that brain injury at any age could be followed by symptomology worsened due to hypopituitarism (Walker and Tesco, 2013). The anterior pituitary gland produces peptide hormones that act peripherally on target organs and in this case the pituitary releases luteinizing hormone (LH) and follicle stimulating hormone (FSH) which act on the gonads (Figure 3). Release of estrogen and testosterone from the gonads requires this interaction from the hormones released from the anterior pituitary; suggesting that alterations in the normal functioning of the pituitary which reduce release of LH and FSH can cause the reduction in estrogen, which ultimately decreases the neuroprotection (Schneider et al., 2007).

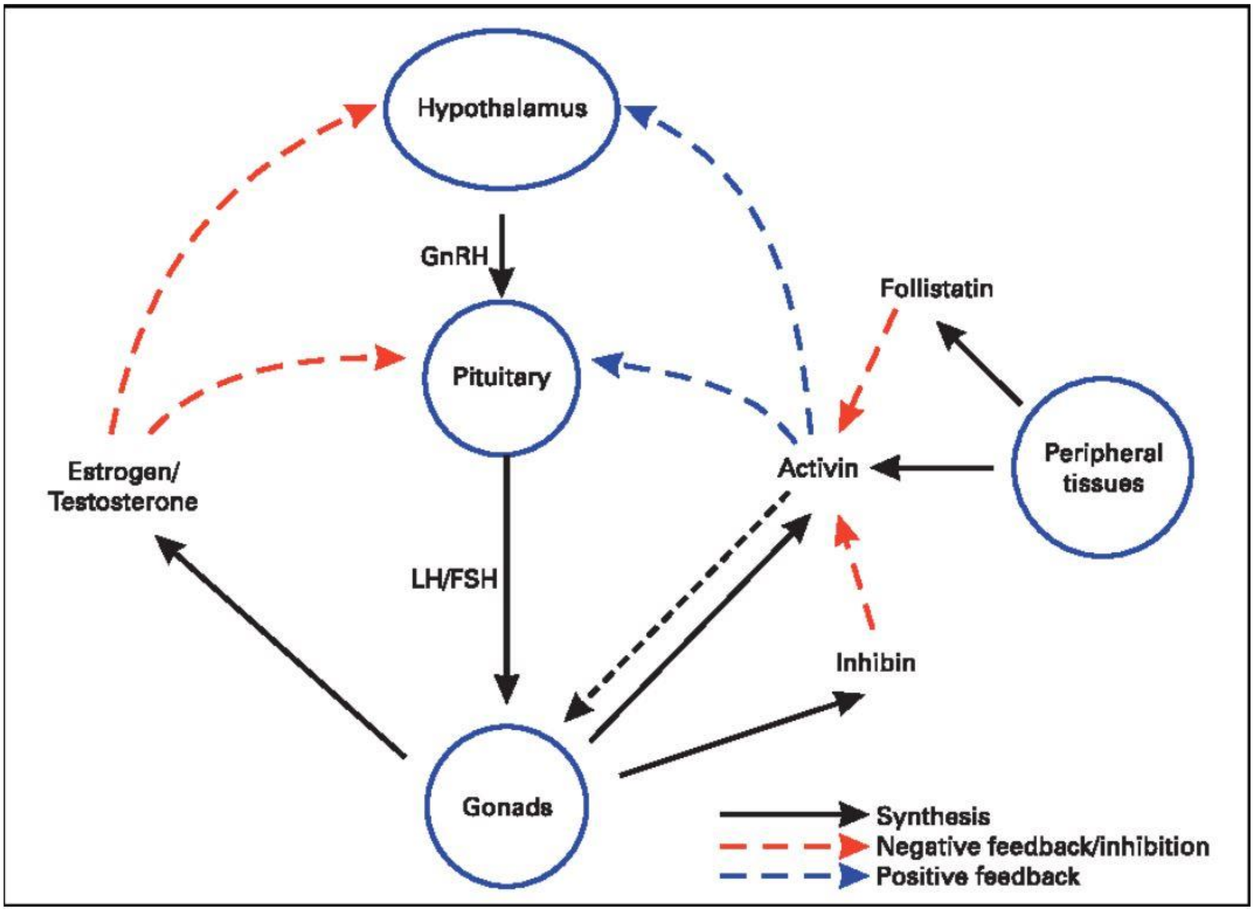


Figure 3: HPG (Hypothalamic-Pituitary-Gonadal) axis as previously established in Bowen et al. (2004). The HPG axis connects the peptide hormones released by the Pituitary (LH and FSH) to the gonads and the hormones released from those organs (estrogen and testosterone).

There are two forms of pituitary dysfunction that can lead to hypopituitarism: functional alternation of the pituitary in the acute phase resulting in changes in hormone concentration or alterations in the hormone secretion that can occur at both acute and chronic time-points (Bondanelli et al., 2005). Two suggestions for the mechanism of these disruptions are either the mechanical disruption of the pituitary gland directly from the impact of the injury, or an interruption of the blood supply to the pituitary gland via compression of the portal veins (Agha et al., 2007).

The first mechanism, mechanical disruption of pituitary gland, is one of the more commonly theorized mechanisms of hypopituitarism post-TBI. The anterior pituitary could be injured due to primary or secondary portions of the brain impact. The stretching and shearing of the brain at the hypothalamic-pituitary axis during the impact could result in the direct injury of the pituitary gland. There is also the possibility that the secondary effects of the injury such as hypotension and brain swelling, could be the cause of injury to the pituitary gland or portal veins in the pituitary stalk (Dusick et al., 2012). Although it could be a combination of primary and secondary injuries, there is evidence that it is mainly due to the secondary injuries after the impact. For example, a study of severe traumatic brain injury found a correlation of increased cranial pressure and swelling to hypopituitarism (Klose et al., 2007). Similarly, a study by Schneider et al., found that axonal injury and other markers of traumatic brain injury to be predictive of hypopituitarism (2008). Although more work needs to be done in this field, there is more evidence for mechanical disruption of the pituitary gland as being the cause of hypopituitarism after traumatic brain injury.

Compression of the portal veins is another possible mechanism of hypopituitarism, and would greatly affect the anterior lobe, which is responsible for releasing gonadotrophins, luteinizing hormone and follicle-stimulating hormone. These gonadotrophs then go to the gonads to initiate the production of mostly estrogen and progesterone in females and mostly testosterone in males from cholesterol (Resch et al., 2017). The portal veins supply the anterior lobe with 70 to 90% of its blood supply, so any alteration of these could drastically alter the anterior lobe’s ability to function.

vi. TBI and Sex Differences

Males and females have been shown to respond differently to TBI. A study done by Russell et al., (2011) showed no significant difference in sensorimotor behavior of juvenile male and female rats post-traumatic brain injury, further suggesting that sex hormones that are found post-puberty may be the causative agents of sex differences. The physiological changes post-TBI are different in males and females as well. In repeat concussions, female mice showed reduced cognitive impairment and reduced reactive astroglial cells (Velosky et al., 2017). After TBI, female rats have had less cortical neuron loss and less contusion volume compared to male rats and ovariectomized females (Bramlett and Dietrich, 2001). In a study done by Gunther et al. (2015) they found that after mild brain injury, males rats had a higher COX-2 response compared with female rats, showing that male rats had an increased inflammatory response and microglial response. COX-2, is a proinflammatory enzyme that catalyzes the first step of synthesis of arachidonic acid derivatives leading to neuroinflammation and both the mRNA levels and the protein levels of COX-2 can be studied to assess inflammatory responses. Furthermore, when male rats were injected with 17β-estradiol before a TBI they showed improvement in both motor function and physiological damage, while females injected with estrogen showed a decreased neurological response; this suggests a threshold of estrogen receptor response to 17β-estradiol post-injury (Emerson et al., 1993). In a large number of additional studies, females have shown higher levels of deficit than males. In a study of mild traumatic brain injury, female rats showed a significantly higher deficit in behavioral tasks including recovery, righting, activity, and spatial memory; suggesting that estrogen could be reduced after injury if there are no neuroprotectant effects (Wirth et al., 2017). Similarly, another study in rodent models of mild traumatic brain injury showed a distinct deficit in females in motor coordination without showing as intense of a deficit in males. On the rotarod, females had a significantly shorter latency to fall off, suggesting that they suffered from more intense motor coordination and balance deficits than the males (Tucker et al., 2016). These sex differences in rodent models need to be assessed more in future studies to gain a full understanding as to the mechanism of the sex differences present. In many of the studies mentioned, the injuries were done on random days of the estrous cycle and were not designated to a specific day, which makes it difficult to assess the possible hormonal effects on traumatic brain injury. Currently there is no study published that assessed sex differences in an animal model of subconcussion. Therefore, this study examined the sex differences in a rodent model of repeat subconcussive injury.

Sex differences are not just present in rodent models. When playing the same sport, women have shown a higher amount of concussions than male players that are in the same position on the field in soccer, basketball, and lacrosse. Concussions are also a greater proportion of injury in female subjects than male subjects, in both high school and college levels (Marar et al., 2012). That said, gender and cause of injury being violent or non-violent were found to be the most significant factors in executive functioning following TBI, with female patients having higher scores in tests of executive function (Niemeier et al., 2007). Additionally, female patients in a post-acute injury rehabilitation center showed significantly higher scores on cognitive tests than male patients that were controlled for age difference and time since injury (Niemeier et al., 2013). In a study by Berry et al. (2009), female patients showed significantly fewer complications and shorter intensive care unit stays after moderate to severe TBI than male patients. However, Bazarian et al. (2010) showed there is a show of slightly higher post-concussion symptoms in female patients after 30 days than male patients, even though they don't show much difference in the time taken to return to normal activity after a mTBI. In soccer players, a study was done comparing head-neck segment mass and neck muscle strength to head-impact kinematics for both male and female players. They found that female players have a lower segment mass and lower muscle strength, which causes greater head impact kinetics when heading a soccer ball. This suggests that sex differences in subconcussive impacts may be caused by physiological differences altering the kinematics of injury (Bretzin et al., 2017).

Currently there is a large amount of non-reported concussions and mild brain injuries that can cause more extensive symptoms in the future. In a study done on female high school athletes, approximately 50% reported having had experienced a concussion from sport-related activities, but 10 out of 31 did not report their concussion to a coach or parent and only 66% had received previous concussion education (McDonald et al., 2016). That said, female players were shown to be more likely to report their concussions than male players, which can cause bias in clinical studies of sport-related concussion (Marar et al., 2012).

Further study is needed to clarify the response to subconcussive events in females compared to males. One of the focuses of this study is to look at the behavioral response to repeat subconcussive events during the highest level of estrogen to determine if there are any sex differences. If sex differences do exist, that knowledge can be further used to increase subconcussion education along with promoting the establishment of better-fit treatments for patients post-injury.

VI. Hypothesis: Experiment 1

The goal of experiment one of this thesis was to finalize the establishment of a clinically relevant closed head model of a repeat subconcussive injury in the adult rat. This was based on a closed-head cortical impact brain injury model created in the lab to model concussions.

Due to the infancy of research in repetitive subconcussions, an animal model has not been established. A goal of creating the animal model is to allow for deeper exploration of mechanisms and physiological responses that underlie important features of subconcussive events in humans. There are animal models in place for repeat concussions (Jamnia et al., 2017), but not yet for repetitive subconcussions. The goal of this part of the project was to finalize an adult rat model of a repeat subconcussive event.

VII. Hypothesis: Experiment 2

The goal of the second experiment is to examine sex differences in the behavioral response to repeat subconcussion. Using the model of repeat subconcussive injuries established in part one of this thesis, the responses of female and male rats to repeat subconcussion with be examined and compared.

VIII. Methods

a. Experimental Design

i. Experiment One: Animals

The animals being used in this study included adult male Hooded Long-Evans rats obtained from Charles River Laboratory. All rats were kept housed two to a cage within DePaul University Animal Facility with food and water available *ad libitum* on a 12:12 hour light/dark cycle. All experiments were approved by the DePaul Institutional Animal Care and Use Committee and were conducted in accordance with rules set by the National Institutes of Health Guide for the Care and Use of Animals.

ii. Experiment One: Experimental Design

Rats were randomly assigned into treatment and control groups including: male sham control group (no injury), male single concussion(sTBI), male single subconcussive injury (sTBIsc), and male 5 repeat subconcussive injuries – 24h apart (rTBIsc; See Table 1). Following injury each behavioral test was performed at a 2h time point after injury along with multiple timepoints on a 30-day timeline shown below. On day 31 the rats were sacrificed and the brains were preserved.

Sex	Impact	# of Rats
Male	Sham (No Impact)	8
Male	Repeat Subconcussion (rTBIsc)	9
Male	Single Concussion (sTBI)	9
Male	Single Subconcussion (sTBIsc)	8

Table 1: Experiment One experimental and Control group sample sizes.

iii. Experiment Two: Animals

The animals being used in experiment two of this thesis included adult male and female Hooded Long-Evans rats obtained from Charles River Laboratory. All rats were kept housed two to a cage within DePaul University Animal Facility with food and water available *ad libitum* on a 12:12 hour light/dark cycle. All experiments were approved by the DePaul Institutional Animal Care and Use Committee and were conducted in accordance with rules set by the National Institutes of Health Guide for the Care and Use of Animals.

iv. Experiment Two: Experimental Design

Rats were randomly assigned into treatment and control groups including: male sham control group (no injury), male single concussion (sTBI), male single subconcussive injury (sTBIsc), male repeat subconcussive injuries (5 injuries– 24h apart) (rTBIsc), female sham control group (no injury), female single concussion (sTBI), female single subconcussive injury (sTBIsc), and female repeat subconcussive injuries (5 injuries – 24h apart) (rTBIsc; See Table 2).

The male rats from experiment one were used as data for corresponding groups in experiment two. Following injury each behavioral test was performed at a 2h time point after injury along with multiple timepoints on a 30-day timeline shown below. On day 31 the rats were sacrificed, and the brains were preserved.

Sex	Impact	# of Rats
Male	Sham (No Impact)	8
Male	Repeat Subconcussion (rTBIsc)	9
Male	Single Concussion (sTBI)	9
Male	Single Subconcussion (sTBIsc)	8
Female	Sham (No Impact)	8
Female	Repeat Subconcussion (rTBIsc)	9
Female	Single Concussion (sTBI)	8
Female	Single Subconcussion (sTBIsc)	7

Table 2: Experimental and control groups for experiment two with the number of rats in each group beside them.

b. Materials and Methodology

i. Closed-Head Controlled Cortical Impact

A controlled closed-head cortical impact was modified to perform the repeat subconcussive injuries at DePaul University. This method was established by Jamnia et al., and it models a single concussion or repeat concussion in rats (2017). Animals were handled for two weeks prior to injury for 15 minutes a day (Becker et al., 2005). The injuries were placed over the right forelimb sensorimotor cortex using the Impact One (Leica Microsystems Inc., Buffalo Grove, IL). The single injury concussion received one injury with a 5mm flat tip at speed of 6.5 m/s and at a depth of 10mm after being anesthetized with isoflurane anesthesia (Figure 4). The sham control group was just anesthetized. The repeat subconcussive impact had the same parameters as the single mTBI, but at an 8mm depth and included 5 hits with 24 hours in between each injury.

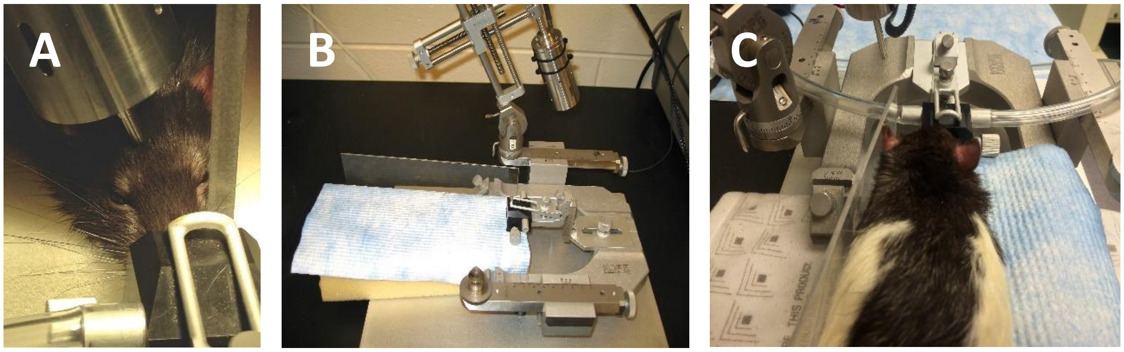


Figure 4: The controlled cortical impact device used to implement the traumatic brain injury. The rat's head is placed on a foam pad underneath the impact device (A) and is rested up against a plexiglass frame (B). The rat's nose is placed in a nose cone that does not have ear bars, so the head is able to move with the impact (C). Taken from Jamnia et al., (2017).

A previous study in our laboratory was used to determine the parameters for the subconcussion and repeat subconcussion. Six groups of rats were compared: sham (no injury) group, single subconcussion group (sTBIsc), single concussion (sTBI), 1st repeat subconcussion group (3 injuries spaced 48 hours apart, 3TBIsc), 2nd repeat subconcussion group (5TBIsc with injuries spaced 48 hours apart), and the 3rd repeat subconcussion group (5TBIsc with injuries spaced 24 hours apart). In this experiment, the data from the Novel Object Recognition test suggests that the group with 5 repeat subconcussive impacts spaced 24 hours apart showed deficits in recognition memory similar to the single concussion group (Figure 5). As previously stated, repeat subconcussive impacts can have deficits similar to or worse than deficits present after a single concussion, so this project used the model of 5 subconcussive impacts spaced 24 hours apart. Behavioral data such as the novel object test (See Figure 5), demonstrated that behavioral deficits were only seen in the group that received 5 subconcussive impacts, spaced 24 hours apart for further study. Therefore, this was the parameter used in this study.

