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ASSESSING THE EFFECTS OF REPETITIVE MILD TRAUMATIC BRAIN INJURY ON RISK/REWARD DECISION MAKING AND CATECHOLAMINE ASSOCIATED PROTEINS

by
Christopher Paul Knapp, B.S.

A Dissertation

Submitted to the
Department of Cell Biology & Neuroscience
Rowan-Virtua School of Translational Biomedical Engineering & Sciences
In partial fulfillment of the requirement
For the degree of
Doctor of Philosophy
at
Rowan University
May 1, 2024

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Acknowledgments

This work was supported by the New Jersey Commission on Brain Injury Research (NJCBIR) grants: CBIR20PIL004 to R.L.N., CBIR17PIL007 and CBIR19IRG025 to B.D.W., Congressionally Directed Medical Research Programs, the United States Department of Defense Traumatic Brain Injury and Physiological Health Research Program grant W81XWH-22-1-0616 to R.L.N. and W81XWH-22-1-0618 to B.D.W., and the Osteopathic Heritage Foundation for Primary Care Research Award to R.L.N.

First, I would like to thank Dr. Rachel Navarra, for her unwavering support and guidance throughout my graduate training. Since joining your laboratory, I have grown tremendously as a scientist and as a person. I am honored to be your first Ph.D. student.

I additionally would like to thank Dr. Barry Waterhouse for his mentorship and guidance throughout my academic journey. You and Dr. Navarra inspired me to pursue a career in neuroscience research and I will be forever grateful to you both.

To Drs. Daniel Chandler (Chair), Daniel Manvich, and Corina Bondi, it has been my utmost pleasure to have received your support, guidance, and expertise throughout my graduate training. Thank you for serving as members of my thesis committee.

I want to thank the members of the Navarra/Waterhouse laboratory, including Douglas Fox and Karen Joyce for their support during my training. To Eleni Papadopoulos and Haven Predale, you both have become life-long friends and I am grateful for your constant support and encouragement. I look forward to having more adventures together.

Most importantly, I am grateful to my family for their unconditional love and support throughout my life. They have always been by my side and I cannot thank them enough for everything they have done for me.

Abstract

Christopher Paul Knapp
ASSESSING THE EFFECTS OF REPETITIVE MILD TRAUMATIC BRAIN INJURY ON RISK/REWARD DECISION MAKING AND CATECHOLAMINE ASSOCIATED PROTEINS
2023 – 2024
Rachel Navarra Ph.D.
Doctor of Philosophy

Mild traumatic brain injury (mTBI) disrupts cognitive processes that influence risk taking behavior; however, the effects of repetitive mild injury (rmTBI) or whether these outcomes are sex specific are unknown. Risk/reward decision making is mediated by the prefrontal cortex (PFC), which is densely innervated by catecholaminergic fibers. Aberrant PFC catecholamine activity has been documented following TBI and may underlie TBI-induced risky behavior. Here, we exposed rats to sham (no injury), single, or three closed-head controlled cortical impact (CH-CCI) injuries to characterize the effects of rmTBI on 1) risk/reward decision making behavior using a probabilistic discounting task (PDT) and 2) levels of catecholamine regulatory proteins within subregions of the PFC using Western blot analysis. Mild TBI transiently increased risky choice preference, more prominently in females. Additionally, rmTBI produced delayed effects on response speed in males only. Mild TBI increased tyrosine hydroxylase (TH) levels in females only, but reduced norepinephrine transporter (NET) levels in both sexes within the orbitofrontal cortex (OFC), indicating this subregion is susceptible to catecholamine instability after mTBI. Overall, the CH-CCI model of mTBI has revealed time-dependent and sex-specific changes in risk/reward decision making and catecholamine regulation following mild head injuries.

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Chapter 1

Introduction

1.1 Traumatic Brain Injury

1.1.1 Epidemiology, Classification, and Pathophysiology

Traumatic brain injury (TBI) is defined as an injury to the brain caused by external trauma, resulting in disruption to normal brain function. In 2013, approximately 2.8 million TBI-related emergency room visits, hospitalizations, and deaths occurred in the United States (Taylor et al., 2017). According to data from 2000, the economic burden for TBI was over $60 billion (Finkelstein et al., 2006) with this estimate increasing to $76.5 billion in the year 2010. The leading causes of TBI include falls (35.2%), motor vehicle accidents (17.3%), and strikes to the head from or against an object, such as in sports injuries (16.5%) (Centers for Disease Control and Prevention, 2015; Faul et al., 2010). Additionally, 10% of TBIs are due to assaults, while 21% are caused by unknown factors. It is important to note that, while these percentages represent the general population, incidence rates vary by age, sex, and race. For example, older adults are at higher risk of fall-related TBIs (Taylor et al., 2017), while women and Black/African Americans are more likely to sustain head injuries from domestic violence and assaults (Black & Breiding, 2008; Jackson et al., 2002; Maldonado et al., 2023).

There are three classifications of TBI severity: mild, moderate, and severe. Multiple assessments are used to determine the severity of TBI in humans, including the duration of loss of consciousness and post-traumatic amnesia, computed tomography (CT) scans, and the Glasgow Coma Scale (GCS) (Centers for Disease Control and Prevention, 2015). The

GCS, which ranges from 3 to 15, is the most widely used assessment for classifying TBI severity and consists of three components: eye opening response, motor response, and verbal response (Teasdale & Jennett, 1974). Patients are scored within each category and the sum of all three scores determines the severity of TBI. A GCS score between 13-15 corresponds with mild TBI (mTBI), while scores between 9-12 and 3-8 correspond with moderate and severe TBI, respectively. In the United States, mTBIs, often labeled concussions, constitute 75% of all TBIs (National Center for Injury Prevention and Control, 2003). This percentage is even higher in certain subpopulations, including military personnel and athletes. The Department of Defense (DOD) recently reported that between 2000 and 2023, mTBIs accounted for 82.2% of all TBIs sustained by service members (Armed Forces Health Surveillance Division, 2024). In South Carolina, a population-based study reported that mTBIs accounted for 91.3% of all sports-related TBIs recorded between 1998 and 2011 (Selassie et al., 2013).

Patients with mTBI often experience a combination of physical, cognitive, and emotional symptoms collectively referred to as post-concussion symptoms (PCS, **Table 1**) (Gouvier et al., 1992). PCS are often self-reported and short-lived; however, in some cases, PCS can persist for months or even years, in which case a patient may be diagnosed with post-concussion syndrome (Ryan & Warden, 2003). The pathophysiological basis behind post-TBI symptoms is associated with damage to neuronal tissues caused by "primary" and "secondary" injuries. Primary injuries occur at the moment of impact due to the external mechanical forces that displace the brain within the skull, whereas secondary injuries occur over time due to ongoing cellular changes following primary insult **(Fig. 1)** (Prins et al., 2013). Primary injuries often include both focal (e.g., intracranial hematomas, skull

fractures, and coup and contrecoup contusions) and diffuse (e.g., diffuse axonal injury) injuries (Kaur & Sharma, 2018) with axonal damage being a hallmark of mTBI (Povlishock et al., 1983). As for secondary injuries, cerebral edema, mitochondrial dysfunction, neuroinflammation, and excitotoxicity are most commonly observed in mTBI (Naumenko et al., 2023). This combination of primary and secondary injuries are directly responsible for the physical, cognitive, and emotional deficits observed in mTBI patients, which can ultimately result in a poorer quality of life if left untreated.

Table 1

List of Post-Concussion Symptoms

Physical	Cognitive	Emotional
Headaches	Memory deficits	Depression
Dizziness	Concentration deficits	Anxiety
Fatigue	Executive function deficits	Irritability
Visual disturbances		
Noise sensitivity		
Light sensitivity		

Note. List of post-concussion symptoms (PCS) that frequently occur after mild traumatic brain injury. Table adapted from Ryan and Warden (2003).

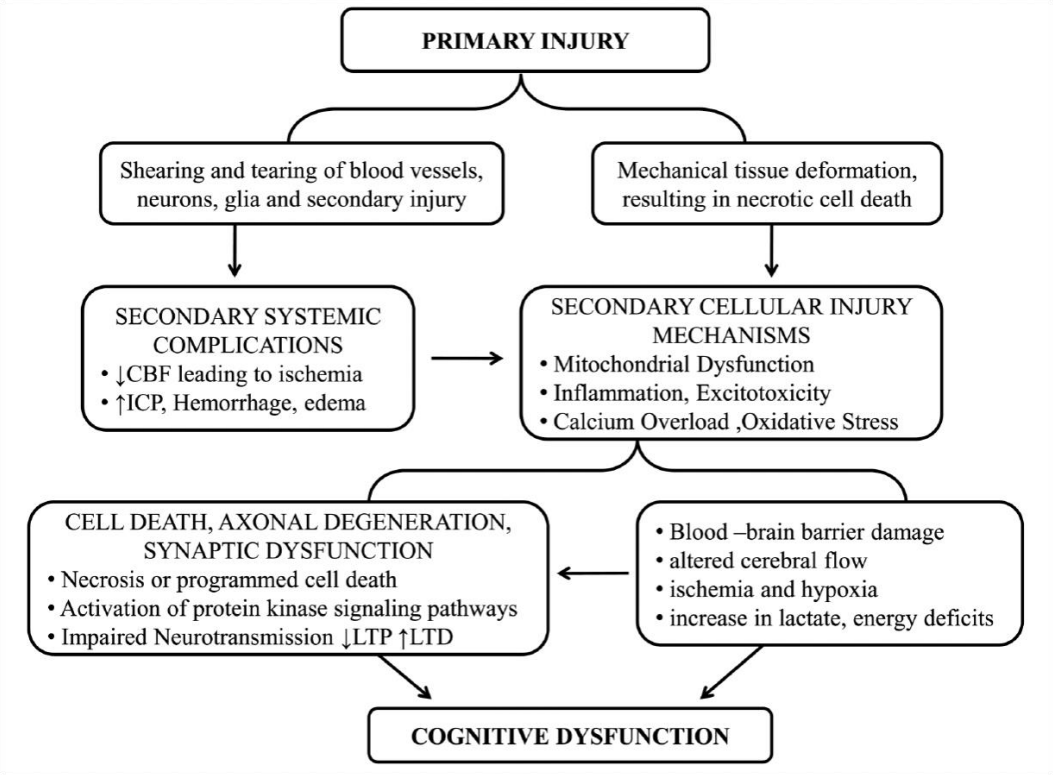


Figure 1. Overview of the pathophysiology of TBI. Abbreviations: cerebral blood flow (CBF), intracranial pressure (ICP), long-term potentiation (LTP) and depression (LTC). Figure from Kaur and Sharma (2018).