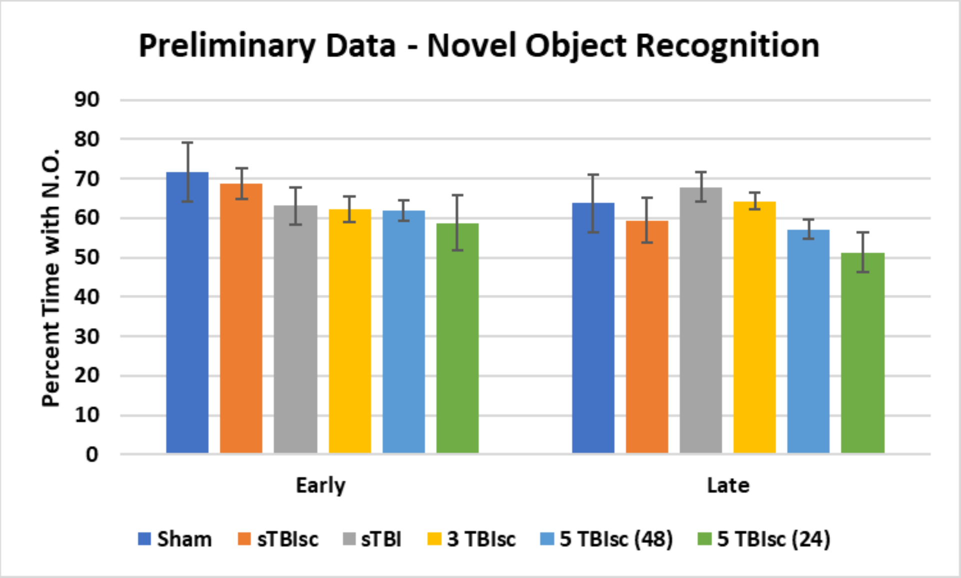


Figure 5: Preliminary Data for Modeling Repeat Subconcussive Impacts. The novel object recognition test is shown as the percent of time spent with the novel object at both an early and late time point.

ii. Estrous Cycle Determination

In experiment two, vaginal smearing was used to monitor the estrous cycles of the female rats. Studying estrous cycle of the female rats allowed us to control the stage of estrous at time of injury and to examine the potential effect of hormones on behavioral changes after brain injury (Eliot and Richardson, 2016). We used the protocol set by Becker et al., for vaginal smears and optimized it for our use (2005). We used bead sterilized scapulas that were immersed in sterile 0.9% saline solution and inserted them into the vagina in order to get the vaginal cells. Only the tip of the scapula was inserted to prevent pseudo pregnancy. These cells were then scraped off onto a labelled microscope slide. The proportion of cells were noted to aid in the determination of the estrous cycle phase. This was determined by measuring the type of cell that is observed from the lavage each day, and two full estrous cycles were mapped before receiving any injury (Becker et al., 2005). All injuries occurred during the same stage of the estrous cycle for all female rats, on the day of proestrus, which has the highest level of estrogen in all the stages of the estrus cycle of the rat.

iii. Behavioral Tests

1. Open-Field Test

In order to study anxiety-like behavior and locomotion we conducted an open-field test (Jamnia et al., 2017; Huang et al., 2013; Lipkind et al., 2004). Two hours post-injury and on post-injury day (PID) 28, rats were placed into the center of an open-field apparatus for ten minutes (San Diego Instruments, San Diego, CA; 40.64 cm x 40.64 cm x 38.1 cm). Amount of locomotion was measured in beam breaks, using the 16x16 beams in the XY plane and defined as the total number of beam breaks. To examine symptoms of anxiety, data from the time in the open field was measured as percent of the total activity that is spent in the center (consisting of 40% of the field in the middle) versus the surround area (Jamnia et al., 2017).

2. Novel Object Recognition Test

The novel-objects recognition (NOR) test was used to measure of working memory. It consists of two parts separated by 24 hours and both trials were videotaped. For the first day of habituation, the rat was placed in the open-field where two like objects are added (for example, red Kong dog toys), and the rat had 5 minutes to explore the objects. On the second day, one of the familiar objects was replaced by one “novel” object (for example, a T-75 flask filled with sand) and the rat interacted with the two objects for 5 minutes. If there is no memory loss, such as the sham control group should show, the natural curiosity of the rat will incline it to spend more time of direct interaction with the “novel” object rather than with the already explored “familiar” object. Time spent with object was determined by watching the video tapes and measuring the amount of time defined as direct interaction, such as touching or sniffing, or the snout facing the object within a few centimeters. Data were measured as percent of total time spent with novel object.

3. Balance Beam

The beam walking test was used to test for vestibular deficit along with balance and locomotion. The balance beam (2 x 2 x 56cm) consisted of a start end and a goal box, with marks at the first 10 cm and 10 cm before the goal box. All data analysis was done on the rats’ performances when the front paws were past the first mark until the back paws were past the second mark. Rats were placed on the first 10 cm of the beam and were tracked with a video camera below the beam until reaching the goal box. Each rat did three trials each testing day and the beam was cleaned between each rat with a 70% ethanol solution. The total time to cross the beam and faults/slips while walking the beam were measured by watching video playback.

4. Foot Fault Test

The foot fault test was used to assess motor coordination and locomotion. The test was done on a test tube rack (33.02 x 25.40 x 7.62 cm, with openings of 2.54 cm) that was raised to decrease the probability of the rat escaping or jumping off. The rat was placed on the foot fault apparatus for two-minutes. There was one trial per day and after each trial the rack was cleaned. During the trial, steps and faults were measured. A step consisted of both front paws being moved, and if the rat was off the side of the rack it was not counted. The number of faults through the wire and number of steps were measured. The data was presented as the number of steps per trial to assess locomotion corrected from baseline measurements and to assess motor coordination the data was presented as the number of faults per trial corrected from baseline measurements.

5. Air-Righting Test

The air-righting test has been used as a test of vestibular deficit in adult rats (Ossenkopp et al., 1990). Rats were held in the supine position at a mark 50 cm above a padded surface. They were dropped onto the padded surface and ability to right themselves in the air and land on their paws was measured for a total of five trials. A trial that the rat "righted" is considered landing on all four paws onto the pad and anything else was not considered perfect righting. Non-perfect landings included those that were not landed on all four paws (Figure 6A), were splayed away from the body (Figure 6B), or were placed unevenly around the body (Figure 6C). The data was analyzed as percent perfect righting out of the five trials.

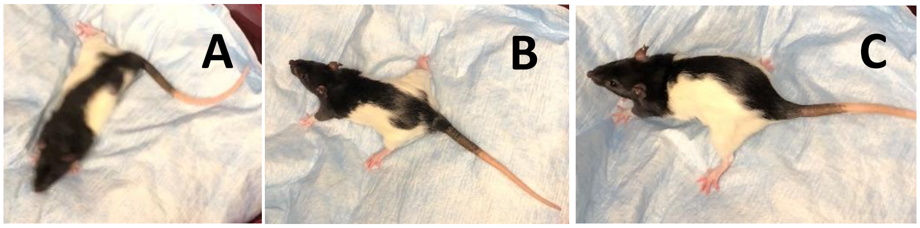


Figure 6: These are three examples of possible landings rats can have in the air-righting task. (A) Not landing on all four paws, but instead landing on the side or on shoulder or hip. (B) Landing is splayed, with legs stretched out and paws a distance away from the body. (C) Landing is uneven, with paws landing at uneven distances from the body.

6. Porsolt Forced Swim Test

The Porsolt (1978) Forced Swim Test (FST) was used as a measure of depression levels in the rodents. The rodents were placed in a tall cylinder filled with water and their behavior was videotaped and observed. Rodents stop swimming and stay "immobile" for a period of time, which has been considered depressive-like behavior, testing for "learned helplessness". The FST was done in a clear plexiglass cylinder (30 x 60 cm; California Plastics) that fits the dimensions outlined in Castagne et al., (2010). Rats were placed in the cylinder for 6 minutes (Milman et al., 2005), where the first 2 minutes was considered habituation and was not counted, but for all time afterwards, trials were timed for frantic swimming and immobility. Frantic swimming was considered swimming, climbing, and movement in a vertical direction with the intent to get out of the cylinder. Immobility was considered lack of swimming and turning, and resembled floating. Data was presented as total amount of time in seconds spent immobile to assess depressive-like behavior and total time in seconds spent swimming frantically to measure anxiety-like symptoms.

iv. Sacrifice/Euthanasia

Animals were euthanized at a 30-day time point after the initial injury. The protocol for euthanasia followed Jamnia et al (2017). The rats were deeply anesthetized with Euthasol, followed by perfion with phosphate buffered saline (PBS) with heparin and 4 % paraformaldehyde in PBS. After perfusion, the skull was examined for cracks and the brain was extracted, post-fixed, cryoprotected, and kept at 4°C.

v. Gross Pathology

After perfusion, an overall observation was made of the skull and brain to assess any gross pathology. In the model previously established and clinical examples there is no bruising, lesions, or skull cracks seen on the brain and skull respectively following a concussion (Jamnia et al., 2017). After perfusion, as the brain was extracted from the skull, the skull and the surface of the brain were assessed.

vi. Statistical Analysis

The statistical analysis was done with a one-way, two-way, or three-way repeated measures ANOVA depending on the number of variables being studied. If there was one variable, groups were compared using a one-way repeated measures ANOVA; if there were two variables being examined, groups were compared using a two-way repeated measures ANOVA; and if there were three variables being examined, groups were compared using a three-way repeated measures ANOVA. In the case of the forced swim test, there was only one time-point being analyzed, so a repeated measures ANOVA was not used. A Tukey's honestly significant difference post-hoc test was used. Statistical analysis was done using R program.

IX. Results: Experiment One

a. Overall Pathology

In the male rats, the overall pathology of the skull and brain were assessed. In all male rats there were no cracks or bruises on the surface of the skull. Once the brain was removed, it was also observed that there were no lesions or bruises on the surface of the brain.

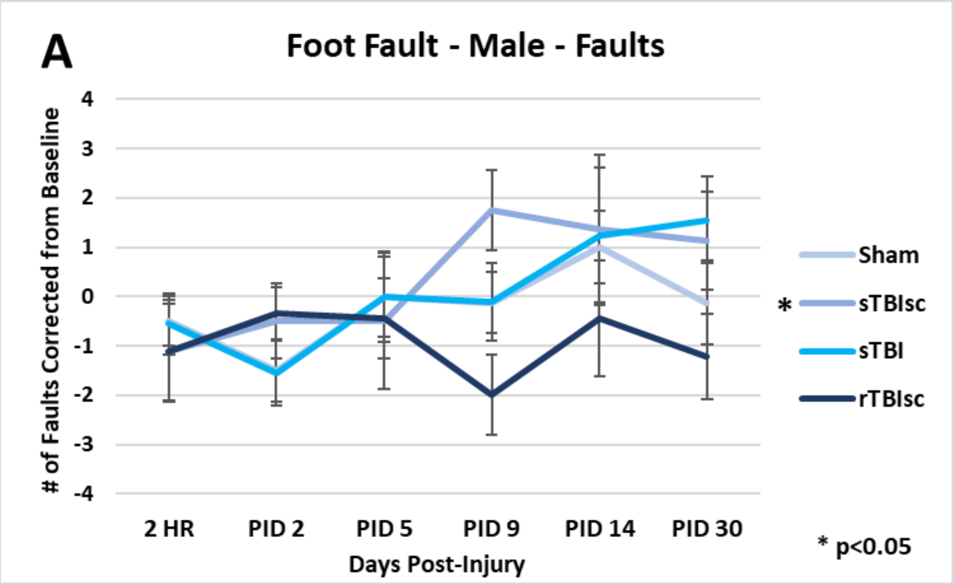
b. Motor Behaviors

i. Motor Coordination

Motor coordination can be measured using the foot fault and balance beam tasks.

Foot Fault: Motor coordination can be revealed using foot fault by assessing the number of faults that occur during a trial. This was corrected to a change from baseline score for the male rats because the baseline data differed between the condition groups (Figure 7A). A two-way repeated measures ANOVA revealed a significant effect of injury type on the number of faults corrected from baseline and a significant effect of day post-injury, but no significant effect of injury type by day post-injury ($F_{3,3}=3.0836$, $p<0.05$; $F_{1,3}=7.1424$, $p<0.01$; $F_{3,194}=1.3291$, $p>0.05$). When examining the faults corrected from baseline, there was a slight increase in faults in the single concussion and single subconcussion groups compared to sham, but there was no significant difference between the sham group and single concussion group. The repeat subconcussion group showed less deficit than both the single concussion group and sham group, which was not what was originally expected ($p<0.05$). The single subconcussion group did not show a significantly different performance than the single concussion group ($p<0.05$).

Balance Beam: The balance beam tests locomotion and vestibular deficits. Time to walk across the beam is calculated as well as the number of times the hindlimbs slip off the beam. Motor coordination was measured in the male rats as a total measure of number of faults or slips per trial (Figure 7B). A rat with more deficit will show more faults and therefore less motor coordination. This was corrected to a change from baseline for the male rats because the baseline data differed between the condition groups. A two-way repeated measures ANOVA of slips corrected to change from baseline showed trends suggesting deficits in the injured groups, but no significant effects of condition, day post-injury, or condition by day post-injury on deficits in faults ($F_{3,18}=0.6107$, $p>0.05$; $F_{6,18}=1.3132$, $p>0.05$; $F_{18,193}=0.9873$, $p>0.05$).



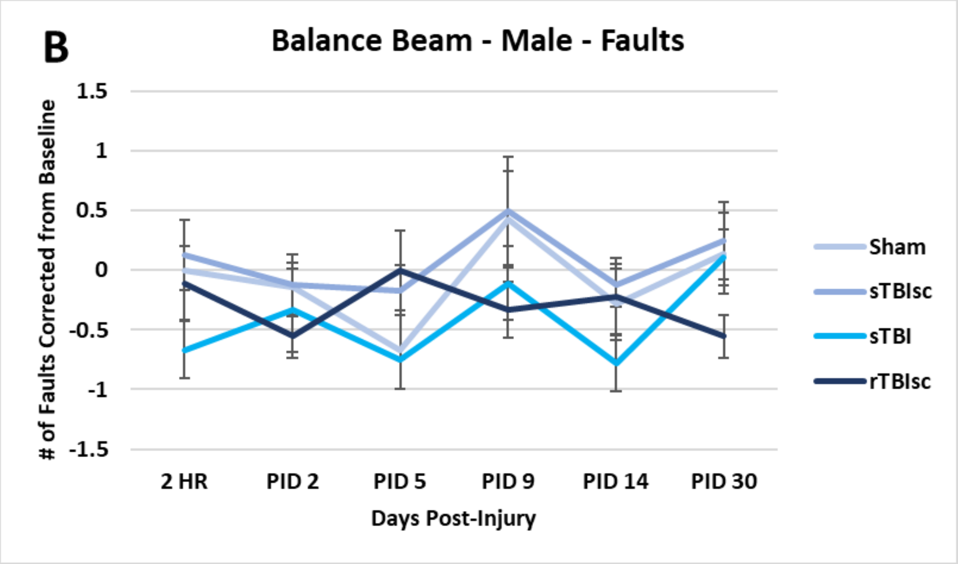


Figure 7: The motor coordination data for the males in both foot fault (A) and balance beam (B). In both graphs the injury groups are represented in different gradients of blue. In the foot fault test (A) there was no increase in faults in the single concussion group, but the single subconcussion group revelaed increased faults compared to sham (*$p<0.05$). In the balance beam test (B) the data is shown as number of faults that were expressed as a change from baseline over the behavioral testing days. There were no statistically significant effects of injury in the balance beam test.

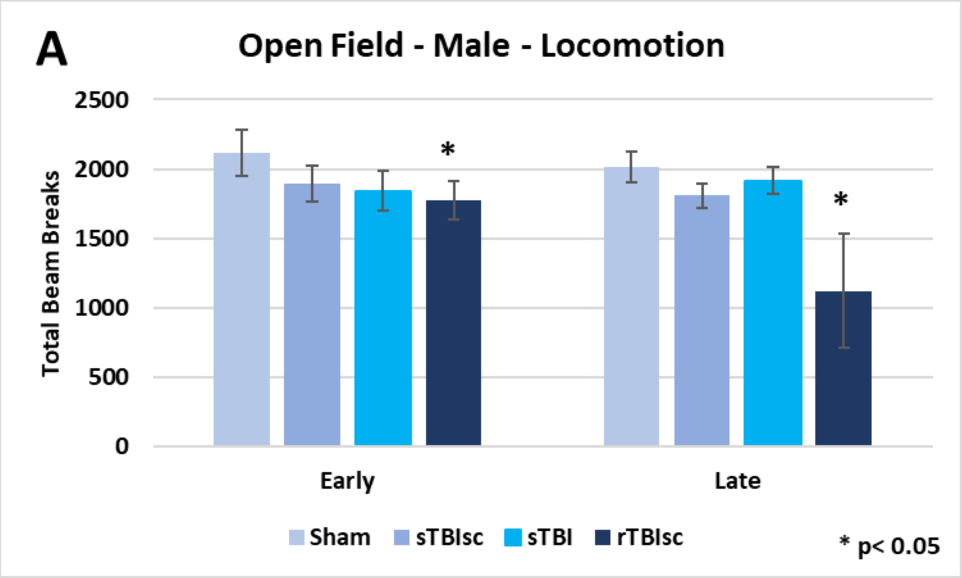
ii. Locomotion

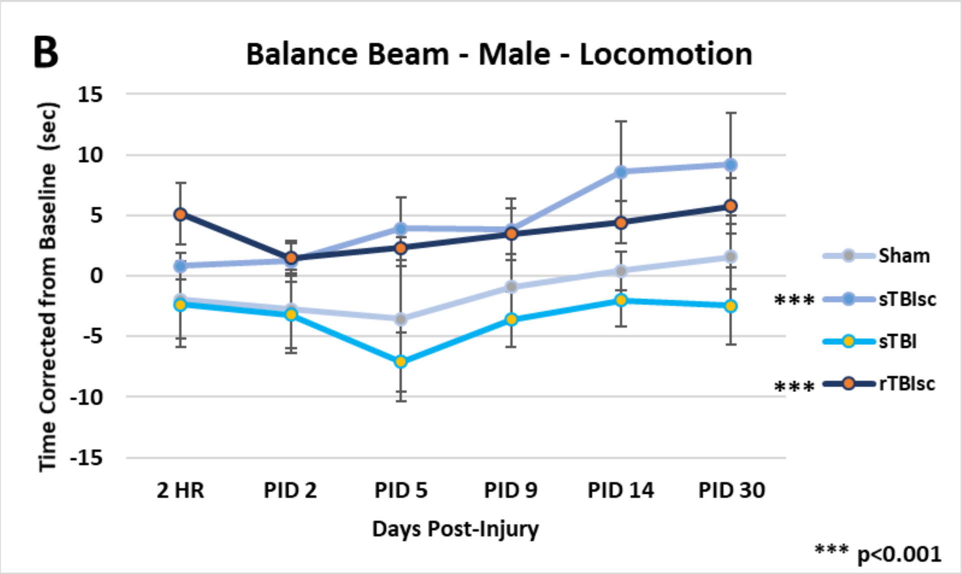
Locomotion was assessed using the open field, balance beam, and foot fault tasks.

Open Field: Open field task can be used to examine both locomotion and anxiety-like behavior. To measure locomotion, the total amount of beam breaks was measured for the duration of the ten-minute trial. This was done at both an early time point (two hours post-injury) and a late time point (28 days post-injury). The data revealed significant effect of injury type on performance. A two-way repeated measures ANOVA revealed a significant effect of injury on performance, but not a significant effect of day post-injury or interaction ($F_{3,3}=3.9212$, $p<0.05$; $F_{1,3}=2.3073$, $p>0.05$; $F_{3,58}=1.7897$, $p>0.05$). The repeat subconcussive group showed significantly lower amount of beam breaks, and therefore decreased locomotion, compared to the sham group at both time points (Figure 8A). Although the single concussion and single subconcussion also showed some deficit, they were not significantly different from the sham or repeat subconcussion performances.

Balance Beam: To assess locomotion using the balance beam test, the time taken to cross the beam was measured. For the male rats, the data was corrected from baseline due to the injury groups having significantly differing baseline averages (Figure 8B). A two-way repeated measures ANOVA revealed a significant effect of injury type, but not a significant effect of day post-injury or injury type by day post-injury ($F_{3,18}=2.9269$, $p<0.05$; $F_{6,18}=2.0409$, $p< 0.01$; $F_3=0.9277$, $p>0.05$). The data show that the single subconcussion group had a significantly worse performance on the balance beam than the sham group, but not significantly worse than the single concussion or repeat subconcussion group ($p<0.05$). Interestingly, the other injured groups were not significantly different from the sham group. There was no significant effect of time shown in this test, but there was a slight increase in the time it took to cross the beam in the later time points for all groups ($p>0.05$). The same trends and deficits are seen when data is transformed to average time across the three trials ($F_{3,18}=2.9293$, $p<0.05$; $F_{6,18}=2.0413$, $p< 0.01$; $F_3=0.9269$, $p>0.05$; data not shown).

Foot Fault: In the foot fault test, locomotor activity can be examined by measuring total steps within a two-minute period. Since there were differences in locomotor activity between groups at baseline we calculated a change from baseline measure for each animal for this analysis (Figure 8C). A two-way ANOVA revealed a significant effect of injury type, day post-injury, and injury type by day post-injury on motor coordination ($F_{3,15}=23.4104$, $p<0.001$; $F_{5,15}=3.278$, $p<0.01$; $F_{15,178}=2.7998$, $p < 0.001$). The repeat subconcussion group showed significant deficits compared to all other groups. The single concussion group and single subconcussion group both show significant deficits compared to sham. At two hours post-injury, both the single concussion group and repeat subconcussion group were already showing significant deficits in locomotion ($p<0.05$). The single subconcussion group did not show deficits as significant two hours post-injury and did not have a specific day of significant deficit. At day 2 post-injury there was not a significant difference between any of the groups and again at post-injury day 30 there was not a significant difference between the groups.


A
Open Field - Male - Locomotion
Total Beam Breaks
2500
2000
1500
1000
500
0
*
*
Early
Late
Sham
sTBIsc
sTBI
rTBIsc
* p< 0.05


B
Balance Beam - Male - Locomotion
Time Corrected from Baseline (sec)
15
10
5
0
-5
-10
-15
2 HR
PID 2
PID 5
PID 9
PID 14
PID 30
Days Post-Injury
Sham
*** sTBIsc
sTBI
*** rTBIsc
*** p<0.001

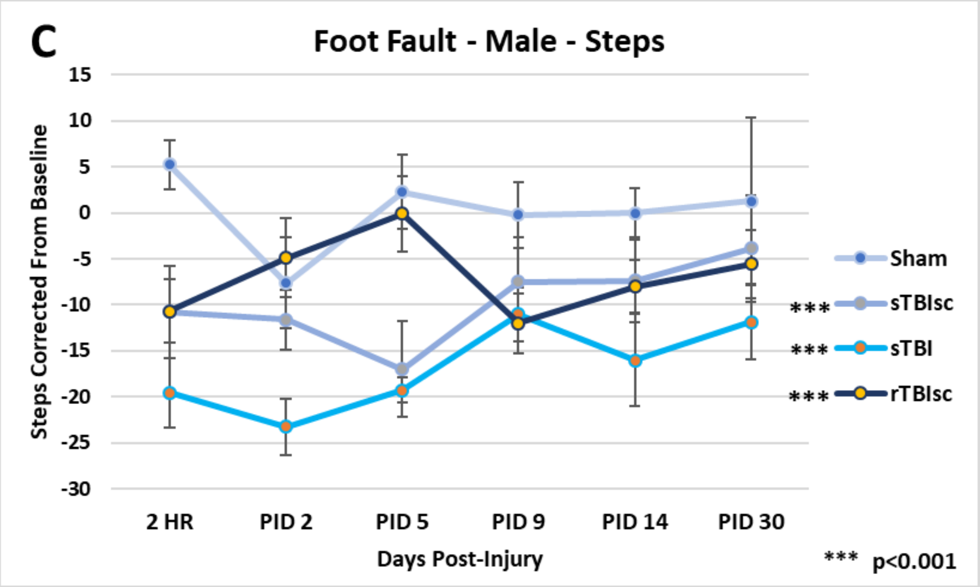


Figure 8: Locomotion was examined using the open field test (A), balance beam (B), and foot fault test (C). In each test the injury groups are shown in gradients of blue. In the open field test (A), the total amount of beam breaks is shown for both the early time point (2 hours post-injury) and late timepoint (PID 28). In the open field test, there were significantly less beam breaks after the repeat subconcussive injury in both the early and late timepoints (*p<0.05). In the balance beam test (B) the time to cross the beam was assessed on each behavioral testing day and was expressed as the time corrected from baseline. On the balance beam both the single subconcussion group and repeat subconcussion group showed significantly longer time to cross the beam overall compared to the sham group. The single concussion group showed slightly lower time to cross the beam compared to sham. In the foot fault test (C) the data was shown as the number of steps corrected from baseline for each behavioral testing day. In foot fault the single subconcussion, single concussion, and repeat subconcussion groups show significantly less steps overall than the sham group (***p<0.001). Compared to the other injury groups, the repeat subconcussion group showed significantly less steps (***p<0.001), suggesting although all groups showed deficits in locomotion in the foot fault test, the repeat subconcussive group showed the worst deficits.

iii. Reflex and Vestibular Deficit – Air Righting

In the air-righting test, which uses righting reflexes to examine vestibular deficits, data demonstrate that overall, the injured animals performed more poorly than the sham group, but that these deficits did recover back to baseline levels by day 30. A two-way repeated measures

ANOVA revealed a significant effect of type of injury on performance and significant effect of day post-injury on performance, along with a significant effect of injury type by day post-injury interaction ($F3,18=33.4574$, $p < 0.001$; $F6,18=16.5466$, $p < 0.001$; $F18,208=2.6272$, $p < 0.001$). A Tukey post hoc test revealed that all injured groups showed a lower percent perfect righting in the righting reflex compared to Sham ($p< 0.05$) (Figure 9). There were no significant differences in performance between the single concussion group and both the single subconcussion group and repeat subconcussion group, but the repeat subconcussion group performed significantly worse than the single subconcussion group ($p<0.05$). At two hours post-injury, all groups performed significantly worse on the task ($p<0.05$). All days before Day 30 show significantly lower performance than baseline, suggesting possible recovery by Day 30 ($p<0.05$).

Baseline performance was not significantly different between groups. At two hours post-injury the rats with a single concussion and the rats with a repeat subconcussion show significant vestibular deficits ($p<0.05$). These deficits are still present out to nine days post-injury and recover by day 30 post-injury. There were no significant differences in vestibular deficits between the single concussion and repeat subconcussion groups in this task. While rats with a single subconcussion showed a trend toward a deficit, it was not significantly different from Sham at any of the time points examined. At the last time point, day 30, no group shows significant deficit compared to the sham group ($p>0.05$)

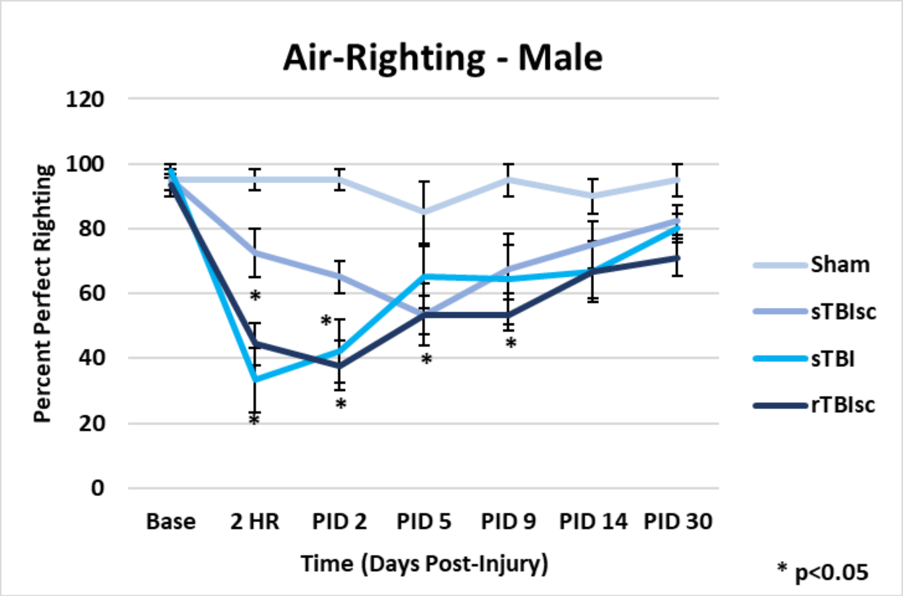


Figure 9: Air-Righting data for male rats. Percent perfect righting was used to compare the amount of trials out of the 5 that were landed without any deviations from perfect. Non-perfect landings included those that were not landed on all four paws, were splayed away from the body, or were placed unevenly around the body. Both the single concussion injury group and repeat subconcussive injury group showed significant deficits (*p<0.05). The single concussion group showed deficits up to PID 2, while the repeat subconcussion group showed significant deficits out to PID 9 (*p<0.05). The repeat subconcussion injury caused deficits that were more chronic than the single concussion.

c. Limbic Behaviors

i. Recognition Memory – Novel Object

In the novel-object task, data demonstrate that injury impacts recognition memory (Figure 10). A two-way ANOVA showed that there was a significant main effect of injury type on percent time spent with the novel object and significant effect of day post-injury, but there was not a significant interaction (F3,3=5.5122, p<0.01; F1,3=41.6317, p<0.001; F3,55=0.8901, p>0.05). The data demonstrate that rats with a single concussion showed significant deficits in recognition memory overall (p<0.05). Both the single subconcussion and repeat subconcussion injury groups showed a poorer performance than the sham group and a higher performance than the single concussion group but were not statistically different from either group. Over time, performance on the task decreased significantly for all groups from the first novel object test at PID 2 to the second test at PID 30 ($p<0.05$).

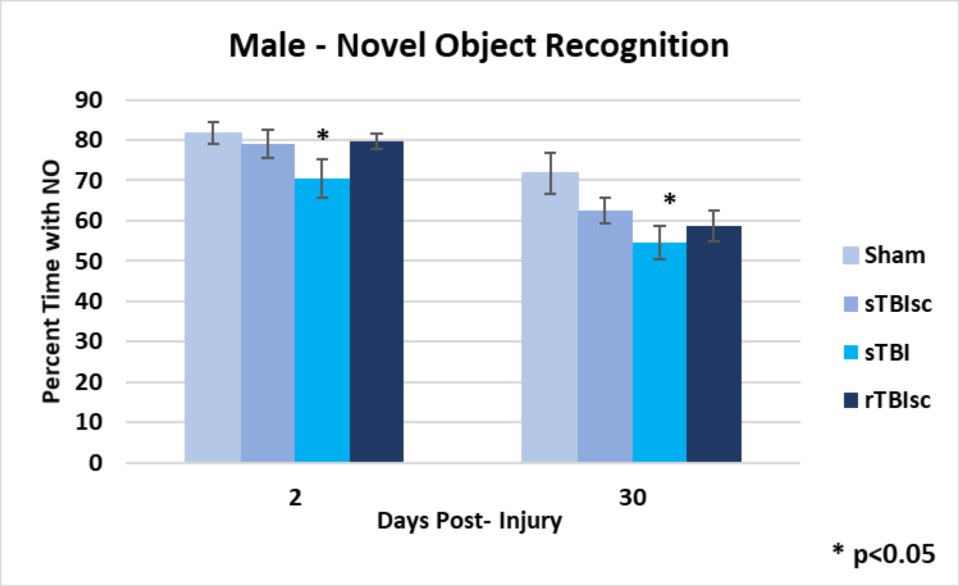


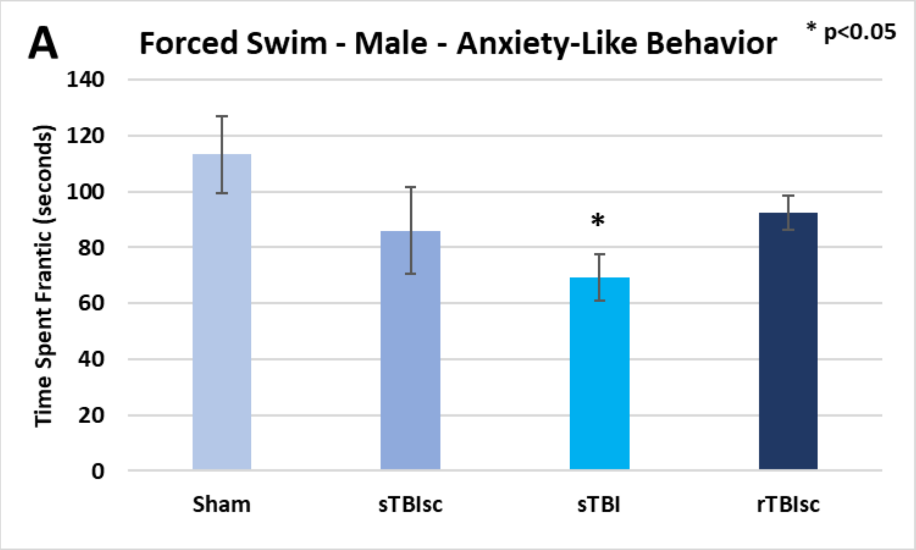
Figure 10: Percent time spent with the novel object on both day 2 and day 30. The different injury groups are shown in gradients of blue. The single concussion group showed significantly lower percent of time spent with the novel object, suggesting deficits in recognition memory, at both time points (*$p<0.05$). The repeat subconcussion injury group showed a trend towards significant deficits in recognition memory in PID 30 as well.

ii. Anxiety-Like Behavior

Symptoms of anxiety were assessed using both the open field and forced swim test.

Forced Swim: In the forced swim test, anxiety-like symptoms are tested by measuring the amount of frantic swimming that the rat does when placed in the forced swim apparatus (Figure 11A). A one-way ANOVA revealed a significant effect of condition on performance ($F_{3,29}=3.1461$; $p<0.05$). The single concussion group showed significantly less frantic swimming than the sham group, suggesting less symptoms of anxiety than the sham. The other injured groups also showed a trend of decreased frantic swimming but not at levels statistically different from sham.