1.1.2 Management and Treatment of Traumatic Brain Injury

Following a TBI, many patients are guided through cognitive and physical rehabilitation for treatment of post-injury symptoms (Centers for Disease Control and Prevention, 2015). In professional sport leagues (e.g., NFL, NBA, NHL), athletes are assessed with concussion diagnosis and management protocols (Davis et al., 2019) and guided through specific return-to-play strategies which begin with symptom-limited activity, followed by aerobic exercises, sport-specific exercises, non-contact training drills, full contact practice, and finally clearance to play (McCrory et al., 2017). The DOD Health Agency also has their own assessments for evaluating concussions, namely the Military

Acute Concussion Evaluation 2 (MACE 2) which encompasses screening tools as well as neurologic and cognitive examinations (Lindberg et al., 2020). Following diagnosis of mTBI, military personnel are also guided through return-to-activity protocols including the Progressive Return to Activities Clinical Recommendation and the Concussion Management Tool (Lindberg et al., 2020). Both of these rehabilitation programs involve rest and a series of light, moderate, and intensive activity stages before unrestricted activity and clearance can be achieved.

In addition to rehabilitation programs, cognitive-enhancing drugs such as methylphenidate (MPH) have been tested clinically as potential treatments for post-TBI symptoms, specifically cognitive deficits. MPH has been shown to improve attention and information processing speed (Al-Adawi et al., 2005; Johansson et al., 2020; Kaelin et al., 1996) as well as depressive symptoms (Johansson et al., 2020; Lee et al., 2005) in patients with mTBI. Although these reports are promising, there are very few MPH studies that focus exclusively on mTBI cases. Trials often recruit patients of varying TBI severities, and there is variation in the dosage (fixed vs. weight-adjusted) and frequency (single vs. multiple) of MPH administration across different clinical studies (Huang et al., 2016). Moreover, other cognitive-enhancing treatments such as amphetamine (AMPH) have yet to be extensively studied in mTBI patients. As such, further research is required to understand the full therapeutic potential of pharmacological agents for treating TBI-induced cognitive dysfunction.

1.1.3 Sex Differences in Traumatic Brain Injury

As mentioned previously, there is often variation in the prevalence of TBI cases across different populations, including sex. Clinical studies have identified clear sex differences in susceptibility, symptoms, and recovery rates in cases of TBI. For example, female athletes are more susceptible to concussive injury in sports such as soccer, basketball, and softball (Cheng et al., 2019; Colvin et al., 2009; Covassin et al., 2016). Women also have a higher prevalence of obtaining a TBI following a domestic violence incident compared to men (Corrigan et al., 2003; Meyer et al., 2021; St Ivany & Schminkey, 2016). Following concussive injury, women experience more PCS (Broshek et al., 2005; Farace & Alves, 2000; Kirkness et al., 2004; Ma et al., 2019) and longer recovery rates than men (Bazarian et al., 1999; Berz et al., 2013; Kirkness et al., 2004; Ma et al., 2019). Despite these differences, women are still overlooked in cases of TBI, particularly those that involve domestic violence incidents. Poor screening tools and a lack of intervention protocols result in women living with untreated PCS and a higher chance of obtaining repetitive head injuries (Karr et al., 2024). Unlike athletes and military personnel who are removed from situations that put them at risk of sustaining additional head traumas, there are very few mechanisms for victims of domestic violence to be removed from abusive situations (Meyer et al., 2021).

1.1.4 Repetitive Mild Traumatic Brain Injury

A major challenge for clinical studies assessing the effects of TBIs is whether a patient has experienced multiple TBI events. Clinical studies have difficulty controlling for the severity, timing, and number of injuries that TBI patients suffer. There is also a wide

variation in the severity and heterogeneity of symptoms that appear in individual clinical TBI cases that can accumulate following repeated head trauma. Furthermore, studying the long-term effects of repetitive injuries in humans is difficult due to the high costs associated with such programs and the unwillingness of mTBI patients to participate in longitudinal studies.

This lack of research dedicated to assessing repetitive injuries has left a gap in our understanding of the full range of effects that can arise following multiple head injuries. Athletes, military personnel, and domestic violence victims often experience multiple traumatic brain injuries over the course of their lives (Ahmed et al., 2017; Bryan & Clemans, 2013; McKee & Robinson, 2014; Pellman et al., 2004; Rosenbaum & Lipton, 2012; Saunders et al., 2009; Selassie et al., 2013; Valera et al., 2019; Wall et al., 2006; Zetterberg et al., 2019; Zieman et al., 2017). In one example, a 2003 study found that 44% of women experienced multiple mTBIs as a result of domestic violence (Valera & Berenbaum, 2003). While the effects of single injuries are often transient, evidence suggests that repetitive mild injuries (rmTBI) can result in more severe and longer-lasting cognitive impairments, including deficits in attention and decision making (Karr et al., 2024), speed of information processing (Collins et al., 1999), and response inhibition (Wall et al., 2006). In addition, veterans with rmTBI have demonstrated greater intra-individual variability (IIV) in assessments of cognitive performance (Merritt et al., 2018), which is a measure of within-person changes in behavioral performance often indicative of cognitive decline (MacDonald et al., 2006). In spite of these observations, many of these studies utilize a battery of neuropsychological tests to evaluate multiple aspects of cognition to

report a general overview of cognitive dysfunction following rmTBI. Specific deficits within individual executive processes have not been thoroughly evaluated.

1.1.5 Animal Models of Traumatic Brain Injury

Animal models of TBI were developed to better understand the consequences of TBI, including secondary injuries described previously, and to test therapeutic strategies applicable to the clinic. There are many types of pre-clinical models of TBI; however, the most commonly used are fluid percussion, weight-drop, and controlled cortical impact (CCI). Fluid percussion injuries (FPIs) involve a rapid injection of saline onto the intact dura mater through a craniotomy to cause displacement of the brain within the skull (Dixon et al., 1987; Namjoshi et al., 2013). Weight-drop injuries involve the release of a weight from a known height onto a closed or open skull to cause brain displacement (Foda & Marmarou, 1994; Marmarou et al., 1994). Both models can produce behavioral deficits, but each has its own limitations that make them less suitable for repetitive TBI studies. For example, the fluid percussion model has a higher mortality rate as compared to other models and requires a separate craniotomy procedure prior to the FPI which can lead to variable outcomes if not consistently positioned (Cernak, 2005; Eakin et al., 2015; Namjoshi et al., 2013). The weight-drop model also has a heightened mortality rate along with the risk of animals obtaining a secondary impact caused by the weight rebounding from the skull (Cernak, 2005; Namjoshi et al., 2013).

The CCI model, which was originally developed to study TBI in ferrets (Lighthall, 1988) and later adapted to be used in rodents (Dixon et al., 1991), allows for complete control over injury parameters such as velocity, depth, duration, and location of impact.

Few limitations exist with this model, the major being mechanical variation and maintenance due to wearing of materials and electrical components that comprise the CCI devices (Osier & Dixon, 2016). The CCI model mimics the pathophysiological consequences observed in clinical TBI cases including inflammation (Acosta et al., 2013), oxidative stress (Lazarus et al., 2015), and axonal injuries (Mohamed et al., 2021) along with behavioral and cognitive impairments (Bondi et al., 2014; Hamm et al., 1992; Huh et al., 2008; Kutash et al., 2023; Lengel et al., 2022; McCorkle et al., 2022). Traditionally, the CCI model has been used with an open-skull design where the exposed cortex is subjected to trauma following a craniectomy. This craniotomy is fairly large compared to those required for the FPI model (Osier & Dixon, 2016). Over time, this model adapted a closed-head design (CH-CCI) which facilitated the assessment of repetitive TBI-induced impairments often observed in athletes, military personnel, and victims of domestic violence, including axonal injury and microglial reactivity (Petraglia et al., 2014), as well as deficits in spatial learning and memory, and increased anxiety-like and depression-like behavior (Lengel et al., 2022; McCorkle et al., 2022; Shitaka et al., 2011). Previous experiments in our laboratory utilized the CH-CCI model to assess the effects of rmTBI on one dimension of executive function, cognitive flexibility. Cognitive flexibility is the ability to adjust one's behavior in response to a changing environment and was assessed using an automated strategy shifting task (Brady & Floresco, 2015). Briefly, this task required rats to learn and shift strategies according to changing task demands. Rats initially acquired a visual cue strategy, in which a light illuminated above one of two levers (left or right) indicated the correct lever press response for reward. Twenty-four hours after initial acquisition, rats were assessed for retrieval of the visual cue strategy followed by a series of strategy shifting and reversal

learning challenges. We found that rmTBI increased overall reaction times during the strategy shifting assay of flexible attention, indicating slower processing speed **(Figure 2A)**. This increase in response latency was independent of whether a correct or incorrect response was made. We also examined throughput scores, which is broadly used in the clinic as a measure of effective performance in behavioral and cognitive tasks (R. Thorne, 2006). Throughput scores blend together accuracy and response speed to yield an effective measure of performance. We observed an overall injury effect in the reduction of these throughput scores **(Figure 2B)**. This study demonstrated the effectiveness of the CH-CCI model to produce rmTBI-induced changes in complex behavior using a rodent assay of cognitive flexibility. These experiments further opened the possibility for evaluating the effects of rmTBI on other dimensions of executive functions using the CH-CCI model.