Open Field: In the open-field test, anxiety-like behavior can be measured by assessing the amount of time spent in the inner 40% of the field ("center") versus the outer 60% of the field ("surround"). The more time spent in the surround, or the less time spent in the center, is considered to be more anxiety-like behaviors (Figure 11B). A two-way repeated measures ANOVA showed that condition, day post-injury, and interaction effects were not significant (F3,3=1.7852, p>0.05; F1,3=0.2325, p>0.05; F3,58=0.2362, p>0.05). There are no statistically significant condition effects, but there were trends of decreased time spent in the center in the single concussion group at both time-points, suggesting possible decreased symptoms of anxiety.



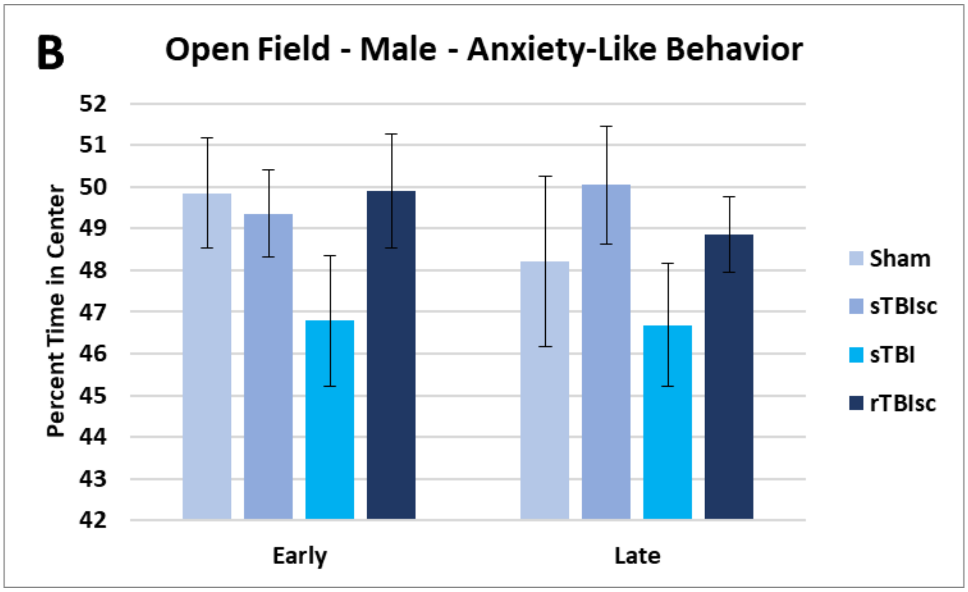


Figure 11: Anxiety-like behaviors were measured using the forced swim test (A) and the open field test (B). Performances of each injury group is shown in gradients of blue for both tests. In the forced swim test (A), the time spent swimming frantically was measured on PID 31. The single concussion group showed a significant decrease in amount of frantic swimming (*p<0.05), suggesting a decrease of anxiety-like symptoms post-injury. Similarly, the single subconcussion and repeat subconcussion injury groups showed decreased amount of frantic swimming that were not statistically significant. In the open field test (B), the percent time spent in the center was analyzed in both the early (2 Hours Post-Injury) and late (PID 28) timepoints. There were no statistically significant effects of injury type or time point on the percent time in the center (p>0.05). There were trends of the single concussion group showing reduced percent of time spent in the center, suggesting higher anxiety levels, but there were trends of higher percent time spent in the center in the single subconcussion group and repeat subconcussion group.

iii. Depression-Like Behavior

The forced swim test can be used to measure symptoms of depression such as learned helplessness. Learned helplessness can be measured by recording the amount of time spent immobile in the forced swim apparatus. A significantly long time spent immobile suggests more depression-like behavior and learned helplessness, and the more time spent immobile, the worse the symptoms (Figure 12). A one-way ANOVA revealed a significant effect of condition on performance (F3,29=4.4325; p<0.05). Both the repeat subconcussive group and the single subconcussion group showed significant deficit compared to sham. The single concussion group showed increased time spent immobile compared to sham, but it was not statistically different from sham.

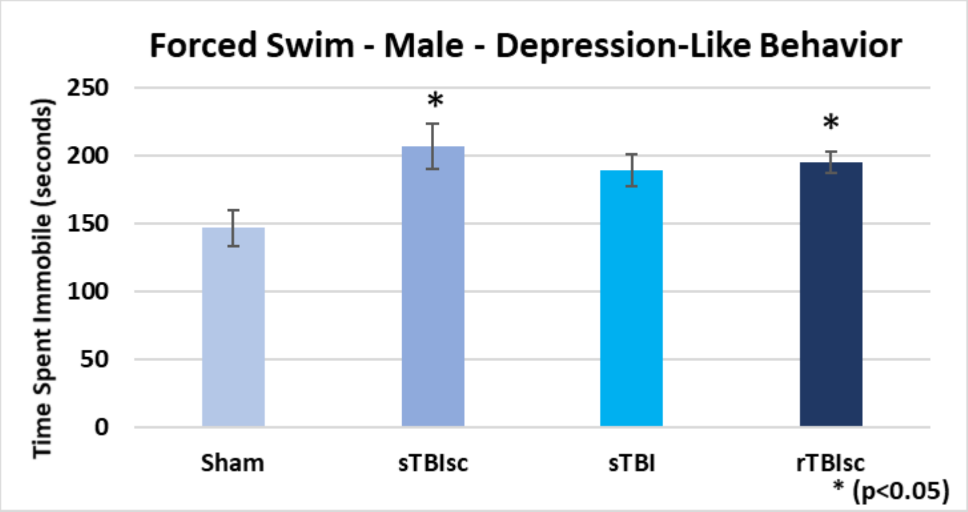


Figure 12: Depression-like behavior was analyzed using the time spent immobile in the forced swim test on PID 31. Each injury group is shown in a gradient of blue. The single subconcussion group and repeat subconcussion group showed significantly higher time spent immobile than the sham group (*p<0.05), suggesting higher levels of depression-like behavior. The single concussion injury showed an elevation in the time spent immobile that was not statistically significant.

d. Summary of Experiment One

The model of repeat subconcussion developed in this study resulted in deficits in both motor and limbic tasks. This model of repeat subconcussion showed deficits that were similar or worse than the single concussion group in “motor” tasks including locomotion and air-righting. The repeat subconcussion injury also showed statistically significant increases in depression-like behavior and deficits in both anxiety-like behaviors and recognition memory. A single subconcussion did not typically result in significant deficits.

X. Results: Experiment Two

In experiment two, sex differences were evaluated by comparing the males from experiment one to a separate group of female rats. In this portion of the thesis, the data of female rats will be presented alone and then will be followed by a comparison of the male and female responses of each injury group.

a. Overall Pathology

In experiment two, the skulls and brains were assessed for any overt pathology during the perfusions. In the female rats, there were no skull cracks or significant marks on the outsides of the skulls. Similarly, on the surface of the brain there were no indents, bruises, or lesions.

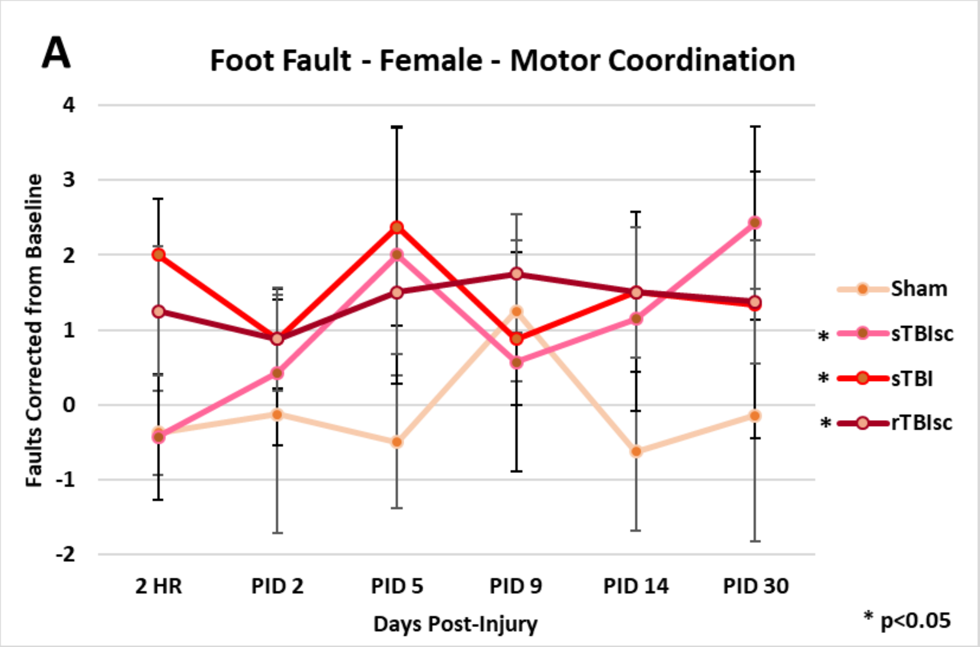
b. Motor Behaviors

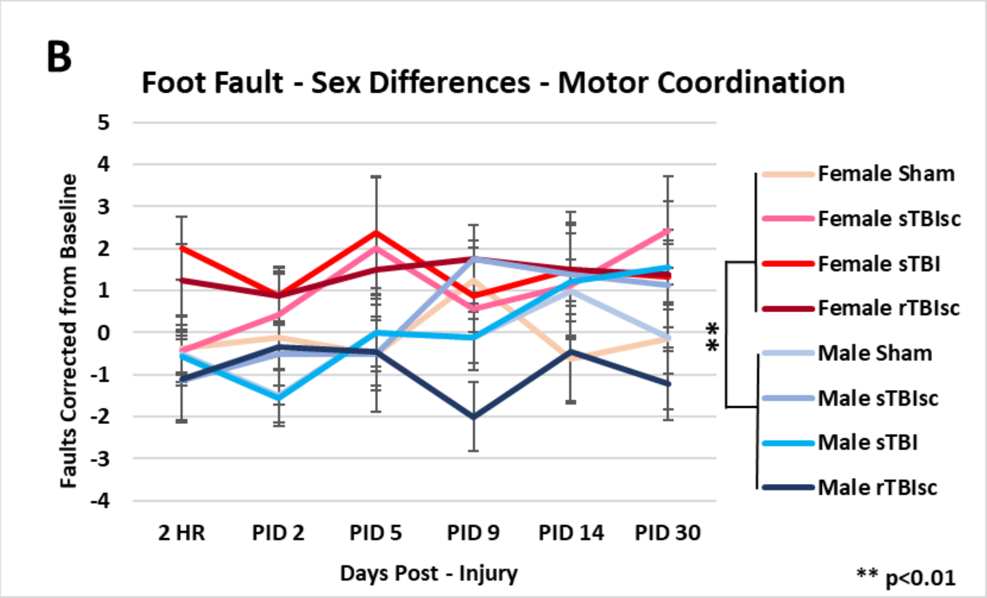
i. Motor Coordination

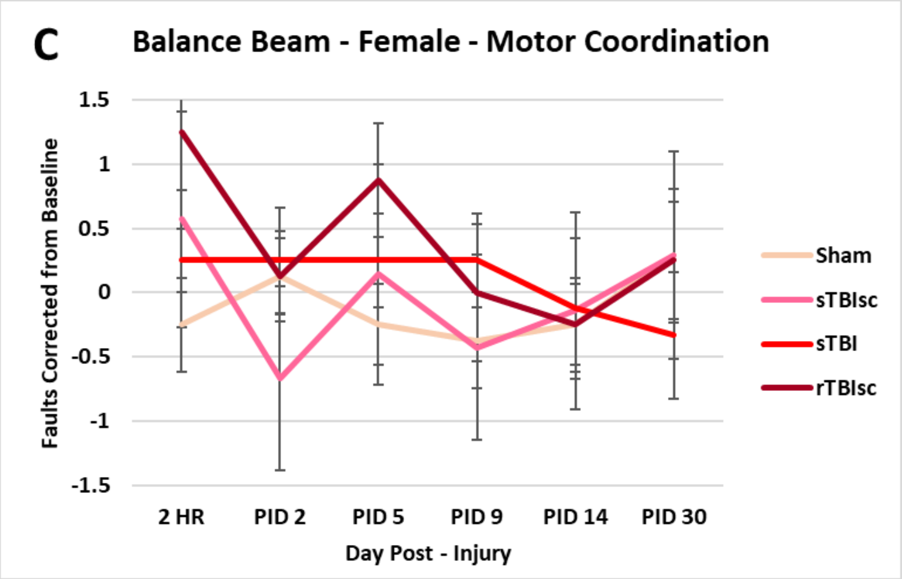
Motor coordination was assessed using the foot fault test and the balance beam test.

Foot Fault: In the female data, faults were measured and were transformed to faults corrected from baseline due to a significant group difference in performance at baseline. A two-way repeated measures ANOVA showed significant effect of injury type on faults, but no significant effect of days post-injury or condition by day post-injury ($F_{3,15}=2.7146$, $p<0.05$; $F_{5,15}=0.3731$, $p>0.05$; $F_{15,159}=0.4391$, $p>0.05$). In females there were significantly more faults in all the injury groups compared to sham ($p<0.05$; Figure 13A). The single concussion group showed slightly higher faults from 2 hours post-injury to PID 5 and the repeat subconcussion group showed slightly higher faults from PID 9 to PID 14. Comparing male and female data, there was a significant difference between the sexes in faults (Figure 13B). A three-way repeated measures ANOVA revealed a significant effect of sex, but not a significant effect of condition, day post-injury, or condition by sex by day post-injury on faults ($F_{3,38}=1.5714$, $p>0.05$; $F_{5,38}=0.5634$, $p>0.05$; $F_{1,38}=7.4914$, $p<0.01$; $F_{38,328}=1.2989$, $p>0.05$). The females showed significantly higher deficit compared to the male rats regardless of type of injury.

Balance Beam: In the females, the balance beam tested motor coordination by looking at the number of faults across the beam, similar to the males (Figure 13C). A two-way repeated measures ANOVA of the faults as a change from baseline showed no significant effect of condition, days post-injury, or condition by day post-injury ($F_{3,15}=1.1383$, $p>0.05$; $F_{5,15}=0.944$, $p>0.05$; $F_{15,158}=0.5332$, $p>0.05$). The repeat subconcussive group showed an increase in the number of faults compared to sham, but this increase was not statistically significant. Similarly, the single concussion and single subconcussive groups both showed an increase in the number of faults on most behavioral days, but did not show significant deficit from sham. Comparing performance of males and females revealed no significant sex differences in motor coordination for the balance beam test (Figure 13D). A three-way repeated measures ANOVA of average faults showed significant effects of condition on motor coordination, but no significant effect of day post-injury, sex, or condition by day post-injury by sex ($F_{3,45}=3.1345$, $p<0.05$; $F_{6,45}=2.0898$, $p>0.05$; $F_{1,45}=1.4866$, $p>0.05$; $F_{45,385}=1.0766$, $p>0.05$).


A
Foot Fault - Female - Motor Coordination
Faults Corrected from Baseline
4
3
2
1
0
-1
-2
2 HR
PID 2
PID 5
PID 9
PID 14
PID 30
Days Post-Injury
Sham
* sTBIsc
* sTBI
* rTBIsc
* p<0.05


B
Foot Fault - Sex Differences - Motor Coordination
Faults Corrected from Baseline
5
4
3
2
1
0
-1
-2
-3
-4
2 HR
PID 2
PID 5
PID 9
PID 14
PID 30
Days Post - Injury
Female Sham
Female sTBIsc
Female sTBI
Female rTBIsc
Male Sham
Male sTBIsc
Male sTBI
Male rTBIsc
**
** p<0.01



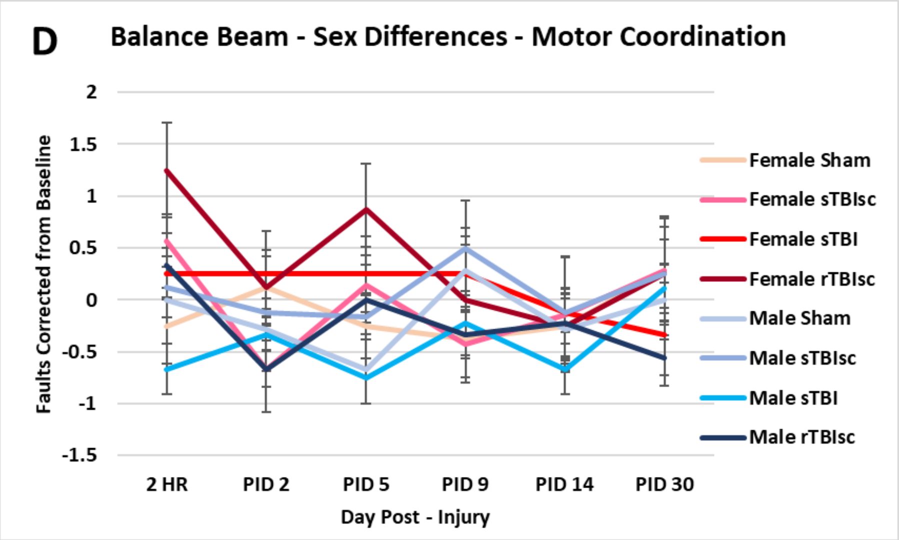


Figure 13: More deficits in females than in males in some motor coordination tasks. Deficits in motor coordination were compared in males and females using the foot fault test (A, B) and the balance beam test (C, D). For the foot fault test (A) the data for the female rats is shown as the number of faults, corrected from baseline, over each behavioral testing day post-injury. The injury groups for the females are seen in gradients of red. In the females, all injury groups showed a significantly more faults than sham (*p<0.05). B) Comparison of male and female deficits, with females shown in gradients of red reflecting type of injury and males having similar gradients of blue. The females showed significantly higher faults than males overall (**p<0.01). In the balance beam test, the female rats (C) did not show a significant effect of injury (p>0.05). The repeat subconcussion group and single concussion group showed an increase in faults, but it was not statistically significant. In the balance beam test, when the females and males were compared (D) they did not show a significant effect of sex (p>0.05).

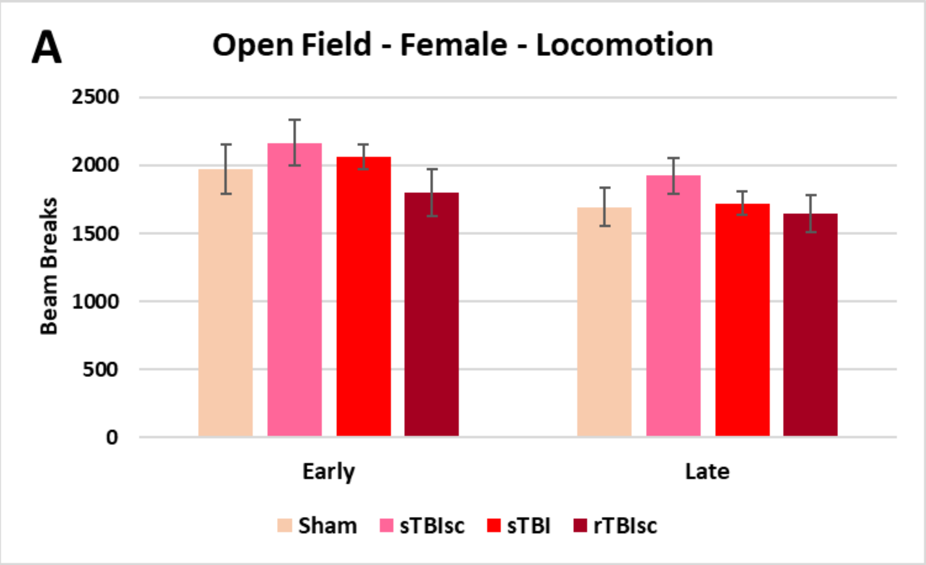
ii. Locomotion

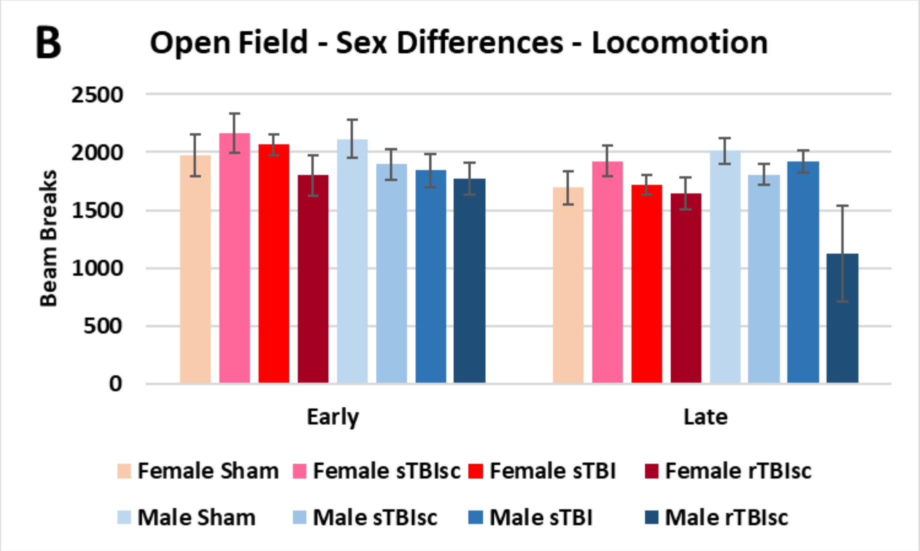
In the female rats, locomotion was measured in the open field test, balance beam, and foot fault. Calculations were conducted similarly to those presented previously for the males.

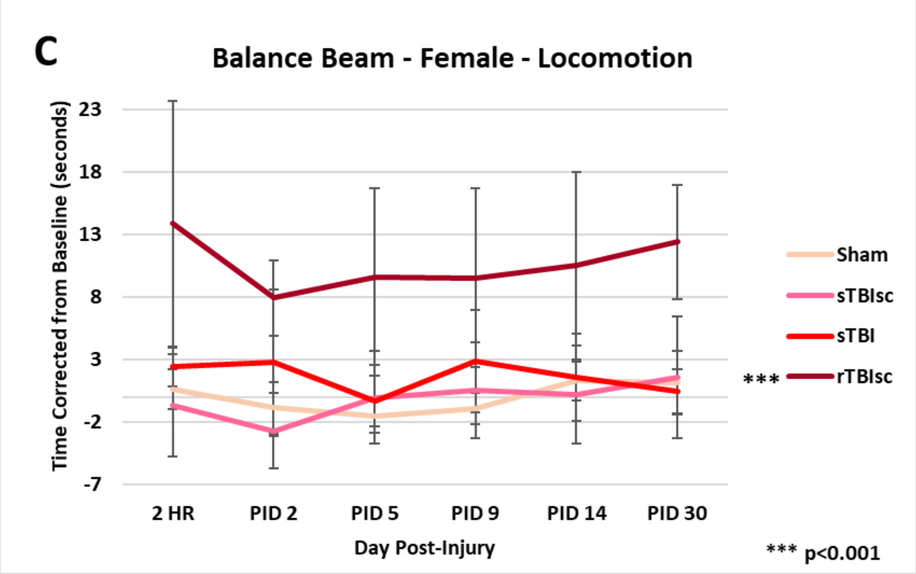
Open Field: A two-way repeated measures ANOVA revealed no significant effect of condition or interaction, but there was a significant effect of day post-injury in the female rats (F3,3=1.6891, p>0.05; F3,53=0.1516, p>0.05; F1,3=6.3804; p<0.05). The female rats did not show a significant effect of condition on locomotion, but there is a trend of lower beam breaks in the repeat subconcussion group, suggesting possible trends towards decreased locomotion (Figure 14A). There was a significant effect of time on the performance in the open-field; in the late time point at day 28, there was a significantly lower amount of locomotion overall, suggesting possible chronic symptomology. Comparing male performance vs female performance unfortunately did not reveal a sex difference in locomotion in the open-field task. A three-way repeated measures ANOVA revealed significant effect of condition and day post-injury, but did not show an effect of sex or interaction (F3,10=4.2463, p<0.01; F1,10=7.4419, p<0.01; F1,10=0.3266, p>0.05; F10,111=1.2148, p>0.05; Figure 14B).

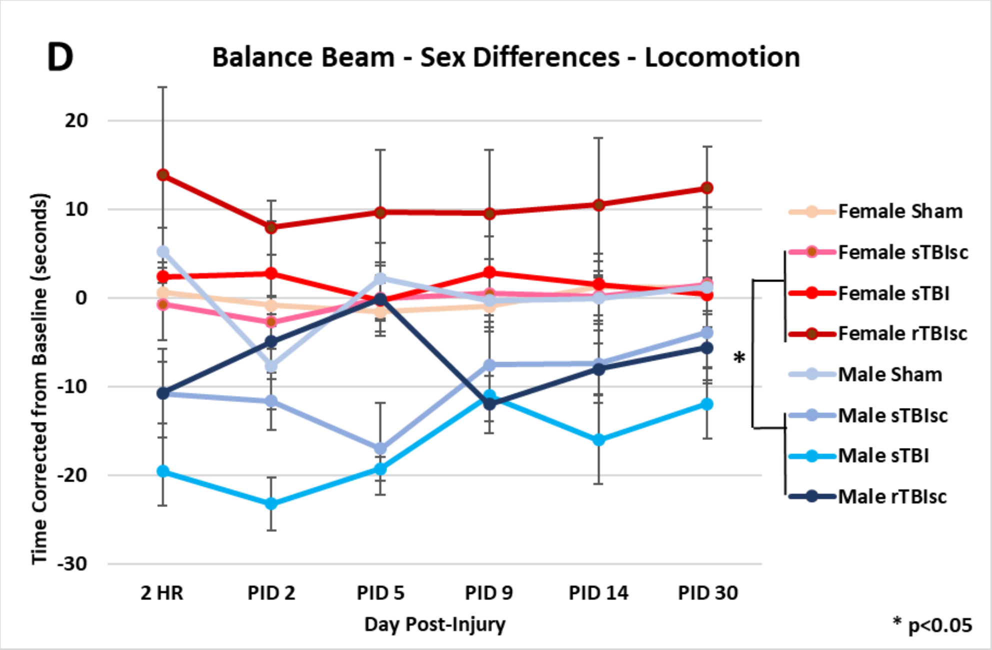
Balance Beam: Time to cross the beam was analyzed as a change from baseline to account for differing baseline values in the males and establish continuity (Figure 14C). In the time to cross the beam, the female rats showed significant effects of condition, but no significant effect of day post-injury or interaction of condition by day post-injury in a two-way repeat measures ANOVA (F3,15=7.9358, p<0.001; F5,15=0.1881, p>0.05; F15,158=0.0976, p>0.05). In the female rats, the repeat subconcussion caused a significant deficit compared to sham. The repeat subconcussive group showed more deficits overall than both the single concussion and single subconcussion groups. These deficits extend to day 30. The single concussive group and single subconcussive group did not show deficits significant deficits compared to the control, but there are trends towards deficits in the single concussion group. We also compared the male and female rats' responses to the balance beam task (Figure 14D). Looking at the time to cross the beam corrected from baseline, there was an effect of condition as well as sex on the performance, but not an effect of day post-injury or interaction effect in a three-way repeated measures ANOVA (F3,38=12.334, p<0.001; F5,38=0.9637, p>0.05; F1,38=4.6466, p<0.05; F38,321=0.6166, p>0.05). Focusing on the sex difference, there is an overall difference between the male and female responses to injury. The female rats did significantly worse, therefore showing more deficits in locomotion than the males did regardless of injury. The baselines did not differ significantly, thus injury results in significantly more deficits in the females than the injured males.

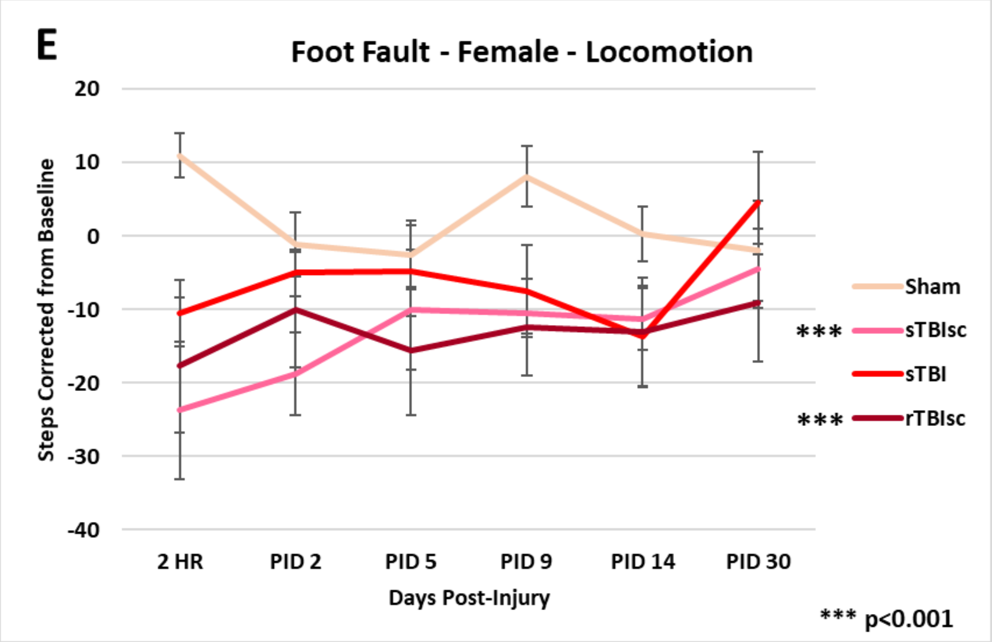
Foot Fault: Locomotor activity measured in female rats using the foot fault test by assessing the number of steps taken, showed similar trends to the male rats (Figure 14E). A two-way repeated measures ANOVA demonstrated that there were significant effects of condition, but not significant effects of day post-injury or condition by day post-injury on performance in the foot fault test (F3,18=8.7483, p<0.001; F6,18=1.6195, p>0.05; F18,185=1.7439, p < 0.05). The repeat subconcussion group, single subconcussion, and the single concussion group both showed significant deficit compared to sham groups. When comparing the males and females, there was a difference between males and females overall (Figure 14F). A three-way repeated measures ANOVA revealed significant effects of condition and sex on the locomotor activity, but not significant effects of day post-injury or condition by day post-injury by sex ($F_{3,38}=23.8861$, $p<0.001$; $F_{5,38}=1.1224$, $p>0.05$; $F_{1,38}=27.3702$, $p<0.001$; $F_{38,328}=1.1543$, $p>0.05$). Among both sexes, all of the injury groups showed significant deficits compared to the sham control group. Repeat subconcussive impacts showed the worst deficit, statistically worse than the single concussion group. The single subconcussion group did not show a statistical difference between the single concussion or the repeat subconcussion groups. Overall females did significantly worse in locomotion than males did.




B
Open Field - Sex Differences - Locomotion
Beam Breaks
2500
2000
1500
1000
500
0
Early
Late
Female Sham
Female sTBIsc
Female sTBI
Female rTBIsc
Male Sham
Male sTBIsc
Male sTBI
Male rTBIsc


C
Balance Beam - Female - Locomotion
Time Corrected from Baseline (seconds)
23
18
13
8
3
-2
-7
2 HR
PID 2
PID 5
PID 9
PID 14
PID 30
Day Post-Injury
Sham
sTBIsc
sTBI
***
rTBIsc
*** p<0.001


D
Balance Beam - Sex Differences - Locomotion
Time Corrected from Baseline (seconds)
20
10
0
-10
-20
-30
2 HR
PID 2
PID 5
PID 9
PID 14
PID 30
Day Post-Injury
Female Sham
Female sTBIsc
Female sTBI
Female rTBIsc
Male Sham
Male sTBIsc
Male sTBI
Male rTBIsc
*
* p<0.05


E
Foot Fault - Female - Locomotion
Steps Corrected from Baseline
20
10
0
-10
-20
-30
-40
2 HR
PID 2
PID 5
PID 9
PID 14
PID 30
Days Post-Injury
Sham
*** sTBIsc
sTBI
*** rTBIsc
*** p<0.001

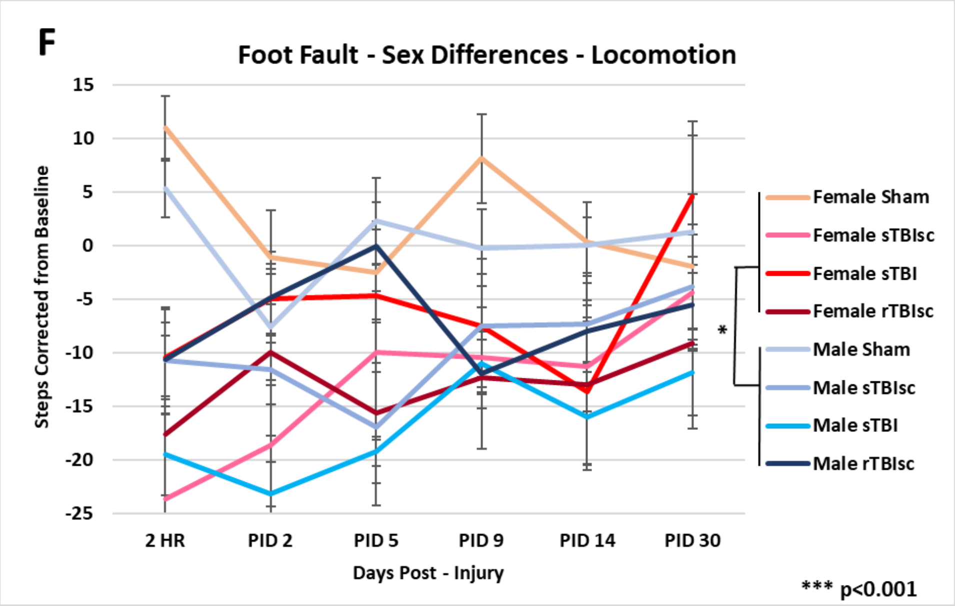


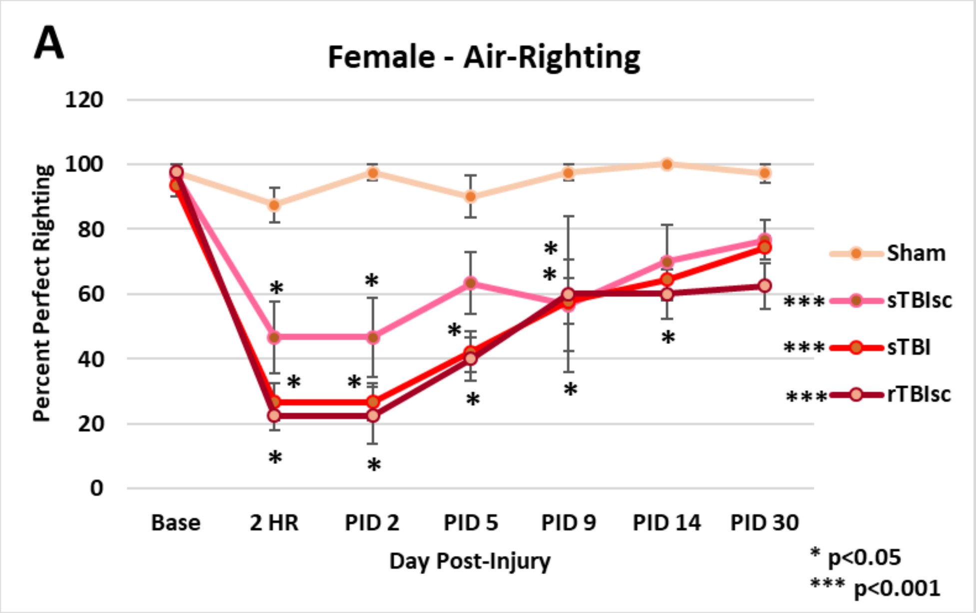
Figure 14: Females show more deficits than males in most tests of locomotion. Locomotion was analyzed by using the open field test (A, B), balance beam test (C, D), and foot fault test (E, F). In each of the tests the female groups are represented by gradients of red and the male injury groups are represented by gradients of blue. Using the open field, the amount of beam breaks was assessed in the female rats (A) at both an early time point (2 hours post-injury) and late time point (PID 28). There were no significant effects of injury type on performance, but there were decreases in the amount of beam breaks in the repeat subconcussion injury group. When comparing males and females (B) there were no statistically significant sex differences in the open field test. In the balance beam test, the female data (C) is shown as the time it took to traverse the beam as a change from baseline values. The data was taken from each behavioral testing day and there was an effect of injury type on performance. The repeat subconcussion group showed significantly longer time to traverse the beam compared to the sham group ($p<0.001$). When comparing the male and female performances in the balance beam test (D), there was a significant difference between the sexes. The females showed significantly longer times to cross the beam than the males overall (*$p<0.05$). This suggests females show more deficits in locomotion in the balance beam test than the males when receiving the same injury because the sham groups were not different. In the foot fault test, female data (E) was analyzed as the number of steps corrected from baseline at each behavioral testing day. The single subconcussion injury group and repeat subconcussion injury group showed significantly less steps than the sham group (***$p<0.001$). The single concussion group had less steps than the sham, but it was not significantly different. When male and female performances were compared in the foot fault test (F) there was a significant difference between male and female performance (*$p<0.05$). Overall, the females showed significantly fewer steps than the males, showing more locomotion deficits than the males.