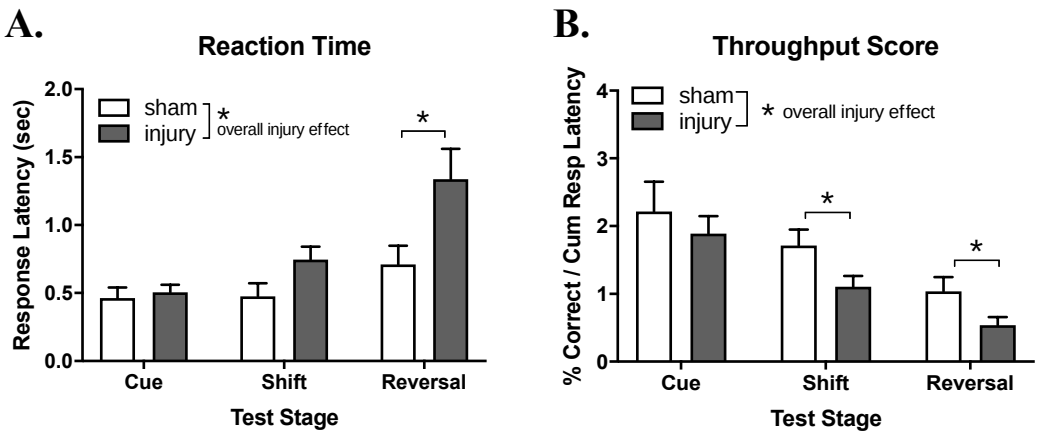


Figure 2. rmTBI effects on strategy shifting performance. A) rmTBI increased response latency with a specific effect in the reversal stage. * denotes $p < 0.05$ for an overall injury group effect on response latency analyzed by two-way ANOVA (adjacent to treatment legends) and between injury groups at specific test stages analyzed by Sidak's multiple comparisons tests. B) Throughput scores are significantly lower in the injured group compared to sham. * denotes $p < 0.05$ for an overall injury effect analyzed by two-way ANOVA and between injury groups at specific test stages analyzed by Sidak's multiple comparisons tests. Bars represent mean ± SEM.

1.2 Prefrontal Cortex

1.2.1 The Organization and Function of the Prefrontal Cortex

The prefrontal cortex (PFC), also known as the "executive center of the brain", mediates complex executive processes that control everyday behavior. The PFC resides at the most anterior portion of the frontal lobe and occupies 30% of the human cerebral cortex. The human PFC is functionally organized into three main domains: dorsolateral (dlPFC), medial (mPFC), and orbital (OFC) cortexes. The dlPFC is involved in many executive processes, including working memory, attentional selection, set shifting, and conceptual planning (Tanji & Hoshi, 2008). The mPFC, which is further sub-divided into the prelimbic, infralimbic, and anterior cingulate (ACC) cortexes, contributes to attention within demanding cognitive tasks, spatial memory, and emotional responses (Siddiqui et al., 2008). Lastly, the OFC is heavily involved in reward processing, social and emotional behavior, motivational behavior such as feeding and drinking, and reversal learning (Izquierdo et al., 2017; Rolls, 2004; Wallis, 2007). Disruptions to PFC-dependent processes can directly lead to cognitive impairments as observed in many neuropsychiatric disorders such as attention deficit hyperactivity disorder (ADHD), schizophrenia, bipolar disorder, and post-traumatic stress disorder.

Similar subdomains of the PFC exist within rodents, including the mPFC (which includes both the prelimbic and infralimbic cortexes), the ACC, and the OFC. In terms of anatomical and functional similarities, the mPFC is most similar to both the dlPFC and ACC-portion of the mPFC in primates (Seamans et al., 2008). The rat OFC also shares a similar organization to that of primates, including two distinct connectivity networks:

medial network (including the medial OFC) and orbital network (including the lateral OFC) (Price, 2007). These similarities have allowed the rat PFC to be an essential model for evaluating executive function.

1.2.2 Catecholamine Modulation of the Prefrontal Cortex

The PFC receives dense innervation from catecholaminergic fibers, containing dopamine (DA) and norepinephrine (NE), which modulate PFC-mediated processes. The primary sources of DA and NE in the PFC are the ventral tegmental area (VTA) (Berger et al., 1976; Fluxe et al., 1974) and the locus coeruleus (LC) (Chandler et al., 2014; Waterhouse et al., 1983), respectively. An optimal balance of catecholaminergic signaling is required for normal operation of all PFC regions and executive functions are robustly manipulated with perturbations of DA and NE concentrations within the PFC (Hamilton & Brigman, 2015; Lapiz & Morilak, 2006; McGaughy et al., 2008; Montes et al., 2015; St Onge et al., 2010; St Onge & Floresco, 2009). While small increases in DA and NE neurotransmission can improve cognitive performance (Berridge & Devilbiss, 2011; Berridge et al., 2006; Bymaster et al., 2002; Devilbiss & Berridge, 2008; Navarra et al., 2017; Spencer et al., 2015), catecholamine activity outside this optimal range can result in impaired cognitive functioning (Grilly & Loveland, 2001).

1.2.3 Effects of Traumatic Brain Injury on the Prefrontal Cortex

The PFC is most frequently affected following TBI events (Bigler, 2001; McAllister, 2011), resulting in cognitive deficits described previously. Previous reports have demonstrated aberrant catecholamine activity within the prefrontal cortex following

TBI (Kobori et al., 2006; Massucci et al., 2004), suggesting that imbalanced DA and NE levels may underlie injury-induced deficits in executive functioning. First, one of the primary pathologies in TBI is axonal injury and the thin unmyelinated fibers of the catecholamine transmitter systems are most vulnerable to such effects (Jenkins et al., 2016). Second, experimental TBI has been shown to alter DA and NE markers and levels within the PFC (Fujinaka et al., 2003; Huger & Patrick, 1979; Kobori et al., 2006; Laskowski et al., 2015; Levin et al., 2019; Massucci et al., 2004; McIntosh et al., 1994; Wagner, Chen, et al., 2005; Wilson et al., 2005; Yan et al., 2001; Yan et al., 2002). Third, expression and function of DA and NE reuptake transporters (DAT and NET), which remove extracellular catecholamines after their release to maintain efficient signaling, are dynamically regulated and affected by a variety of drugs and pathologies (Bradshaw et al., 2016; Gray et al., 2007; Mandela & Ordway, 2006; Somkuwar et al., 2015; Wagner et al., 2007). Finally, clinical studies have reported efficacy of low-dose MPH, which blocks NET and DAT to elevate extracellular catecholamine concentrations (Solanto, 1998; Volkow et al., 2002), to improve fatigue and components of chronic executive dysfunction following TBI (Al-Adawi et al., 2020; DeMarchi et al., 2005; Ekinci et al., 2017; Huang et al., 2016; Johansson et al., 2017; Kurowski et al., 2019; LeBlond et al., 2019; Levin et al., 2019; Traeger et al., 2020; Warden et al., 2006; Yamamoto et al., 2018) as well as reduce the rate of subsequent TBIs during medicated periods (Ghirardi et al., 2020; Raman et al., 2013). These findings clearly indicate that DA and NE neurotransmission to the PFC is compromised following TBI and restoring normal catecholamine function following injury is beneficial. In addition, preclinical reports have demonstrated that cognitive impairments following more severe injury can be remediated by low-dose psychostimulants (Kline et

al., 2000; Leary et al., 2017; Wagner et al., 2007), potentially via neuroprotective effects, which may enhance recovery (Rau et al., 2016).

The reasons for these alterations are unclear; however, some studies have reported changes in catecholamine-associated regulatory protein levels following TBI, including tyrosine hydroxylase (TH), the rate-limiting enzyme for catecholamine synthesis. Increased levels of TH have been observed in the PFC, suggesting a surge in catecholamine production; however, one report observed these increases 14 days after injury (Kobori et al., 2006) whereas another reported them at 28 days post-injury (Yan et al., 2001). These differences may be due to different injury parameters used by each laboratory. While both groups performed an open-skull CCI injury lateral of the midline between bregma and lambda, Kobori et al. used a depth of 1.7mm and a velocity of 6m/s, indicative of a moderate injury (Dixon et al., 1991; Hamm et al., 1992), whereas Yan et al. used a depth of 2.6mm and a velocity of 4 m/s, which has also been described as a moderate-level injury (Ciallella et al., 1998). While these differences in injury parameters highlight the interlaboratory variation among different CCI models, it is clear that TH levels rise following moderate TBI. However, it has yet to be determined whether these changes occur following mild injury. Decreased levels of DAT have also been reported at the same 28-day time point (Yan et al., 2002) as the observed increases in TH (Yan et al., 2001), suggesting a compensatory response to the surge of extracellular DA levels due to increased TH activity. These results further suggest that potential changes in the expression of other catecholamine-associated proteins may also be identified if the same injury parameters are consistently used. These regulatory proteins include: vesicular monoamine transporter-2 (VMAT2), norepinephrine transporter (NET), catechol-O-methyltransferase

(COMT), and monoamine oxidase-A (MAO-A). As described further in **Chapter 9**, these regulatory proteins also maintain optimal activity within the PFC; however, it has not been determined how mTBI and, specifically, repetitive injuries affect these proteins.

1.3 Risk/Reward Decision Making

1.3.1 Risk-Taking Behavior

Decision making is a PFC-mediated executive function that depends upon multiple cognitive processes, personal motivation, proper catecholamine activity, and the environmental context of situations to make choices involving various costs and benefits associated with each decision. Risk-taking is the voluntary participation in behaviors that have probabilistic outcomes and can either be positive or negative. Positive risks are often characterized by three features: 1) beneficial to one's well-being, 2) socially acceptable, and 3) carry mild costs (Duell & Steinberg, 2019). Some of these positive behaviors include participating in team sports, experimenting with different value systems and identities, and seeking out challenging tasks such as advanced academic courses (National Academies of Sciences et al., 2019). Negative risk-taking, which includes behaviors such as driving under the influence of alcohol, provoking a physical fight, and substance abuse, pose adverse consequences to an individual (National Academies of Sciences et al., 2019). A mix of positive and negative risk-taking is considered constructive for personal development; however, disruptions to proper PFC functioning can lead to inappropriate decision making and increased risk-taking as observed in cases of pathological gambling disorder (Cavedini et al., 2002; Kertzman et al., 2011; Potenza et al., 2003), attention deficit hyperactivity

disorder (ADHD) (Pollak et al., 2019; Yang et al., 2019), substance use disorder (Bechara et al., 2001; Petry, 2001), and TBI (Bechara et al., 1994).

1.3.2 Iowa Gambling Task

Risk/reward decision making is assessed clinically through the Iowa Gambling Task (IGT) (Bechara et al., 1994). Developed in 1994, this task models real-life decisions involving reward, punishment, and uncertainty of outcomes. Specifically, the IGT requires participants to choose cards from four different decks in an opportunity to win money. Two decks are associated with large gains but also pose risks of losses that are more frequent or of higher magnitude, and two decks are associated with small gains, but pose less risk of loss. During performance of the task, healthy individuals learn that choosing the small/safe decks are advantageous to maximize overall net gain.