iii. Righting and Vestibular Deficit – Air Righting Task

In the female rats there were similar responses to injury as seen in the male rats. A two-way repeated measures ANOVA revealed a significant effect of type of injury, day post-injury, along with a significant interaction between injury type and day post-injury ($F_{3,18}=66.9943$, $p < 0.001$; $F_{6,18}=31.1901$, $p < 0.001$; $F_{18,187}=3.3182$, $p < 0.001$; Figure 15A). The data show lower percent perfect righting in all three injury groups compared to the sham control ($p<0.05$). There were also significant deficits in the repeat subconcussion group and single concussion group compared to the single subconcussion group, but there was no significant difference between the single concussion and repeat subconcussion injuries.

Baseline performances were not significantly different between groups. These significant deficits in the repeat subconcussion group last from two hours post-injury until day 30 compared to baseline levels and day 14 compared to sham. The deficits in the single concussion group last until post-injury day 9. Single subconcussive injuries showed significant deficits compared to sham two hours post-injury, two days post-injury, and on day 9. The data suggest recovery, because no group shows significant deficit at post-injury day 30.

In the air-righting test, the data comparing the females to the males demonstrate that overall there is significant increase in deficits in the female population compared to the males ($p<0.05$; Figure 15B). A three-way repeated measures ANOVA showed a significant effect of injury type, day post-injury, sex, and an interaction effect of injury type by day post-injury by sex on righting reflexes ($F_{3, 45} =94.3535$, $p<0.001$; $F_{6,45}=44.7053$, $p<0.001$; $F_{1,45}=6.6441$, $p<0.05$; $F_{45,386}=2.6935$, $p<0.001$). There was a significant increase in deficit in females compared to males. The data did not show a specific time point for there being a significant difference between male and female responses, the females in the repeat subconcussion group did show worse performance on the air-righting task than the males in all time points except day 9. In the single concussion groups, the females did worse than the males until day 30, and the single subconcussion group for females had a worse performance than males on all days except day 5. Overall, females showed worse performance than males with each injury type, although they showed a better performance than the males on all days except the day of the injury in the sham groups.



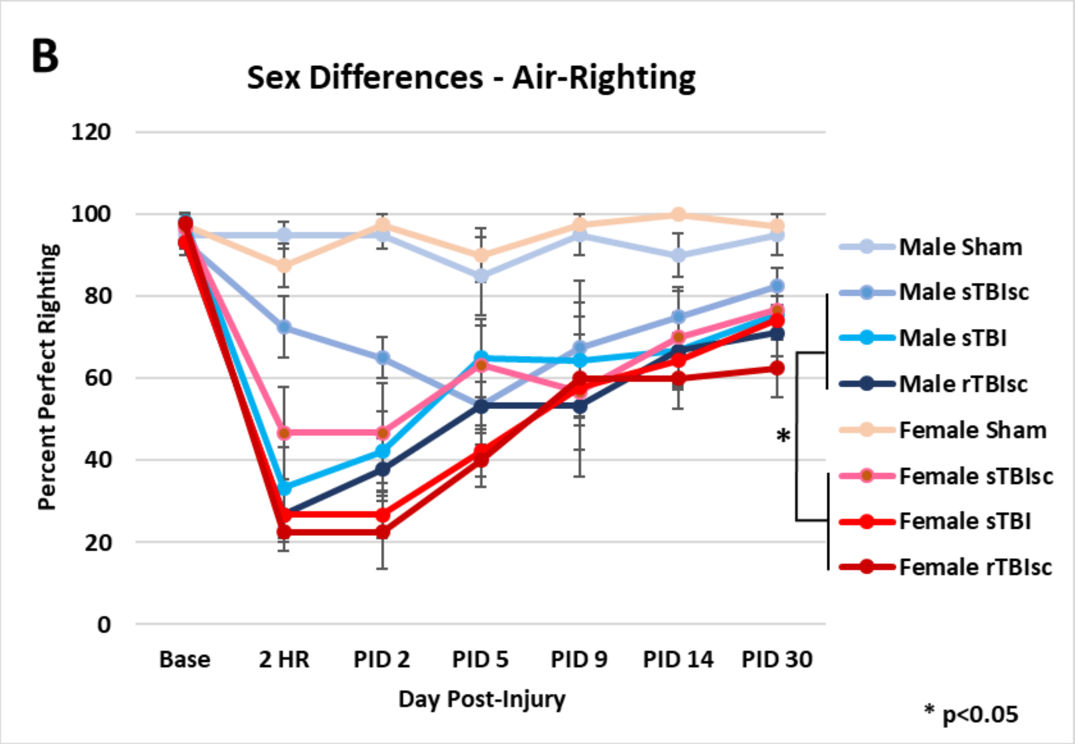


Figure 15: The air-righting test was used to assess righting reflexes and vestibular deficits. Percent perfect righting was used to compare the amount of trials out of the 5 that were landed without any deviations from perfect. The data for female rats (A) compares each injury group's percent perfect righting at each behavioral time point. All injury groups show significantly lower percent righting than the sham group (***p<0.001). The repeat subconcussion group and single concussion group had significantly worse performances than the single subconcussion group (p<0.001). The deficits in the single concussion group last until PID 9 and the deficits in the repeat subconcussion group last until PID 14. When comparing the male and female performances in the air-righting task (B) there were significant differences between the sexes. The females showed significantly lower percent righting than the males with sham groups that were not significantly different, suggesting all differences are in the injury groups (*p<0.05). Females show significantly worse deficits in righting reflexes and vestibular deficits than males after similar injuries.

c. Limbic Behaviors

i. Recognition Memory – Novel Object

The novel object recognition test was used to assess recognition memory in the female rats (Figure 16A) A two-way repeated measures ANOVA of the data from the female rats showed there was a significant effect of condition and day post-injury on performance, but not a significant interaction effect ($F_{3,3}=15.727$, $p<0.001$; $F_{1,3}=13.6494$, $p<0.001$; $F_{3,50}=0.3665$, $p>0.05$). The data demonstrate that the repeat subconcussion group showed deficits compared to the sham group over both days. The single concussion group and single subconcussion group showed trends towards deficits on day 30, but these were not statistically significant. Over time, performance on the task decreased significantly for all groups from day 2 to day 30.

When comparing the males versus females in the novel object task, there was not a statistically significant difference between the sexes, but there were different trends in the data (Figure 16B). A three-way repeated measures ANOVA revealed significant main effects of injury type and day post-injury, but not sex. It also revealed a significant interaction of condition by day post-injury by sex ($F_{3,10}=10.8750$, $p<0.001$; $F_{1,10}=59.3326$, $p<0.001$; $F_{1,10}=0.5824$, $p>0.05$; $F_{10,105}=3.3891$, $p<0.001$). There was not a statistical difference overall between the male and female responses to the novel object recognition test, there was a statistical difference between the repeat subconcussion groups' responses. The female repeat subconcussion group showed significantly more deficits than the male group on day 2, which was supported by the Tukey post-hoc ($p<0.05$). There was significant deficit in the male single concussion group on day 30, but not in the female rats. The data also suggests decreased performance on the test after time for both sexes and in all groups regardless of injury. The main sex difference between the groups is presented with the females producing the biggest deficits in the repeat subconcussion group, whereas the males produced the biggest deficits in the single concussion group.

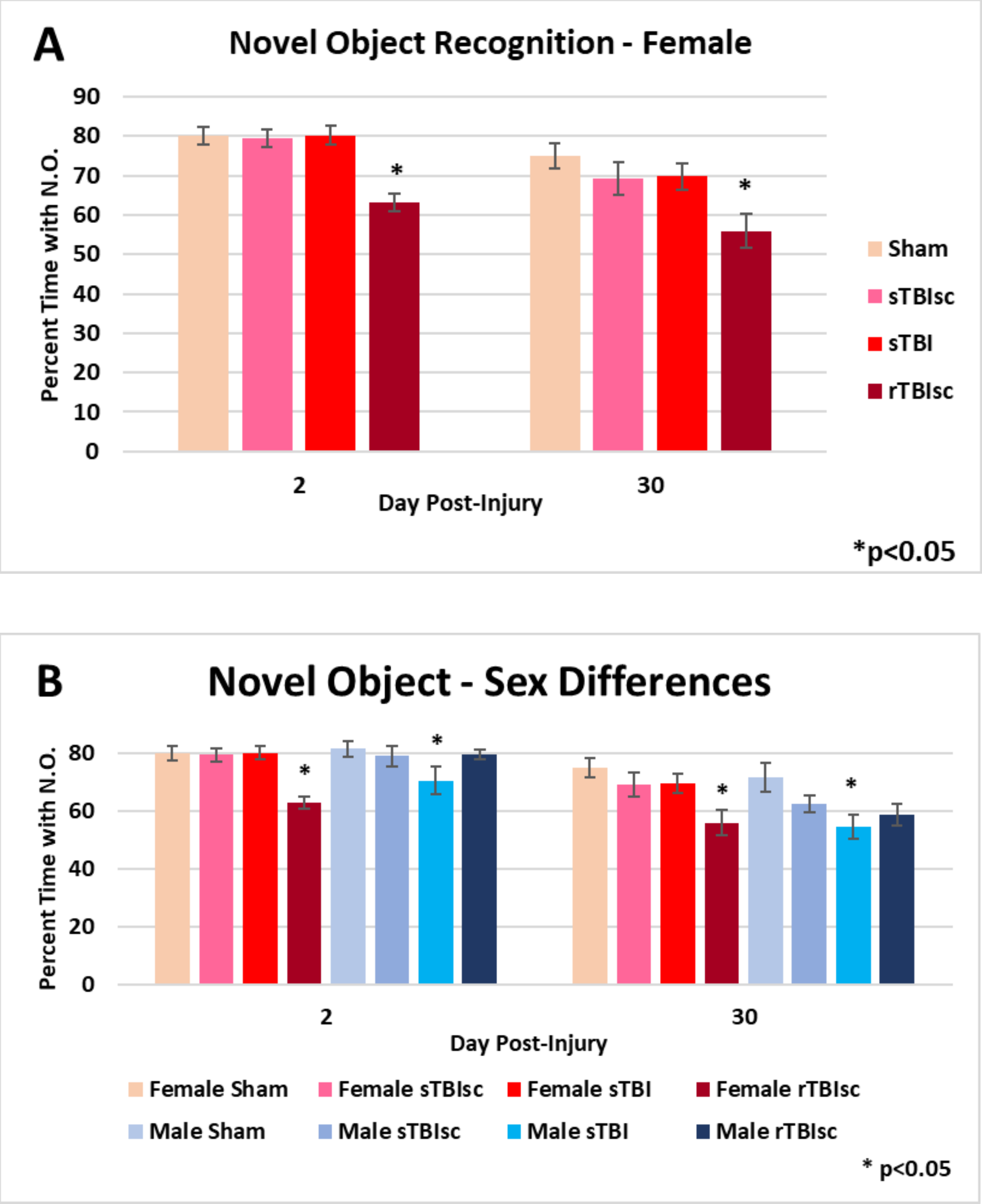


Figure 16: The novel object recognition test was used to assess recognition memory and data is shown as percent time that was spent with the novel object on PID 2 and PID 30. The groups for the females

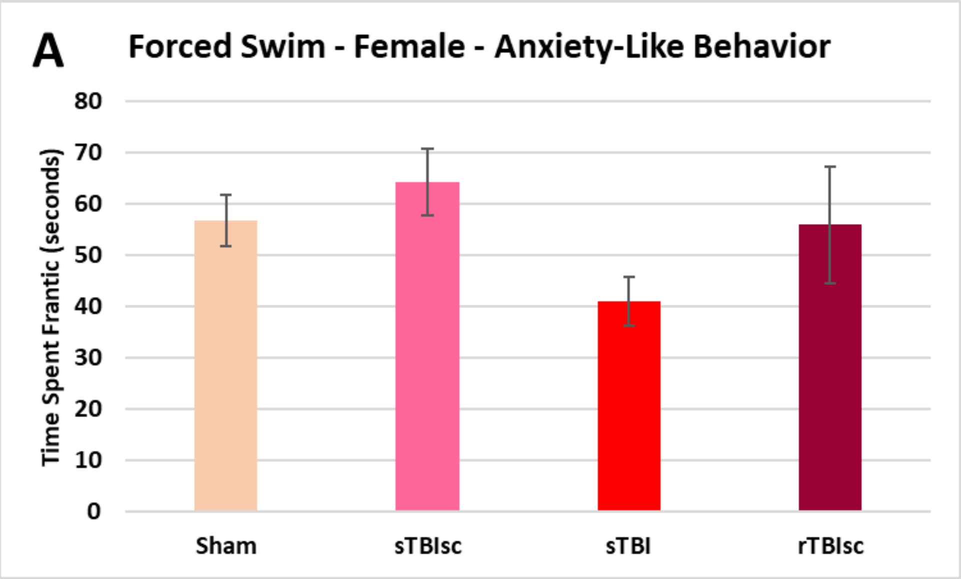
are shown in gradients of red and the males are shown in gradients of blue. The female data (A) shows significant effects of injury type on performance (*$p<0.05$). The repeat subconcussion group showed significantly lower percent of time spent with the novel object than the sham group, suggesting higher deficits in recognition memory. When comparing the male and female performances (B) there were no statistically significant sex differences overall. Nevertheless, the males showed significant deficits in recognition memory in the single concussion group while females showed significant deficits in the repeat subconcussion group.

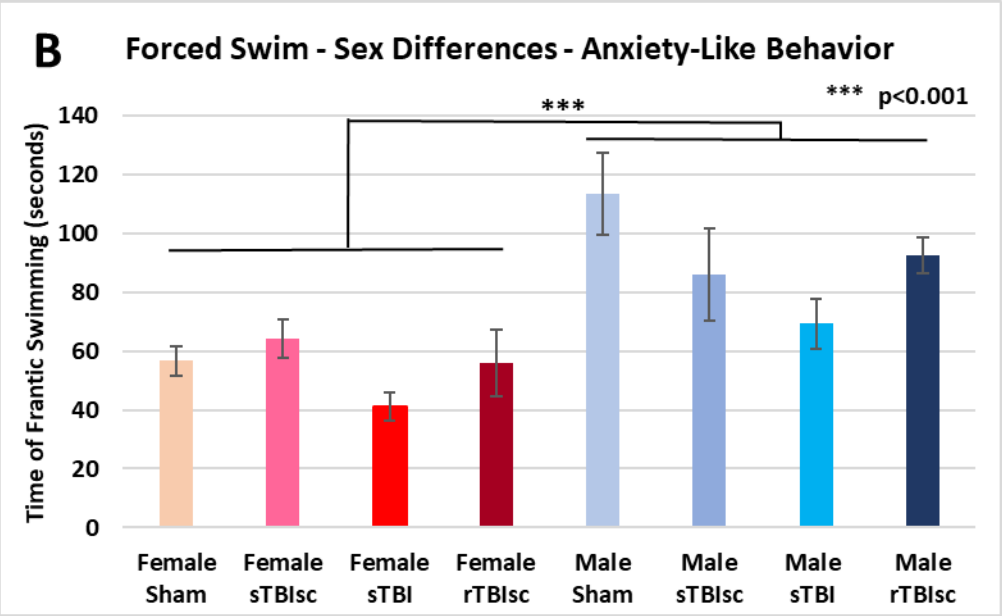
ii. Anxiety-Like Behaviors

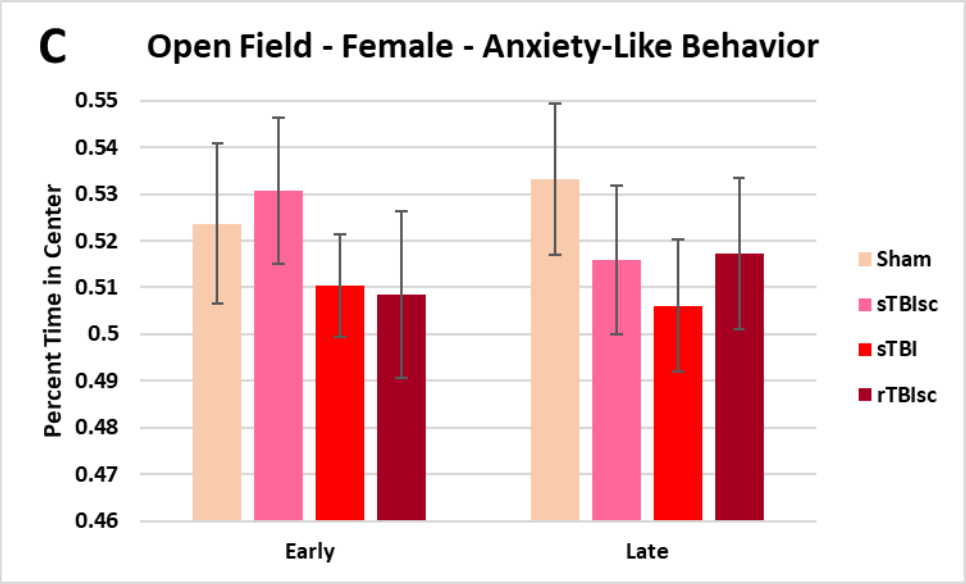
To assess sex differences in anxiety-like symptoms, both the forced swim test and open field test were used. The measurements of symptoms of anxiety in the females were calculated the same way as was done in the males which is explained above.