Initial studies established that patients who have experienced TBI, especially with PFC damage, exhibited greater loss of money and impaired decision making capabilities in the IGT (Bechara et al., 1994; Bechara et al., 1998; Bechara et al., 2000). The OFC, in particular, was among the first regions to be identified as a major mediator of risk/reward decision making (Bechara et al., 1994). In 1999, the OFC's involvement was further confirmed through studies involving the Cambridge Gambling task (CGT), which is a computerized decision making task that offers neuroimaging compatibilities (Rogers, Everitt, et al., 1999; Rogers, Owen, et al., 1999). In 2002, a study involving the IGT and CGT revealed that patients with specific lesions to the OFC demonstrated longer deliberation times when making risky choices (Manes et al., 2002), suggesting a deeper involvement of the OFC in multiple aspects of the risk/reward decision making process.

Following the establishment of the PFC's importance in risk/reward decision making, additional investigations revealed the relationship of mPFC and ACC in working with the OFC to facilitate efficient risk/reward decisions (Broche-Pérez et al., 2016; Hadland et al., 2003; Jenni et al., 2021; Krawczyk, 2002; Labudda et al., 2010; O'Doherty et al., 2001; Rogers, Owen, et al., 1999; Rogers et al., 2004; Rolls, 2004; Rushworth et al., 2004; Schweimer & Hauber, 2005; Seamans et al., 2008; St Onge et al., 2011; St Onge & Floresco, 2010; Stopper et al., 2014). Individual damage to any of these regions has been shown to impair reward-guided learning and action selection (Hadland et al., 2003; Schweimer & Hauber, 2005), increase perseverative behavior (Bechara et al., 1994; Muir et al., 1996), reduce sensitivity to consequences of risky decisions (Bechara et al., 1994; Bechara et al., 2000), and impair information processing relating to new and/or changing risk/reward contingencies (Manes et al., 2002; Rogers, Everitt, et al., 1999; St Onge & Floresco, 2010). TBI often damages multiple areas of the PFC, which causes collective impairments to decisional processes, leading to the reported increases in risk taking behavior. While the effects of repetitive TBI have not yet been explored, it is likely that multiple head injuries exacerbate existing damage to prefrontal areas and cause new injuries in previously uninjured regions.

1.3.3 Probabilistic Discounting Task

The probabilistic discounting task (PDT) was developed as a rodent analogue of the IGT to pre-clinically assess risk/reward decision-making (St Onge & Floresco, 2009). As described further in **Chapter 3**, the PDT requires rats to choose between small/certain rewards delivered with 100% certainty and large/risky rewards delivered with decreasing

probabilities across a session (i.e. 100% probability → 50% → 25% → 12.5% → 6.25%) (**Fig. 3**). Similar to the IGT, performance in the PDT relies on intact PFC functioning, and changes in neural (St Onge & Floresco, 2010; Stopper et al., 2014) or catecholaminergic (Jenni et al., 2021; St Onge et al., 2011) activity within the PFC have been shown to alter risk taking behavior. This sensitivity to changes in PFC activity makes the PDT a viable assay for detecting potential impairments to risk/reward decision-making following TBI.

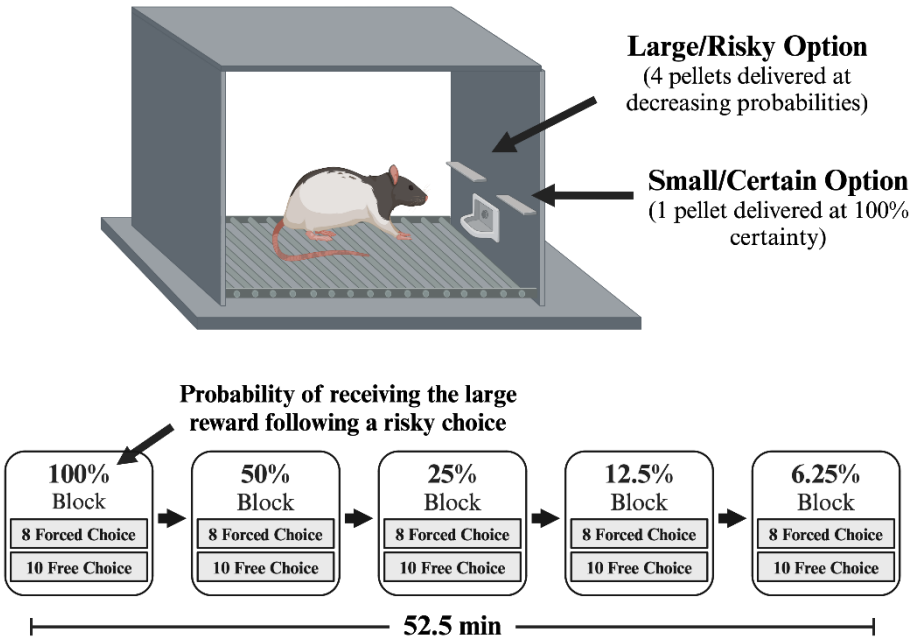


Figure 3. Schematic of the probabilistic discounting task Top: Cost/benefit contingencies associated with each lever. Bottom: Task design depicting the decreasing large reward probabilities across five trial blocks. *Made with Biorender.com*.

1.4 Goal of the Studies

To date, the effects of repetitive mild head injuries on risk/reward decision making have not been explored. The goal of this dissertation was to investigate how rmTBI affects

risk/reward decision making behavior, what processes contribute to TBI-induced catecholamine imbalances, and whether these outcomes can be differentiated by sex. *The central hypothesis tested was that rmTBI produces greater increases in risk-taking behavior compared to single injury and uninjured animals, and that these effects are more severe and longer-lasting in females compared to males. It was further hypothesized that these effects are driven by TBI-induced alterations of catecholamine regulatory protein levels.* Each aim described in **Chapter 2**, outlines a collection of experiments designed to address this overarching hypothesis. The results of these studies will serve to provide greater insight into potential sex differences in risk/reward decision making following rmTBI and may reveal novel mechanisms leading to future therapeutic targets.

Chapter 2

Hypothesis and Specific Aims

2.1 Hypothesis

Clinical assessments of decision making have reported increased risk-taking behavior following single traumatic brain injury (TBI) events. While the effects of single injuries are often transient, evidence suggests that repetitive injuries can result in more severe and longer-lasting cognitive impairments. The effects of injury can also vary depending on the sex of the patient. Women have been reported to suffer worse outcomes following a TBI event compared to men, including greater post-concussive symptoms and longer recovery rates. *Therefore, the central hypothesis tested in the current work is that repetitive mild traumatic brain injury (rmTBI) produces greater increases in risk-taking behavior compared to single injury, and that these effects are more severe and longer-lasting in females compared to males.* These findings would suggest that patients who have sustained multiple concussions may have greater difficulty evaluating and executing decisions involving uncertain risk/reward outcomes compared to single injured patients, and that separate therapeutic approaches should be considered when treating the differential effects of rmTBI in men and women.

2.2 Specific Aims

2.2.1 Aim 1: Validation of a Rodent Assay of Probabilistic Discounting

Disruption or enhancement of prefrontal cortex (PFC) activity can result in changes in risk-taking behavior within the probabilistic discounting task. Pharmacological

manipulations of catecholamine transmission have been shown to substantially alter choice behavior within this task. The goal of this aim was to validate our laboratory's ability to manipulate an animal's discounting behavior by evaluating the effects of three pharmacological agents that enhance catecholamine transmission on risk/reward decision making. Given that pharmacological treatments which elevate catecholaminergic activity have been shown to increase risk preference within this task, it was predicted that we would also observe increased risk-taking behavior across all treatment conditions. Aim 1 is addressed in Chapter 4.

2.2.2 Aim 2: Characterize the Effects of rmTBI on Risk/Reward Decision Making Using the Probabilistic Discounting Task

Damage to the PFC as a result of a single mTBI has been shown to impair dimensions of executive function including decision making; however, no clinical studies have examined the effects of repetitive injuries. The goal of this aim was to evaluate the effects of three mild head injuries on risk/reward decision making in rats using a probabilistic discounting task. Given clinical evidence that TBI impairs performance and slows processing speed when evaluating cost/benefit contingencies associated with risk-based decisions, it was hypothesized that rmTBI would increase risk-taking behavior and slow reaction times to making a risk/reward decision in comparison to animals with a single or sham injury. Aim 2 is addressed in Chapters 5, 6, 7, and 8.

2.2.3 Aim 3: Examine Levels of Proteins that Affect Catecholamine System Function Following rmTBI Within Prefrontal Cortical Regions that Contribute to Risk/Reward Decision Making

Optimal balance of catecholamine signaling is necessary for normal operation of all regions of the PFC. Aberrant catecholamine activity has been observed following TBI, suggesting that imbalances within the PFC may underlie TBI-induced behavioral outcomes. Catecholamine regulatory proteins work to maintain optimal catecholamine concentrations within the PFC; however, no studies have examined the levels of these proteins following rmTBI. The goal of this aim was to use Western Blot analysis to investigate changes in catecholamine synthetic, packaging, degradation, and reuptake transporter proteins within three prefrontal cortical regions that contribute to risk/reward decision making. The hypothesis tested here was that levels of these catecholamine regulatory proteins would be altered in the mPFC, OFC, and ACC following rmTBI. Aim 3 is addressed in Chapters 9, 10, and 11.

Chapter 3

Materials and Methods

3.1 Animals

A total of 217 male and 153 female Long-Evans rats were used in these studies. Animals were obtained at 3-7 weeks old/50-250g from either Charles River or Envigo Laboratories and housed in a 12h:12h reverse light/dark cycle facility. Following a week of acclimation, rats were single housed into separate cages, and placed on a food regulated diet (5g per 100g body weight/ day) with *ad libitum* access to water. They were maintained to 85% of their free feeding weight throughout the duration of these studies. All experimental procedures were in accordance with the Rowan University School of Osteopathic Medicine Institutional Animal Care and Use Committee and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

3.2 Probabilistic Discounting Task

3.2.1 Apparatus

Behavioral studies were conducted in 16 operant chambers [29cm (L) x 24cm (W) x 29cm (H); Med-Associates, Albans, VT] enclosed within sound attenuating boxes. Operant chambers were equipped with a fan, a house light, and 2 retractable levers located on either side of a food dispenser where sucrose pellet rewards (45 mg; Bio-Serv, Flemington, NJ) were delivered. A photo beam was located at the dispenser entry point to detect reward collection. Custom built Med Associates Nexlink computer packages controlled training, testing, and data acquisition during performance.

3.2.2 Lever-Pressing Training

Initial training protocols were adapted from St. Onge and Floresco (2009). Rats were first trained to press a single lever (either the left or right) using a fixed-ratio one (FR1) schedule to a criterion of 50 presses within 30 minutes. Once criterion was achieved, rats repeated this procedure for the opposite lever. Rats then trained on a simplified version of the probabilistic discounting task (PDT) (90 trials per session) which required them to press one of the two levers within a 10 second period for a sucrose reward delivered with a 50% probability. This procedure familiarized them with the probabilistic nature of actions and outcomes. Rats were trained for at least 3 days to a criterion of 75 or more successful trials (i.e., $\leq$ 15 omissions) on the simplified PDT.

3.2.3 PDT Training and Testing

The PDT was used to assess changes in risk/reward decision making and has been described previously (Floresco & Whelan, 2009; Jenni et al., 2021; Montes et al., 2015; St Onge et al., 2011; St Onge et al., 2010; St Onge & Floresco, 2009, 2010; Stopper & Floresco, 2011; Stopper et al., 2014) (**Fig. 3, Chapter 1**). This task required rats to choose between levers that result in either a small/certain reward (1 pellet) delivered with 100% certainty and large/risky rewards (4 pellets) delivered with decreasing probabilities across a series of five trial blocks (i.e. 100% probability $\rightarrow$ 50% $\rightarrow$ 25% $\rightarrow$ 12.5% $\rightarrow$ 6.25%). Each session took 52.5 minutes to complete and consisted of 90 trials, separated into 5 blocks of 18 trials. These 18 trials consisted of 8 forced-choice trials where only one lever was extended allowing rats to learn the relative likelihood of obtaining the larger reward

in each block. This was followed by 10 free-choice trials, where both levers were extended allowing rats to freely choose between the small/certain or the large/risky lever. Each session began in darkness with both levers retracted. A trial began every 35 seconds with the illumination of the house light and extension of one or both levers. Once a lever was chosen, both levers retracted, rats were rewarded 1 pellet if they chose the small/certain lever or a possible 4 pellets if they chose the large/risky lever, and the house light turned off. If the rat did not respond within a 10 second period, levers retracted, the house light turned off, and the trial was recorded as an omission.

Rats were trained 5-7 days per week until they achieved baseline criteria, which included choosing the risky lever in >80% of trials in the 100% block and maintaining stable patterns of choice for 3 consecutive sessions. Determining stable baseline performance involved analyzing 3 consecutive sessions using a repeated measures analysis of variance (ANOVA) with two within-subjects factors (day and trial block). Rats were required to demonstrate a significant main effect of block ($p < 0.05$), but not a main effect of day nor a day x block interaction ($p > 0.1$). If animals, as a group, met these 3 requirements, they were determined to have achieved stable baseline levels of choice behavior.

3.2.4 Behavioral Measures

The primary measure of interest obtained from the PDT is choice behavior, which is the percentage of choices directed towards the large/risky option. Choice behavior is visualized through a probabilistic discounting (PD) curve, which illustrates a rodent's discounting behavior across the five trial blocks. In normal animals, the percent choice of

the risky option is higher in the first two blocks (100% and 50%) and lower in the last three final blocks (25%, 12.5%, and 6.25%). This is because the relative value of the large, probabilistic reward is decreasing as the chances of obtaining it are getting progressively lower. Following different experimental manipulations, this PD curve may "rise" or "fall" in shape depending on whether an animal demonstrates an increased or decreased preference for the risky option, respectively (**Fig. 4A and 4B**). Animals may also demonstrate a sub-optimal choice pattern in the form of a "flattened" curve where they exhibit a decreased preference for the riskier option in the high probability blocks (100% and 50%) and an increased preference for the riskier option in the low probability blocks (12.5% and 6.25%) (**Fig. 4C**). By displaying this behavior, an animal is exhibiting no discernible discounting upon changes in reward probabilities (St Onge et al., 2011).

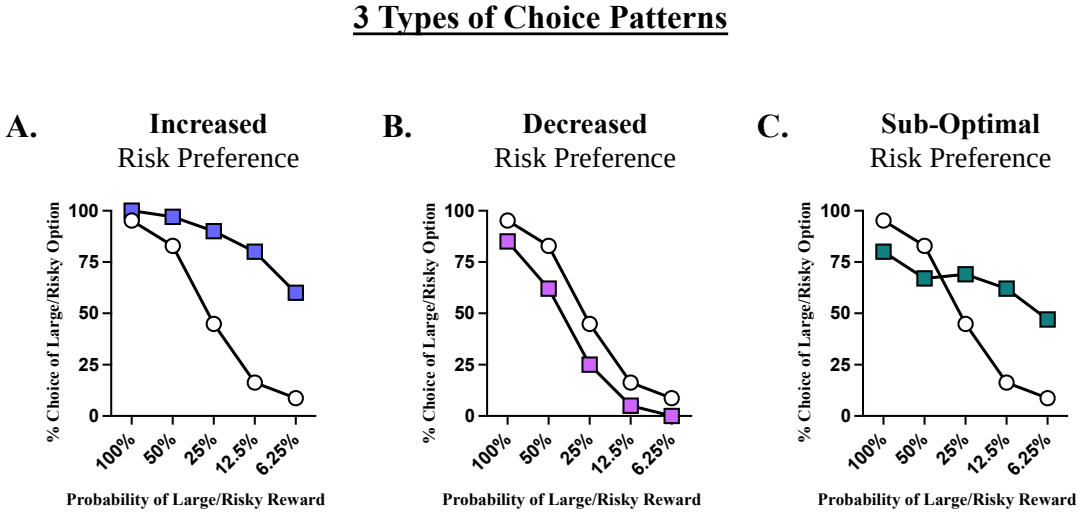


Figure 4. Visual examples of 3 types of choice patterns**.** A) Increased Risk Preference (blue squares). B) Decreased Risk Preference (purple squares). C) Sub-Optimal Risk Preference (teal squares). All graphs illustrate a comparison to untreated animals (white circles).

In order to understand why changes in choice behavior are occurring, a supplementary win-stay/lose-shift analysis can be conducted (Jenni et al., 2021; St Onge et al., 2011; St Onge et al., 2012; Stopper & Floresco, 2011; Stopper et al., 2014). "Win-stay" values are calculated as the proportion of trials a rat stays with the risky option after obtaining the larger reward on the previous trial. "Lose-shift" values are calculated as the proportion of trials a rat switches to the certain option after a non-rewarded risky choice. This analysis helps clarify whether changes in choice preference are caused by altered sensitivity to positive or negative feedback. For example, increases in risky choice may be attributed to increases in win-stay behavior, which reflects an animal's positive response to obtaining the large rewards (**Fig. 5A, Left Panel**). However, increases in risky choice may also be attributed to decreases in lose-shift behavior, reflective of an animal's reduced sensitivity to losses (**Fig. 5A, Right Panel**). Alternatively, decreases in risky choice may be attributed to decreases in win-stay behavior (**Fig. 5B, Left Panel**), indicative of reduced sensitivity to rewarded outcomes, or increased lose-shift behavior, indicative of increased sensitivity to losses (**Fig. 5B, Right Panel**).

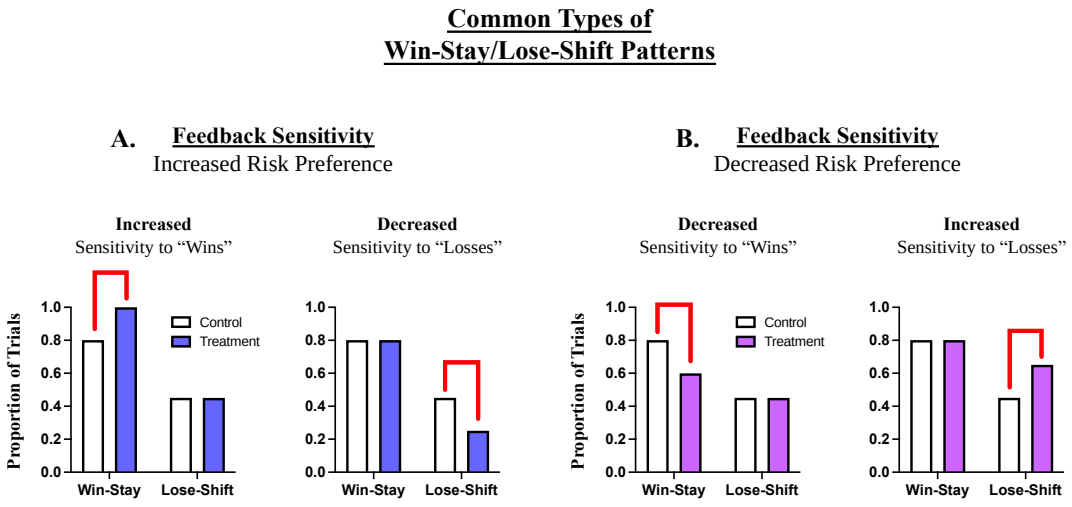


Figure 5. Visual examples of common win-stay/lose-shift patterns. A) Increased Risk Preference can be the result of increased win-stay behavior (Left) or decreased lose-shift behavior (Right). B) Decreased Risk Preference can be the result of decreased win-stay behavior (Left) or increased lose-shift behavior (Right). All graphs illustrate a comparison to untreated animals (white bars).

The latency to make a probabilistic decision (response latency) can also be measured with the PDT. Following the presentation of both risky and certain options, animals utilize previously established information including cost/benefit contingencies and outcome history to make the most advantageous decision. This deliberation period can be improved or impaired by experimental treatments, resulting in faster or slower response times to probabilistic choices.

Lastly, the latency to collect rewards (magazine latency) and the amount of omitted trials made across each block (omissions) are also recorded. Since this task operates on a food-reward system, magazine latency can provide information relating to any treatment-induced effects on satiety that might occur. A lack of motivated response can be identified by the length of magazine latency and the omission count.

3.3 Drugs

Amphetamine (AMPH) and methylphenidate (MPH) were purchased from Sigma-Aldrich (St. Louis, MO). SK609 was gifted from PolyCore Therapeutics (Philadelphia, PA). All drugs were dissolved in sterile saline and injected intraperitoneally (i.p.) at a volume of 1 mL/kg either 10 min (AMPH), 15 min (MPH) or 5 min (SK609) before testing.