Forced Swim: Anxiety-like behavior of frantic swimming was measured in females using the forced swim test. A one-way ANOVA test revealed no significant effect of condition on performance ($F_{3,30}=1.6721$, $p>0.05$). There was an increase in the amount of frantic swimming in the single subconcussive group, suggesting possible increases in anxiety-like symptoms, but it was not statistically different from the sham (Figure 17A). Similarly, the single concussion group showed less frantic swimming than the sham, but not a statistical difference, while the repeat subconcussive group did not seem to differ from the sham. Changes in frantic swimming were compared between males and females to determine if there was a significant sex difference (Figure 17B). A two-way ANOVA revealed a significant effect of sex on performance, but no effect of condition or interaction effect ($F_{1,3}=32.013$, $p<0.001$; $F_{3,3}=0.07835$, $p>0.05$; $F_{3,60}=1.003$, $p>0.05$). Overall there was a significant increase in the amount of frantic swimming in the males compared to the females, suggesting that male rats experienced more anxiety-like behaviors than the female rats. This is probably not due to injury considering that the sham groups are also different from each other.

Open Field: In the female rats, the percent of time spent in the center was evaluated to determine anxiety-like behaviors post-injury. A two-way repeated measures ANOVA revealed no significant effect of condition, injury, or interaction (F3,3=0.7283, p>0.05; F1,3=0.001, p>0.05; F3,53=0.2645, p>0.05). Although there was not a statistical difference, there is a slight decrease in the amount of time spent in the center in both the single concussion and repeat subconcussion at both time points, along with a decrease in the single subconcussion at the late time point (Figure 17C). Both the female rats and male rats were compared to see any differing responses to the open-field (Figure 17D). A three-way repeated measures ANOVA revealed a significant effect of sex, but not a significant effect of condition, time-point, or interaction (F1,10=16.5668, p<0.001; F3,10=2.01, p>0.05; F1,10=0.1406, p>0.05; F10,111=0.3115, p>0.05). Overall, males spent significantly less time in the center than the females, suggesting higher levels of anxiety-like symptoms in the males than the females. In this test the sham groups were not significantly different, suggesting that all differences in response between males and females were in the injury groups.


A
Forced Swim - Female - Anxiety-Like Behavior
Time Spent Frantic (seconds)
80
70
60
50
40
30
20
10
0
Sham
sTBIsc
sTBI
rTBIsc


B
Forced Swim - Sex Differences - Anxiety-Like Behavior
*** p<0.001
***
Time of Frantic Swimming (seconds)
140
120
100
80
60
40
20
0
Female Sham
Female sTBIsc
Female sTBI
Female rTBIsc
Male Sham
Male sTBIsc
Male sTBI
Male rTBIsc



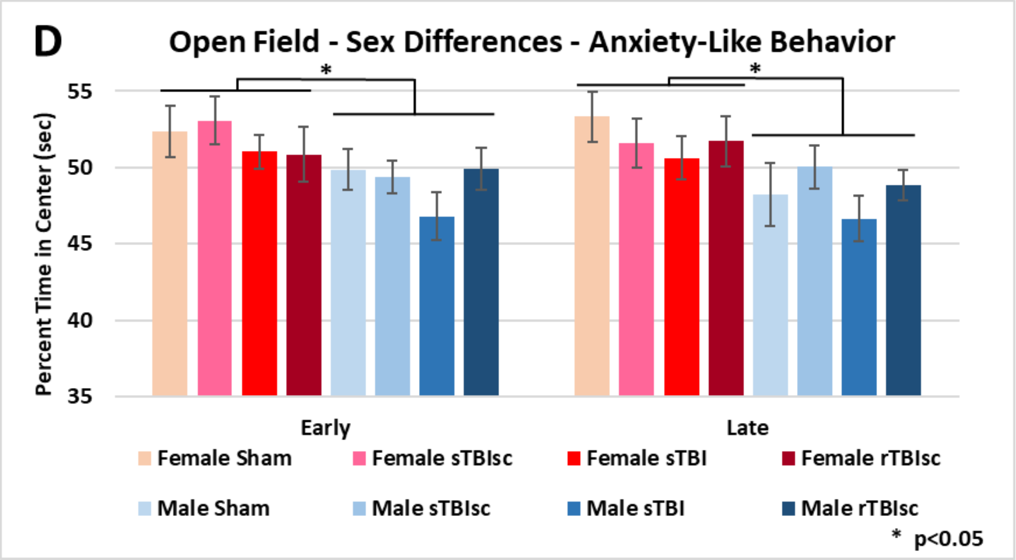


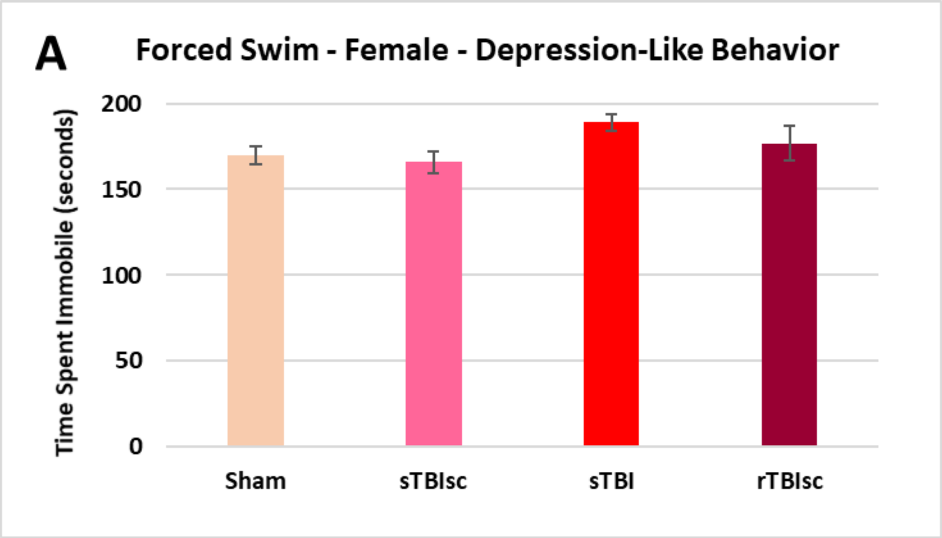
Figure 17: Males showed more anxiety-like behavior than females. The forced swim test (A, B) and the open field test (C, D) were used to assess anxiety-like behaviors. For all tests the female data was shown in gradients of red and the male data was shown in gradients of blue. In the forced swim test the female data (A) was assessed by looking and the time spent swimming frantically on PID 31. The females did not show a significant effect of injury type on time spent frantic ($p>0.05$). The single concussion and repeat subconcussion group did show a reduced amount of time frantically swimming, but it was not statistically significant. Comparing the male and female performances in the forced swim test (B) the males and females showed significant sex differences. The males showed significantly longer time spent frantically swimming compared to females, suggesting that males showed more anxiety-like symptoms ($p<0.05$). In the open field test, the performance of the injury groups in the females (C) was compared by assessing the percent of time spent in the center of the field. There were no statistically significant

deficits in any injury groups, but there were reductions in percent time spent in the center in the single concussion and repeat subconcussion groups ($p>0.05$). When comparing males and females (D) there were significant differences between the sexes. The males showed significantly less time spent in the center than the females (*$p<0.05$). Both tests suggest that males had more anxiety-like symptoms post-injury.

iii. Depression-Like Behavior

A one-way ANOVA revealed no significant effect of condition on time spent immobile in the forced swim task ($F3,30=2.1632$, $p>0.05$). This suggests that injury in the female rats did not create depressive-like symptoms (Figure 18A).

When comparing the males and females, there were no significant sex differences in learned helplessness and depression-like behavior although there was a trend for injury to increase time spent immobile more in males than females. A two-way ANOVA did reveal a significant effect of condition and interaction, but not sex ($F3,59=4.0755$, $p<0.05$; $F1,59=0.9144$, $p>0.05$; $F3,59=3.7435$, $p<0.05$). When looking at overall condition, there was a significant increase in immobility in the single concussion group and repeat subconcussion groups (Figure 18B). This suggests that these two injuries do cause depression-like symptoms such as learned helplessness in the rats. There was also an increase in symptoms in the single subconcussion group, but not a significant difference compared to sham.



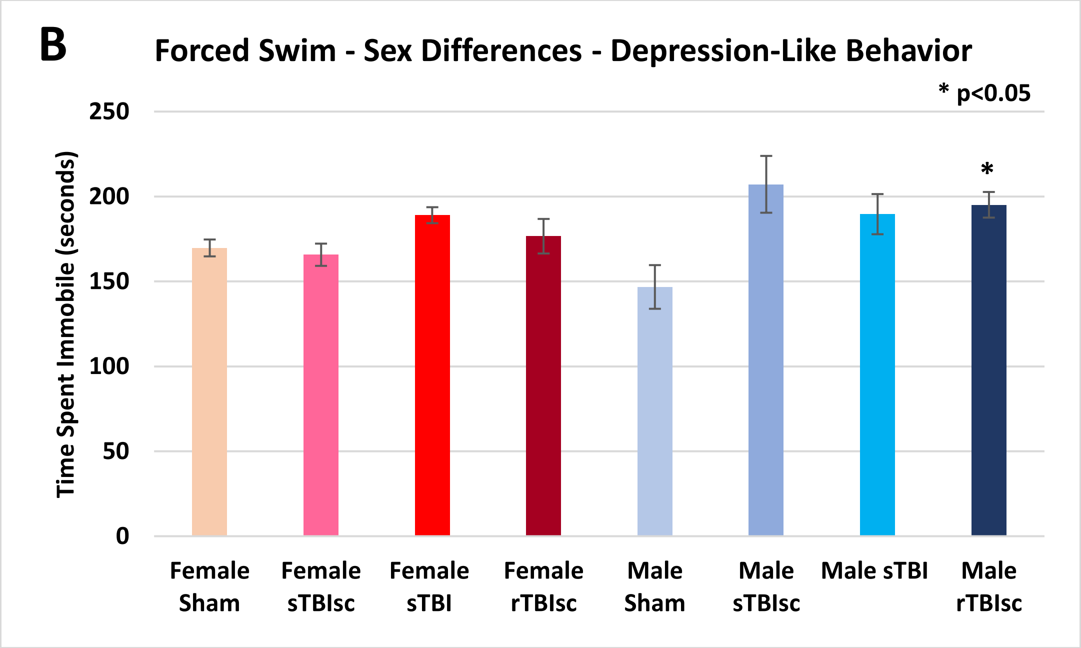


Figure 18: The forced swim test was used to assess depression-like behavior. In these tests the time spent immobile was assessed on PID 31. The female data are shown in gradients of red and the males' data are shown in gradients of blue. (A). In the females, there were no significant effects of injury type on performance ($p>0.05$). The single concussion group did show a longer time spent immobile than the sham, but it was not statistically significant. When comparing the male and female performances (B) there were no statistically significant sex differences. There were differences in which injury groups showed deficits between males and females. In males, the repeat subconcussion group showed significant increases in symptoms of depression (*$p<0.05$), but in females no groups showed significant increases in depression-like symptoms.

d. Summary of Experiment Two

Sex differences were present in many of the responses to injury. In tasks that are more “motor” or vestibular, the females showed more significant deficits in behavioral responses after injury than the males. Females show more deficits in motor coordination, locomotion, and righting reflexes. In the tasks that are more “limbic” tasks, the males showed more significant deficits in responses after injury than the females. The males showed increased levels of anxiety-like behaviors and depression-like behaviors compared to the females.

XI. Discussion and Conclusions

a. Experiment One: Rodent Model of Repeat Subconcussion

Experiment one of this thesis was focused on creating a model of repeat subconcussion. Our study was successful in establishing a clinically relevant model of repeat subconcussion in the adult rat using a closed head injury device. Using the model already established in the lab by Jamnia et al. (2017), the first experiment of my thesis altered the depth and rate of injury to produce a repeat subconcussive impact.

Overall, rats with repeat subconcussive injury show similar or worse deficits than rats in the single concussion group, with those receiving single subconcussive impacts not showing many deficits. This resembles what is seen in the human condition. In a clinical study done by Bazarian et al. (2012), changes in white matter were assessed using diffusion tensor imaging. The patients with repeat subconcussive head blows showed changes in white matter that were three times the changes in the control group in both fractional anisotropy and mean diffusivity. The subjects with a single concussion showed similarly high changes in white matter compared to the subjects with multiple subconcussive injuries. Similarly, in a study done in high school football players the symptomology and neurological performance of the players post injury was assessed. The injuries were characterized into three groups: no clinically diagnosed concussion with no changes in behavior, no clinically diagnosed concussion with changes in behavior, and a group with a clinically diagnosed concussion with changes in neurological behavior. These different groups were then diagnosed as patients with a single subconcussion, repeat subconcussive events, and a clinical concussion, respectively (Talavage et al., 2014). The results of this study reflect the results seen in our injury groups very closely. It is also important to note that this model did not produce pathology on the surface of the brain in the repeat subconcussion, single concussion, or single subconcussion groups, similar to clinical patients (Tagge et al., 2018).

There is an extensive list of symptoms associated with traumatic brain injury which varies between patients, but is typically broken down by physical, cognitive, emotional, and motor symptoms. In the experiments in this thesis, behavioral tests were consolidated into two categories: motor skills and limbic skills. Motor skills rely on the motor cortex, vestibular system, and cerebellum. These include behaviors regarding posture, balance, and controlling biomechanics and movement (Adolph and Franchak, 2017). Limbic skills rely on the amygdala, hypothalamus, and thalamus, and are considered more “emotional” or “cognitive” behaviors. These behaviors include anxiety, depression, and processing of memory (Rajmohan and Mohandas, 2007; Kruetzer et al., 2001; van der Horn et al., 2016). Our model shows deficits in many of the behaviors that have been outlined above and are associated with symptoms of concussions.

i. Motor Skills

Neuromotor impairment is a common symptom of concussions, falling into the more physical category of symptoms, and can last for longer than weeks in clinical patients (CDC, 2010). In our model, the foot fault test did not reveal significant deficits in the repeat subconcussion group or the single concussion group. The repeat subconcussion resulted in less motor coordination deficits in this test, which was unexpected. Similarly, the balance beam did not show expected results. In the balance beam test, there were no statistically significant deficits in motor coordination due to injury type. In a previously published model established of single concussion and repeat concussion, there were significant deficits seen in the injury groups compared to the sham group, unlike our data (Jamnia et al., 2017). Our model of injury could be too mild to show deficits in the less-sensitive tasks used to assess motor coordination which are typically used in more moderate injuries seen in other models. It is also possible that the foot fault test and the balance beam task are not completely effective means of studying motor coordination post-concussion. There are separate motor coordination tasks such as the rotarod task that could be used in future studies to try to better assess motor coordination.

In clinical settings, deficits in locomotion are seen in patients with traumatic brain injury. Patients with moderate to severe TBI showed slower gait speeds in multiple tests than the control patients without neurological problems in group comparison study (Vallee et al., 2006). In previous mouse models of repeat subconcussion there has been no significant evidence of locomotion deficits, as measured by swimming in the Morris Water Maze, which does not assess the same locomotion as the tests in this study as swimming does not require the same type of somatosensory feedback as does locomotion on hard surfaces (DeFord et al., 2002). The current study used the open field test, balance beam test, and foot fault test to examine locomotion deficits. In the open field test, our model revealed significant deficits in locomotion in the repeat subconcussion group. There were also non-significant deficits seen in the single concussion group and single subconcussion group. In the foot fault test, there were significant locomotor deficits in the repeat subconcussion, single concussion, and single subconcussion groups. The repeat subconcussion group showed the most deficits in locomotion compared to all other groups, and those deficits were chronic up to post-injury day 30. Similarly, the single concussion group showed deficits from the acute time points up to the chronic time points. The balance beam test also showed decreased locomotion in the repeat subconcussion group, but deficits were not statistically significant. Overall, there were significant deficits on locomotion in the repeat subconcussion group, with some deficits being also seen after the single concussion injury. Similar results were seen in a clinical study that assessed the locomotor effects of traumatic brain injury in highly functional young adults. In the study there were significant decreases in walking speed and stride lengths, which was translated as deficits in locomotion compared to the healthy no-concussion controls (McFadyen et al., 2003). In the model that was previously established in this lab, the rats with head injuries showed a slight increase in the activity levels in the first day post-injury, but then on post-injury day 5 there was hypoactivity that was similar to the deficits seen in this model (Jamnia et al., 2017).