3.4 Surgical Procedures

On the day of surgery, each group of rats underwent a total of three surgeries over the course of one week separated by 2 days using the closed head-controlled cortical impact (CH-CCI) model (Custom Design & Fabrication Incorporated, Glenn Allen, VA). Animals were anesthetized at 4% isoflurane in 95% oxygen/5% carbon dioxide, then maintained at ~2.0% isoflurane while a midline incision of 2cm was made to expose the skull. Anesthesia was discontinued and the rat was transferred to a stage under the CCI device with its head resting on a foam pad [10cm (L) x 5cm (W) x 1 cm (H)]. A 5mm diameter metal impactor tip was positioned on the skull surface along the sagittal suture with the edge of the tip aligned with bregma. The tip was then electronically driven at a velocity of 5.5m/s and a depth of either 2.5mm or 3.5mm below the surface point of contact with a dwell time of 100ms. Following injury, righting reflex times were recorded by measuring the latency for animals to regain normal posture after being placed in the supine position. Animals were then re-anesthetized and the incision was closed with wound clips. rmTBI rats received an impact on all 3 surgery days whereas smTBI rats received sham surgeries on the first 2 surgery days and an impact on the 3rd day. Sham rats underwent the same surgical procedures but were not impacted on any day.

3.5 Western Blotting of Prefrontal Sub-Regions

Rats were lightly anesthetized with 4% isoflurane in 95% oxygen/5% carbon dioxide and decapitated. The medial prefrontal cortex (mPFC), anterior cingulate cortex (ACC), and orbitofrontal cortex (OFC) were dissected using a 2mm tissue puncher (**Fig. 6**), immediately frozen on dry ice, and stored at -80°C until use. Tissue was then homogenized in lysis buffer (consisting of 10mM HEPES, 2mM EDTA, 2mM EGTA, 1% Triton X-100, 1X Protease Inhibitor I, and 1X Protease Inhibitor II), followed by refrigerated centrifugation at 20,000 x g for 2 minutes at 4°C. The supernatants were then aliquoted and stored at -80°C until use. Protein concentrations were determined using a Bio-Rad DC Protein Assay Kit (Bio-Rad, Hercules, CA). Protein samples were prepared in 4X sample buffer (Bio-Rad, Hercules, CA) and heated at 90° for 3 minutes. Equal amounts of protein (15ug) were loaded into each well of a Criterion XT Bis-Tris Protein Gel (Bio-Rad, Hercules, CA). Following gel electrophoresis, protein were transferred to Immuno-Blot PVDF membranes (Bio-Rad, Hercules, CA). Membranes were blocked with 5% non-fat dry milk in TBS-T (20X TBS and 10% Tween) for 1 hour and then probed with either rabbit anti-TH (1:1000; MilliporeSigma, Temecula, CA), rabbit anti-VMAT2 (1:1000; Abcam, Waltham, MA), rabbit anti-NET (1:1000; Abcam, Waltham, MA), rabbit anti-MAO-A (1:1000; ThermoFisher Scientific, Waltham, MA), or rabbit anti-COMT (1:1000; ThermoFisher Scientific, Waltham, MA) antibody at 4°C overnight followed by goat anti-rabbit secondary antibody conjugated with peroxidase (1:10,000; Rockland Immunochemicals, Inc., Limerick, PA) for 1 hour the next day. β-actin (1:2000; MilliporeSigma, Temecula, CA) was used as the loading control. Chemiluminescence was

detected using Clarity Western ECL substrate (Bio-Rad, Hercules, CA), imaged using Azure c400 Biosystems imaging system (Azure Biosystems, Dublin, CA), and analyzed using AzureSpot Analysis Software (Azure Biosystems, Dublin, CA).

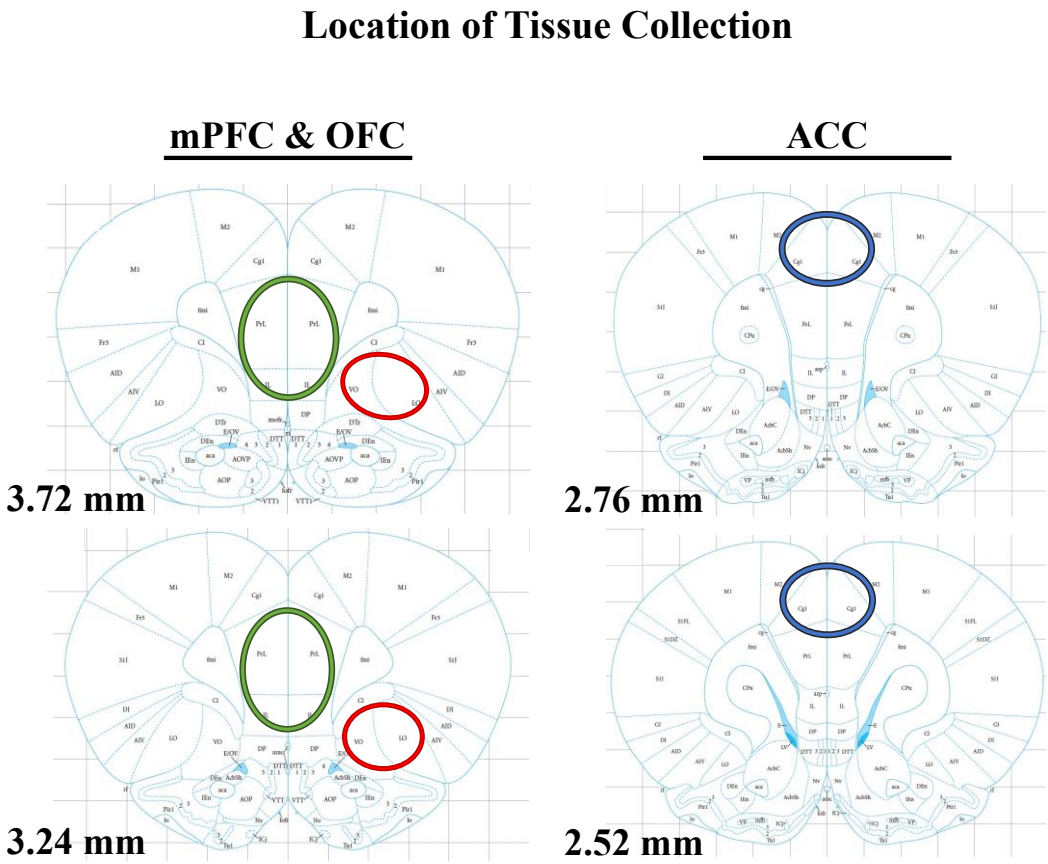


Figure 6. Location of tissue collection. Tissue was collected from the medial prefrontal (mPFC), orbitofrontal (OFC), and anterior cingulate (ACC) regions for Western Blot assays (adapted from Paxinos & Watson, 2007).

3.6 Immunohistochemistry of the OFC

Rats were deeply anesthetized with 4% isoflurane in 95% oxygen/5% carbon dioxide and perfused transcardially with 0.9% saline and 10% formalin. Brains were

removed and stored in 10% formalin at 4°C for at least 24 hours and then transferred to 30% sucrose until use. Brains were then sectioned along the coronal plane at a thickness of 40 µm using a freezing microtome. Tissue was permitted to freely float in small wells and a series of 3-4 sections per animal containing the OFC were selected (coordinates: 4.20mm to 3.24mm from bregma). Sections were washed (4 x 10min) in 1X PBS (phosphate buffer saline) and blocked in 5% donkey serum in PBS-t (phosphate buffer saline triton) with 1% BSA (bovine serum albumin) for 90 minutes before incubation in mouse anti-DBH (1:2000, MilliporeSigma, Temecula, CA) antibody at 4°C overnight. After incubation, tissue was washed in PBS and incubated in Alexafluor 488 donkey anti-mouse (1:250, Invitrogen, Waltham, MA) for 1 hour. Sections were then washed in PBS (4 x 10min), mounted on gelatin-coated glass slides, and covered with vectashield mounting medium with DAPI. Fluorescent photomicrographs of the OFC were taken at 20X using a Keyence BZ-X710 microscope. Images were acquired using the Keyence image acquisition software and were processed with Image J (initial protocol provided by Dr. Daniel Chandler and modified by CPK).

3.7 Statistical Analysis

All data analyses were performed using GraphPad Prism (GraphPad Software, San Diego CA) or SPSS (IBM, SPSS Inc.) software. P values were generated using unpaired Student's t-tests or ANOVA tests: NS $p > 0.05$ or $*p < 0.05$. Multiple comparisons tests, when appropriate, were used to compare individual differences when overall significance was found. All error bars indicate mean ± SEM.

Chapter 4

Validation of the Probabilistic Discounting Task

4.1 Introduction

As described in the **Behavioral Measures** section of **Chapter 3**, the probabilistic discounting task (PDT) offers a range of measurements that allow for a comprehensive analysis of risk/reward decision making in rodents. Our aim was to use this well-established assay to characterize the effects of repetitive mild traumatic brain injury (rmTBI). However, we first had to validate the PDT in our laboratory. Thus, the experiments described in **Chapter 4** were designed to validate our lab's ability to manipulate an animal's discounting behavior by replicating previous reports.

The PDT has previously been used to evaluate pharmacological treatments that augment or reduce DA and NE activity to reveal the specific mechanisms in which catecholaminergic circuits mediate risk/reward decision making (Floresco & Whelan, 2009; Montes et al., 2015; St Onge et al., 2010; St Onge & Floresco, 2009; Stopper et al., 2013). Amphetamine (AMPH), which blocks reuptake and enhances transmitter release, was the first drug tested in the PDT and established a precedent for how the shape of a probabilistic discounting (PD) curve can change when catecholaminergic transmission is altered (St Onge & Floresco, 2009). Since then, AMPH has been used as a baseline for comparing the effects of other pharmacological treatments on risk/reward decision making; specifically, the receptor-mediated mechanisms that modulate probabilistic discounting. It has been reported that systemically activating D1 and D2 receptors (St Onge & Floresco, 2009) or blocking noradrenergic α2 receptors (Montes et al., 2015) increases risky choice

when the likelihood of obtaining a probabilistic reward decreases over time. Thus, enhancing DA and NE activity, such as with AMPH, increases the preference for larger, probabilistic rewards even when their long-term value decreases. In contrast, systemically blocking D1 and D2 receptors (St Onge et al., 2010) or stimulating D3 and α2 receptors, reduces risky choice in the PDT (with the latter effects potentially driven by autoreceptor activation) (Montes et al., 2015; St Onge & Floresco, 2009). Therefore, reducing activity at certain catecholamine receptors can reduce the allure of larger yet riskier rewards.

Although other receptor mechanisms such as the remaining adrenergic receptors have yet to be evaluated, these collective findings have established a framework for how catecholamine-based treatments can alter probabilistic discounting. Using this information, we designed experiments to validate the PDT in our laboratory using three pharmacological agents that enhance catecholamine transmission to compare their individual potentials to increase risky choice.

4.2 Rationale

Psychostimulants block reuptake and elevate extracellular concentrations of both DA and NE, and are common pharmacological strategies used to improve PFC-dependent behavioral dysfunction associated with neuropsychiatric disorders like attention deficit hyperactivity disorder (ADHD). However, this approach can be problematic given the side effect liability and abuse potential of psychostimulants. One of these potential side effects, risk taking, can be evaluated using the PDT. AMPH has been shown to increase risky choice behavior in the PDT (Floresco & Whelan, 2009; St Onge et al., 2010; St Onge & Floresco, 2009), whereas methylphenidate (MPH), another ADHD-approved

psychostimulant, has not yet been evaluated. SK609 is a novel and non-stimulant NE reuptake blocker that selectively activates DA D3 receptors without affinity for the DA transporter (Xu et al., 2017). SK609 has been shown to improve sustained attention similar to MPH but does not produce MPH-like increases in spontaneous locomotor activity associated with DA transporter activity (Marshall et al., 2019). These findings suggest SK609 may help ameliorate neurocognitive impairments without psychostimulant-like side effects. The objective of **Chapter 4** was to first confirm our ability to manipulate standard PD curves using AMPH, a drug known for producing reliable and robust increases in risky choice behavior in the PDT. We then aimed to evaluate MPH and SK609 for their potential to increase risky choice. *We hypothesized that MPH would produce similar increases in risky choice as AMPH, but that SK609 would only marginally increase the preference for large/risky rewards.*

4.3 Methods

4.3.1 Animals

Thirty male Long-Evans rats were used in this study. Animals were obtained at 225-250g from Charles River Laboratories and underwent the housing, acclimation, and food regulation conditions described in **Chapter 3**.

4.3.2 Probabilistic Discounting Task

4.3.2.1 Lever-Pressing and PDT Training. Animals underwent the training protocols described in **Chapter 3**. Rats required ~18 days of PDT training before stable criterion performance was achieved. They then received approximately 10 additional days

of training to maintain stable performance. A counter-balanced drug dosing design was then used to assign an order of drug or vehicle (saline) tests to each rat.

4.3.3 Drug Testing

A within-subjects design was used for all drug tests with AMPH (0.25, 0.50 and 1.0 mg/kg; Sigma-Aldrich), MPH (2.0 and 8.0 mg/kg; Sigma-Aldrich), and SK609 (4.0 mg/kg; and PolyCore Therapeutics). As described in **Chapter 3**, all drugs were administered intraperitoneally (i.p.) either 10 min (AMPH), 15 min (MPH) or 5 min (SK609) before testing. Following a drug test day, rats were retrained until they, as a group, displayed stable patterns of choice for 3 consecutive sessions, after which subsequent drug tests were administered. These 3 days also served as a washout period between drug doses. This procedure was repeated until all rats had received each of their designated treatments.

4.3.4 Statistical Analysis

All data analysis was performed using GraphPad Prism software (GraphPad Software, San Diego CA). For AMPH and MPH, choice behavior, along with response and magazine latencies, were analyzed using two-way mixed-design ANOVAs with trial block (100%, 50%, 25%, 12.5%, and 6.25%) and treatment (AMPH – 0, 0.25, 0.5, and 1mg/kg; MPH – 0, 2.0, and 8.0 mg/kg) as the within-subjects factors. For SK609, choice behavior, along with response and magazine latencies, were analyzed using two-way repeated measures ANOVAs with trial block (100%, 50%, 25%, 12.5%, and 6.25%) and treatment (0 and 4.0 mg/kg) as the within-subjects factors. For all drug treatments, Win-Stay/Lost-Shift behavior was analyzed using two-way repeated measures ANOVAs with feedback

(win-stay and lose-shift) and treatment (AMPH – 0, 0.25, 0.5, and 1mg/kg; MPH – 0, 2.0, and 8.0 mg/kg; SK609 – 0, 4.0 mg/kg) as the within-subjects factors. Dunnett's or Sidak's multiple comparisons tests, when appropriate, were used to compare individual differences when overall significance was found. For all results, statistical significance was determined by a p value < 0.05.

4.4 Experimental Results

4.4.1 Producing Characteristic Probabilistic Discounting Curves

To establish and validate the PDT within our laboratory, rats were initially trained on the task to verify our ability to produce characteristic probabilistic discounting (PD) curves as reported by other laboratories (**Fig. 7**). Analysis of choice behavior across the last 3 consecutive training sessions revealed a significant main effect of block [$F (2.732, 79.23) = 184.2$, $p < 0.0001$], but not a main effect of day [$F (1.713, 49.67) = 0.9351$, $p = 0.3865$] nor a block x day interaction [$F (5.866, 168.6) = 1.424$, $p = 0.2092$], indicating that stable baseline levels of choice behavior were achieved.

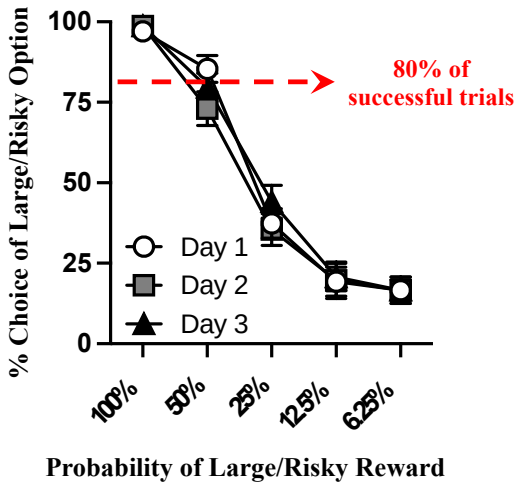


Figure 7. Last 3 consecutive pre-treatment sessions on the probabilistic discounting task. Rats (n = 30) chose the risky lever in >80% of trials (red dashed line) in the 100% block and maintained stable patterns of choice. Symbols represent mean ± SEM.

4.4.2 Effects of Amphetamine

After successfully producing PD curves, we then assessed our ability to reproduce pharmacological manipulations on standard PD curves using doses of AMPH that have previously been shown to increase risky choice in the PDT (Floresco & Whelan, 2009; St Onge et al., 2010; St Onge & Floresco, 2009) (**Fig. 8A**). We tested a low dose (0.25 mg/kg, i.p.- squares) that has been shown to improve cognition (Andrzejewski et al., 2014; Berridge & Stalnaker, 2002), a moderate dose (0.5 mg/kg, i.p.- triangles) (Grilly & Loveland, 2001), and a high dose (1.0 mg/kg, i.p.- upside-down triangles) that is above the therapeutic window of the drug and has been shown to promote hyperactive behavior in rats (Grilly & Loveland, 2001). Analysis of choice data revealed a significant main effect of block [$F(2.931, 84.99) = 235.5$, $p < 0.0001$], and treatment [$F(2.437, 70.68) = 58.28$, p

< 0.0001] as well as a significant block x treatment interaction [F (5.181, 148.9) = 20.84, p < 0.0001]. Dunnett's multiple comparisons analysis determined that rats treated with all doses of AMPH displayed a significant increase in risky choice preference ($p < 0.05$) in the 50%, 25%, and 12.5% blocks in comparison to vehicle (saline), whereas only the 0.5 and 1.0 mg/kg doses resulted in a significant increase in risky choice ($p < 0.05$) in the 6.25% block.

Analysis of win-stay/lose-shift data (**Fig. 8B**) revealed a significant main effect of both feedback [F (1.000, 29.00) = 914.2, p < 0.0001], and treatment [F (2.501, 72.54) = 21.63, p < 0.0001] as well as a significant feedback x treatment interaction [F (2.469, 71.61) = 36.34, p < 0.0001]. Dunnett's multiple comparisons analysis determined that rats treated with all doses of AMPH displayed a significant decrease in lose-shift tendencies ($p < 0.001$) compared to vehicle-treated rats, whereas only the 0.5 dose resulted in an increase in win-stay behavior ($p < 0.05$).

Analysis of both response and magazine latency data (**Fig. 8C** and **8D,** respectively) revealed a significant main effect of block [F (2.302, 66.75) = 6.501, p = 0.0017; F (2.646, 76.73) = 5.736, p = 0.0021, respectively], but did not yield a significant main effect of treatment [F (2.347, 68.06) = 0.3345, p = 0.7505; F (2.273, 65.91) = 0.7875, p = 0.4738, respectively] or a block x treatment interaction [F (5.836, 167.8) = 2.000, p = 0.0703; F (2.953, 79.72) = 2.551, p = 0.0624, respectively].

Overall, this experiment validated our lab's ability to manipulate an animal's discounting behavior, which is represented by the changing shape of their discounting curves.