Righting reflexes are affected after traumatic brain injury. Measuring righting reflexes in rats is a method for testing vestibular deficit, loss of consciousness, and issues with physical coordination that can occur in clinical patients (Frank et al., 1963). Although contact righting is commonly used to assess these deficits, air-righting is also another method of testing righting reflex (Ossenkopp et al., 1990). In previous models, mice with traumatic brain injury via a fluid percussion model showed significantly reduced righting reflexes compared to the sham controls (Carbonell et al., 1999). In our model, righting deficits were seen in all injured rats overall, but the most prominent deficits were in the repeat subconcussion group and the single concussion group, with minimal deficits seen in the single subconcussion group. These deficits were both acute and chronic with significant decreases in performance seen starting at post-injury day 2 and continuing until post-injury day 30. Reflecting similar conclusions to our results, a study done by Bolton and Saatman used righting reflex to assess coordination and loss of consciousness in mice with single and repeat concussive injuries. In this model, the righting reflexes showed significant deficits in the injury groups compared to sham, and the intensity of injury correlated with the extent of the deficits (Bolton and Saatman, 2014). In a blast model of traumatic brain injury, injured rats showed deficits in contact righting compared to the sham comparison group (Readnower et al., 2010). Righting reflexes are a common assessment of loss of normal functioning in rats, and our model presents deficits in righting reflexes.

Compared to the symptoms seen in humans, we have been able to capture similar motor deficits in rats using this model of repeat subconcussions. This model produced deficits in both locomotion and righting reflexes, which involves deficits in vestibular functioning.

ii. Limbic Skills

One of the more common deficits seen in patients with traumatic brain injury is memory loss (Andriessen et al., 2010). In a previous model of single concussion and repeat concussion there were significant deficits in recognition memory in the injury groups shown by decreased percentage of time spent with the novel object (Jamnia et al., 2017). In our study, deficits were present in recognition memory in the repeat subconcussive group, similar to the previously established model. Also, performance decreased over time in all rats including sham rats, suggesting that deficits got worse over time. We can attribute our results as a study of recognition memory with very little interference from stress placed upon the rat because the test does include a habituation period to the arena and familiar objects, along with utilizing natural behavior of the animals (Akkerman et al., 2012). This provides a reference supporting our model as replicable and helps establish that our model showed clinically relevant recognition memory deficits.

There is strong evidence suggesting that there is a link between repetitive traumatic brain injury and anxiety (CDC, 2016; Lipkind et al., 2004). Using the forced swim test, our study showed decreased anxiety-like behavior in the single concussion group compared to the sham group, which was unexpected. The other injury groups showed changes that were not statistically significant, but still showed decreased anxiety levels and not increased. In our model, the open field test showed an increase in anxiety-like symptoms in only the single concussion group. In the other model previously established in this lab of a repeat concussion, the repeat concussion group showed significantly higher levels of anxiety as measured by lower percent time spent in the center compared to the sham group in the open field test (Jamnia et al., 2017). Our results in the single concussion group do not replicate these and contradict many other studies in the field. It is possible that the increased amount of handling could have altered the rats' responses to the tests assessing anxiety-like behavior. It also could be possible that there is variability regarding the anxiety response following injury. Although definitely present, there are studies that show clinical patients respond to mild traumatic brain injury with anxiety in only 27-49% of cases, with the higher percentage happening 3 days post-injury (Dischinger et al., 2009). Our tests could have been done too early and too late for the effects in anxiety to have shown or it could have also not had a prevalent effect in a small population.

Depression has been a common symptom of traumatic brain injury, with 27-42% of patients showing enough depression symptoms to constitute major depressive disorder (Kreutzer et al., 2009; Seel et al., 2003; Harmon et al., 2012). Using the forced swim test, our model shows increases in learned helplessness or depression-like behavior in the single subconcussion group and repeat subconcussion group. Although the symptoms seen in the single subconcussion group are unexpected, the presence of depressive-like behavior in the repeat subconcussion group reflect expected results. This model of repeat subconcussion reflects the depression symptoms seen in clinical patients with mild traumatic brain injury. The data in this thesis also reflects data seen in animal models. In a fluid percussion model of repeat mild traumatic brain injury in adult rats, the repeat impact group showed a significantly longer amount of time spent immobile in the Porsolt forced swim test compared to sham, suggesting higher levels of depression-like behavior (Shultz et al., 2012).

There are multiple methods of modeling closed head traumatic brain injury in rodents including fluid percussion, weight-drop, and controlled cortical impact. The controlled cortical impact is a well-studied and published method of traumatic brain injury that regularly demonstrates reproducible deficits in behavior and cognition (Walker and Tesco, 2013). We have previously adapted the controlled cortical impact model so that it produces an impact on the surface of the head. In this study, we were able to further adapt a commercially available controlled cortical impact device to produce a repeat subconcussion that showed behavioral and cognitive deficits similar to a single concussion. There is previous evidence that repeat subconcussive injuries can produce similar effects as a single concussion, which provided a reference point for our expected behavior when choosing the rate of injury (Talavage et al., 2014; Bailes et al., 2013). Also, in this model the single subconcussion injury did not show deficits in most tasks, which is similar to previously published work by Shultz et al., (2012) who found a lack of difference in behavioral tasks between the subconcussive injury group and sham control in measures of anxiety-like behavior, cognition, sensorimotor functioning, and locomotion. These deficits are clinically applicable to symptoms seen in patients who have incurred a mild brain injury and some of the tests have a better translation to human symptomology than other. In this study the tests for locomotion, righting reflexes, recognition memory, and depression were important in determining extent of injury in our model. Each of these behavioral and cognitive symptoms are commonly assessed in patients that present with traumatic brain injury and can be a way to make animal models more translatable to the clinic.

b. Experiment Two: Sex Difference in Repeat Subconcussions

Studying differences between males and females in research has become a prime topic in neuroscience and the NIH. As a large majority of attention to the field of TBI has been the result of examining athletes, it's important to note that the NCAA reported that athletic participation was 56.6% male and 45.4% female in the season of 2013 to 2014 (Johnson, 2014). Having an almost even representation of both genders in sports supports the need to find treatments that have been tested in both men and women instead of using a treatment that does not consider gender. The NIH now mandates that scientists account for the possible role of sex as a biological variable and requires sex be studied in almost all studies of neurodegeneration (NIH, 2015). Understanding sex differences in the response to repeat subconcussion will not only allow for more clinical application but will also add to the expanding field of sex differences research in traumatic brain injury.

There are many published works that examine sex differences in traumatic brain injury, but less examples in subconcussive injuries. A study by Bazarian et al. assessed sex differences in clinical patients of mild traumatic brain injury by looking at symptomology in general and giving patients a post-concussion symptom (PCS) score. There was a significant difference in the odds of females having poorer outcomes post-injury with regards to the PCS score than males (Bazarian et al., 2010). When looking at a study of symptomology after traumatic brain injury, there was a significant difference in which symptoms were reported by male and female patients. In male patients there were significantly higher numbers reporting restlessness, sensitivity to noise, and sleep disturbance. In females there were more issues reported in headaches, dizziness, and lack of initiative/anxiety (Colantonio et al., 2010). In a study of soccer players who did not receive concussions but received subconcussive events from ball heading, there were significant differences between males and females on impact kinematics. In females, there was a significant increase in the rotational velocity after impact with the soccer ball than males, which was correlated with less neck strength through flexor strength (Bretzin et al., 2017). Although clinical studies are beginning to focus on sex differences following subconcussion there is a lack of studies that examine sex differences in behavioral and cognitive responses after subconcussive injury in an animal model.

Using the model of subconcussion previously established in experiment one of the thesis, we assessed where sex differences exist in behavioral responses to repeat subconcussive injuries. Overall, female rats showed more deficits in motor coordination, locomotor functioning, and vestibular reflexes than males, but male rats showed increases in anxiety-like symptoms and depression like-symptoms. In previous studies in a clinical setting, females showed higher scores in Post-Concussive symptom (PCS) scores compared to males, which was more prominent in females before menopause (Bazarian et al., 2010). Women with higher levels of estrogen than the women in a later age group showed the worse effects, suggesting that disruption in normal estrogen levels could be responsible for differences in responses to similar mild traumatic brain injury.

i. Motor Skills

In the current study, females showed more deficits overall than males in some of the tests of motor coordination. In the foot fault test, data revealed there were significantly more deficits in motor coordination seen in female rats than male rats. On the contrary, in the balance beam test the females did not show significantly different performance in motor coordination than the males. In another study done on mice, motor coordination was assessed using rotarod. In this study, the males showed a longer latency to fall than the females, suggesting that the females showed more intense deficits in motor coordination (Tucker et al., 2016). This suggests that post-traumatic brain injury, females tend to show more deficits in motor coordination than males.

Females also demonstrated more deficits than the males in some of the tests for locomotion. In the balance beam and foot fault tests, females performed significantly worse than males following injury. Despite the lack of significant effects in the open field test, overall there were more deficits in locomotion seen in the female rats compared to the male rats with a similar injury. This is supported by studies showing higher effects of injury on locomotion in females as opposed to males (Wirth et al., 2017). However, in another study, female mice showed higher levels of locomotion than the males when measured with swimming speeds in the Morris Water after traumatic brain injury (Velosky et al., 2017). The contrasting results of this study could be a result of the fact that swimming is not an on-land activity requiring different somatosensory feedback, unlike the tests done in this thesis. In locomotion, there is some question as to whether females show worse or better locomotion than males after traumatic brain injury, but more study needs to be done to truly assess the differences in locomotor activity. It may be that different types of locomotion are differentially affected by TBI.

Female rats showed vestibular deficits that were significantly worse than the males, showing decreased righting reflexes. Another study did find results similar to the current study using a mild traumatic brain injury model that produces a rapid head rotation. In this model, the female rats showed more widespread effects on activity and spatial memory; these effects expanded to include righting reflexes where the females showed more deficits after the mild traumatic brain injury than the males (Wirth et al., 2017). Although more study needs to be done on righting reflexes, the evidence thus far provides validation that females show significantly more severe symptomology in righting reflexes after traumatic brain injury.

ii. Limbic Behaviors

Recognition memory following repeat subconcussion did not show overall significant sex differences, but there were different responses of the groups to specific injuries in each sex. In the female rats there were significant deficits seen in the repeat concussion group, with smaller deficits seen after the single concussion and single subconcussion. In male rats, there were more memory deficits in the single concussion group, than with the deficits in the repeat subconcussion group. Although not statistically significant, these differences show that instead of an overall increase or decrease in deficits between males and females, there was a difference in the pattern of responses based on sex. In a clinical study done comparing male and female athletes who had previously had concussions, there were no sex differences seen in a test of visual recognition memory (Majerske et al., 2008). On the other hand, a study done on male and female soccer players showed that females reported lower scores on visual memory than the males, but another study using the same methods of examining visual memory did not find sex differences with regard to visual memory impairments (Covassin et al., 2013; Zuckerman et al., 2012). This suggests that our data relates to clinical sex differences and that there are not always significant differences in recognition memory post-injury. To our knowledge there is currently no other work looking at sex differences in recognition memory after traumatic brain injury in animal models and all the work is done in spatial memory tasks. This further supports the need for more work in cognitive differences between males and females after traumatic brain injury.

Using the forced swim test, this model showed significant sex differences in anxiety-like behaviors. Interestingly, overall the male rats showed significantly higher levels of anxiety-like behavior than females in all groups. Similarly, using the open field test, the males showed significant increases in anxiety-like symptoms compared to females. These baseline differences in anxiety-like behavior were not differentially affected by the injury. In humans, although women generally present with higher levels of anxiety than men without injury, men tend to have higher responses to the HPA axis than women. The HPA axis is the mechanism responsible for increases in anxiety through corticotropin after stress or injury, and it is possible that women are more resilient to increasing corticosterone effects than males (Kudielka et al., 2004; Altemus, 2006). This suggests that the males could have shown a higher increase in the injury groups compared to sham because alterations in the HPA axis cause more intense reactions in the males than females.

We also used the forced swim test, to examine depression-like behavior. The data showed no statistically significant sex differences in immobility, but the model did show different trends between the sexes. In the females there was no significant effect of injury on time spent immobile, but in the males, there was an increase in time spent immobile seen in both the single subconcussion and repeat subconcussion injuries. The sham groups between males and females were not significantly different, therefore male rats seem to be more affected by the injury than females. In clinical research, females report higher levels of depressive symptoms in the first 6 months after mild to moderate traumatic brain injury, but after that point there were no significant sex differences (Bay et al., 2009). It is possible that our timepoint at PID 31 was too late to see sex differences in the rat model of repeat subconcussions, but it is also possible that examining a complex symptom like depression is difficult in an animal model. Similar to anxiety, at normal baseline, without injury, women have also had higher levels of depression than men, but after one of many forms of stress females seem to have a relative resistance to increasing depression levels while males seem to have a greater HPA axis response (Altemus, 2006). This suggests the possibility that after injury, female rats were more resilient to increases in depression symptoms, while the males were more sensitive to them. Further research is needed to elucidate sex differences in anxiety and depression.

There are multiple studies that have shown sex differences in responses to TBI, but there are also some that have found none. In a study done of patients following mild traumatic brain injury looking at neuropsychological tests there were no significant differences between male and female performances (Tsushima et al., 2009). That said, this study was done two years post-injury, making it hard to compare to our data in which sex differences were assessed at a more acute time point. More often though, there are studies that further establish the prevalence of sex differences post-traumatic brain injury. For example, in male and female collegiate athletes, the female athletes showed 1.7 times greater deficits in reaction times and more post-concussion symptoms than male athletes in sports of the same caliber (Broshek et al., 2005). More study is needed to further understand sex differences after traumatic brain injury overall. Nevertheless, the current study provides evidence of sex differences in repeat subconcussive injuries.

c. Conclusions:

This thesis produced a clinically relevant closed-head model of repeat subconcussive events in the adult rat using a controlled cortical impact device. Our model results in clinically relevant deficits in locomotor function, reflexes, recognition memory, anxiety like-behavior, and depression- like behavior. Our model also produces no pathology on the exterior of the brain, suggesting further similarities to a clinical repeat subconcussion.

Using this model, we were able to demonstrate some differences between male and female behavioral responses to repeat subconcussive events. Females showed significantly worse performance in motor-based tasks post-injury and males showed significantly worse performance on limbic-based tasks post-injury. This additional evidence of sex differences following TBI is important to advise further research and provide support for updating treatment plans for traumatic brain injury in a clinical setting so that they take into account gender.

d. Future Directions:

The current study focused primarily on the behavioral responses to subconcussive events. Future studies need to assess the cellular and neuroinflammatory responses in this model to further characterize the cellular response to injury. Although we do have extensive behavioral data supporting deficits in this model, it was beyond the scope of this thesis to examine cellular responses. Microglia and astrocytes play an important role in neuroinflammation after neurotrauma, while neuronal death would be an important measure of intensity of injury (Chui et al., 2016). Similarly, the tau protein has been identified as a significant long-term consequence of traumatic brain injury. In the tissue that has been preserved from this study, it would be interesting to assess the neuroinflammation, neuronal death, and tau levels found in the injured tissue.

The mechanisms underlying the sex differences presented in this study need further study. For example, studies examining biomechanics of the injury that occurred could be done to provide evidence for physical differences between sexes, such as size of the animal or size of neck muscles. These have been shown to effect soccer players who are regularly heading the ball. Female athletes have been shown to have up to a 32% increase in linear acceleration than males which could be a reason for increased behavioral and cognitive deficits post-injury (Tierney et al., 2008). It would also be interesting to compare the data of this study to an impact that is more severe and assess the scale of sex differences depending on the gradient of injury type. The influence of hormones on the sex differences need further examination. A study could be done with this model and include ovariectomized females to assess reaction to injuries when there is a lack of estrogen completely along with an increase in estrogen. The tissue taken from this study could possibly be used to assess the expression of sex hormones and their receptors (Taves et al., 2011). Then when assessing sex differences in anxiety levels, it would be interesting to assess cortisol levels in the rats to compare the behavioral responses to levels of cortisol in the blood, both at rest and following exposure to a stressful environment.

Animal model studies can be used to further inform the scientific community of the effects of concussions and inform future research in traumatic brain injury. There is a lack of education of the symptoms and consequences of concussions in athletic groups that needs to be remedied. In a cross-sectional study of over 300 varsity football players, 25% of the high school students were ignorant to concussion education and did not have an appropriate knowledge of the symptoms (Cournoyer and Tripp, 2014). Even further, in a study done on female athletes from metropolitan areas, 33% of female athletes did not report signs and symptoms of concussions that they incurred (McDonald et al., 2016). Examining the differences seen in between sexes could be important to ensuring female athletes also are educated in concussion symptoms and could lead to an increase the percent of reported concussions in athletes.

XIII. References

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