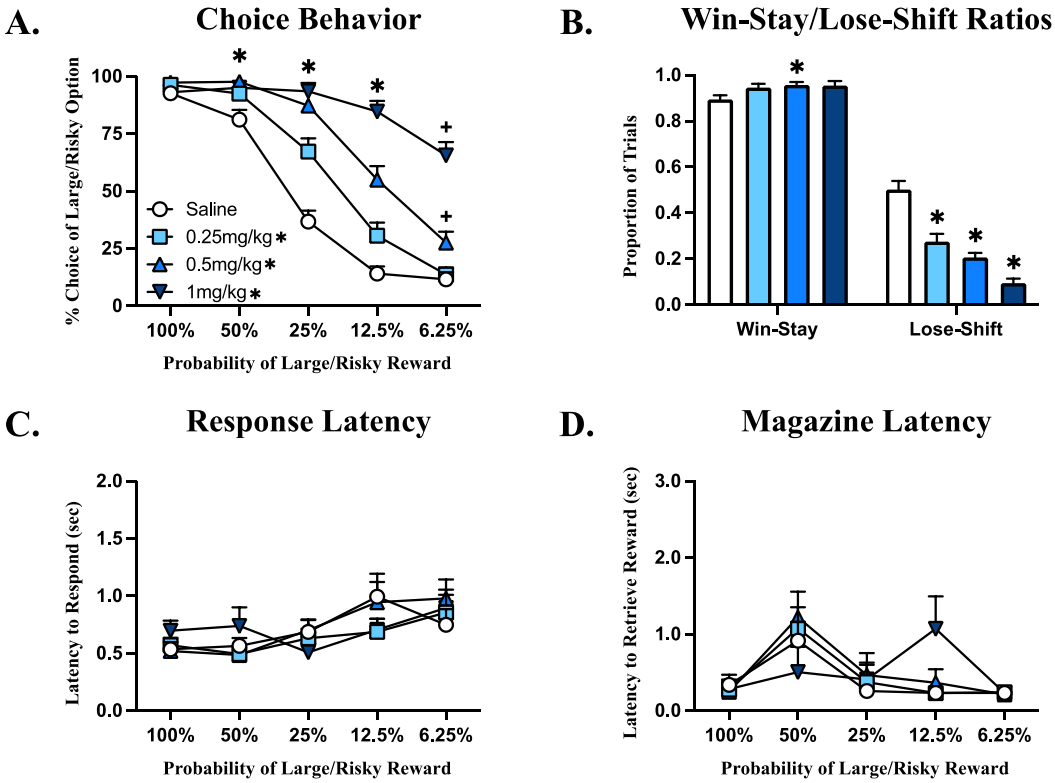


Figure 8. Effects of d-amphetamine on PDT performance. (n = 30) * on symbol key denotes $p < 0.05$ for an overall treatment effect of each dose versus vehicle. A) Choice Behavior- * on graph denotes $p < 0.05$ for the 0.25, 0.5, and 1 mg/kg doses versus vehicle at a specific block analyzed by Dunnett's multiple comparisons tests. + denotes $p < 0.05$ for the 0.5 and 1 mg/kg doses versus vehicle at a specific block analyzed by Dunnett's multiple comparisons tests. B) Win-Stay/Lose-Shift Ratios- * on graph denotes $p < 0.05$ versus vehicle analyzed by Dunnett's multiple comparisons tests. C) Response Latency- no specific effects observed. D) Magazine Latency- no specific effects observed. Symbols and bars represent mean ± SEM.

4.4.3 Effects of Methylphenidate

We further evaluated the effects of MPH (**Fig. 9A**) using a dose that has been shown to improve cognitive performance (2.0 mg/kg, i.p.- squares) (Arnsten & Dudley, 2005; Berridge et al., 2006; Navarra et al., 2017) as well as a high dose (8.0 mg/kg, i.p.- triangles)

that exceeds the therapeutic window of the drug and that we suspected would promote large increases in risk taking behavior. Analysis of choice data revealed a significant main effect of block [$F(2.810, 39.33) = 92.76$, $p < 0.0001$], and treatment [$F(1.491, 20.88) = 20.12$, $p < 0.0001$] as well as a significant block x treatment interaction [$F(4.217, 57.99) = 14.79$, $p < 0.0001$]. Dunnett's multiple comparisons analysis determined that rats treated with the 8 mg/kg dose of MPH displayed a significant increase in risky choice preference ($p < 0.05$) in the 25%, 12.5%, and 6.25% blocks in comparison to vehicle (saline).

Analysis of win-stay/lose-shift data (**Fig. 9B**) revealed a significant main effect of both feedback [$F(1.000, 14.00) = 93.84$, $p < 0.0001$], and treatment [$F(1.682, 23.55) = 12.58$, $p = 0.0004$], but did not yield a feedback x treatment interaction [$F(1.511, 21.16) = 1.203$, $p = 0.3078$]. To determine whether the observed main effect of treatment was specifically driven by either win-stay or lose-shift behavior, subsequent one-way ANOVA analyses were conducted. Analysis of win-stay data only failed to reveal a main effect of treatment [$F(1.258, 17.62) = 0.8829$, $p = 0.3845$]; however, analysis of lose-shift data revealed a main effect of treatment [$F(1.766, 24.73) = 9.559$; $p = 0.0012$] with Dunnett's multiple comparisons analysis determining that rats treated with the 8 mg/kg dose of MPH exhibited a significant decrease in lose-shift tendencies ($p = 0.0007$) compared to vehicle-treated animals.

Analysis of response latency data (**Fig. 9C**) failed to reveal a main effect of block [$F(1.965, 27.51) = 0.2085$, $p = 0.8093$], a main effect of treatment [$F(1.798, 25.17) = 1.706$, $p = 0.2036$] or a block x treatment interaction [$F(2.750, 37.81) = 0.8910$, $p = 0.4471$].

Analysis of magazine latency data (**Fig. 9D**) revealed a significant main effect of block [F (1.476, 20.66) = 7.658, p = 0.0060], but did not yield a significant main effect of treatment [F (1.779, 24.90) = 0.5905, p = 0.5426] or a block x treatment interaction [F (2.992, 38.53) = 1.936, p = 0.1401].

Overall, these results show that MPH, another psychostimulant with cognition-enhancing properties, also shows potential to increase risky choice behavior.

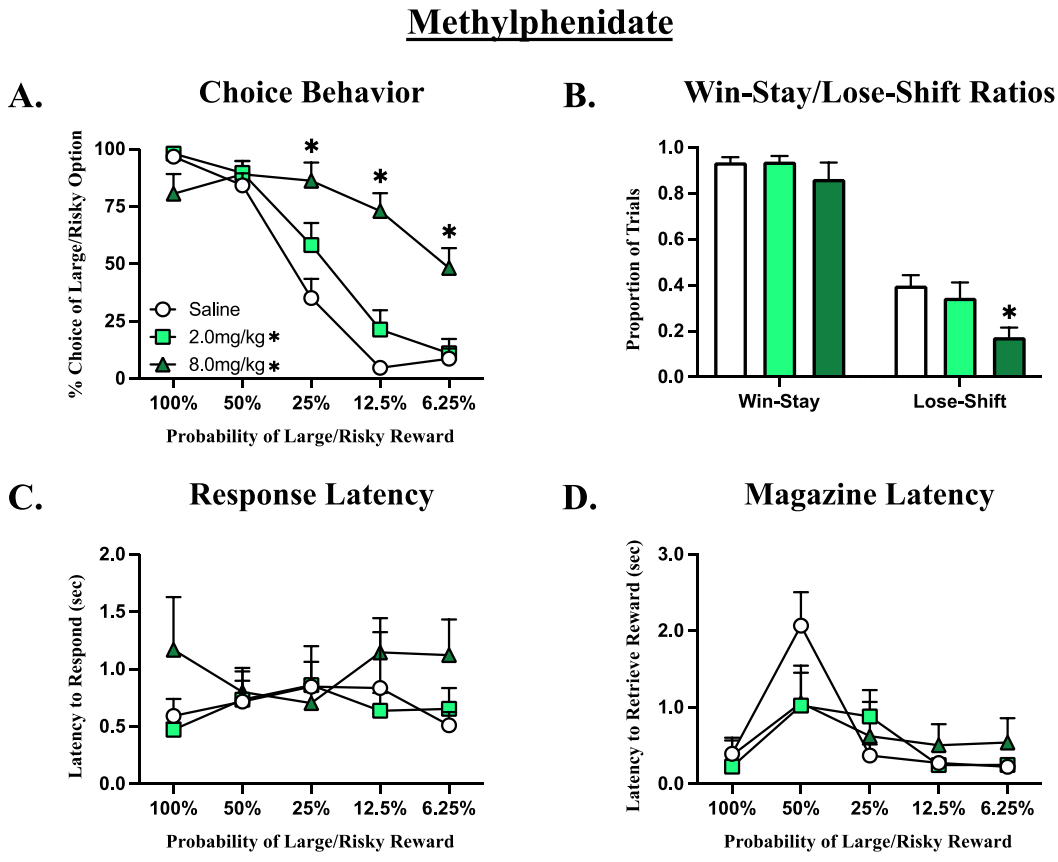


Figure 9. Effects of methylphenidate on PDT performance. (n = 15) * on symbol key denotes $p < 0.05$ for an overall treatment effect of each dose versus vehicle. A) Choice Behavior- * on graph denotes $p < 0.001$ for the 8 mg/kg doses versus vehicle at a specific block analyzed by Dunnett's multiple comparisons tests. B) Win-Stay/Lose-Shift Ratios- * on graph denotes $p < 0.001$ versus vehicle analyzed by Dunnett's multiple comparisons tests following one-way ANOVA analysis. C) Response Latency- no specific effects observed. D) Magazine Latency- no specific effects observed. Symbols and bars represent mean ± SEM.

4.4.4 Effects of SK609

We then assessed SK609 (**Fig. 10A**) at its cognitive enhancing dose (4.0 mg/kg, i.p.- squares) (Marshall et al., 2019). Analysis of choice data revealed a significant main effect of block [$F (2.057, 22.62) = 105.1$, $p < 0.0001$], but did not yield a significant main effect of treatment [$F (1.000, 11.00) = 1.430$, $p = 0.2569$] or a block x treatment interaction [$F (2.326, 25.58) = 0.01624$, $p = 0.9909$].

Analysis of win-stay/lose-shift data (**Fig. 10B**) revealed a significant main effect of feedback [$F (1.000, 11.00) = 53.69$, $p < 0.0001$] and a strong trend towards a significant main effect of treatment [$F (1.000, 11.00) = 4.049$, $p = 0.0693$]. There was no significant feedback x treatment interaction [$F (1.000, 11.00) = 1.247$, $p = 0.2879$] observed.

Analysis of response latency data (**Fig. 10C**) revealed a significant main effect of block [$F (1.373, 15.10) = 5.503$, $p = 0.0246$], but did not yield a significant main effect of treatment [$F (1.000, 11.00) = 0.01082$, $p = 0.9190$] or a block x treatment interaction [$F (1.982, 21.80) = 0.2375$, $p = 0.7887$].

Analysis of magazine latency data (**Fig. 10D**) failed to reveal a main effect of block [$F (1.061, 11.67) = 0.9101$, $p = 0.3657$], a main effect of treatment [$F (1.000, 11.00) = 2.460$, $p = 0.1450$] or a block x treatment interaction [$F (1.035, 11.38) = 2.189$, $p = 0.1661$].

Overall, these results show that SK609 does not increase risky choice behavior at its cognitive enhancing dose as represented by the lack of change in the shape of the PD curve.

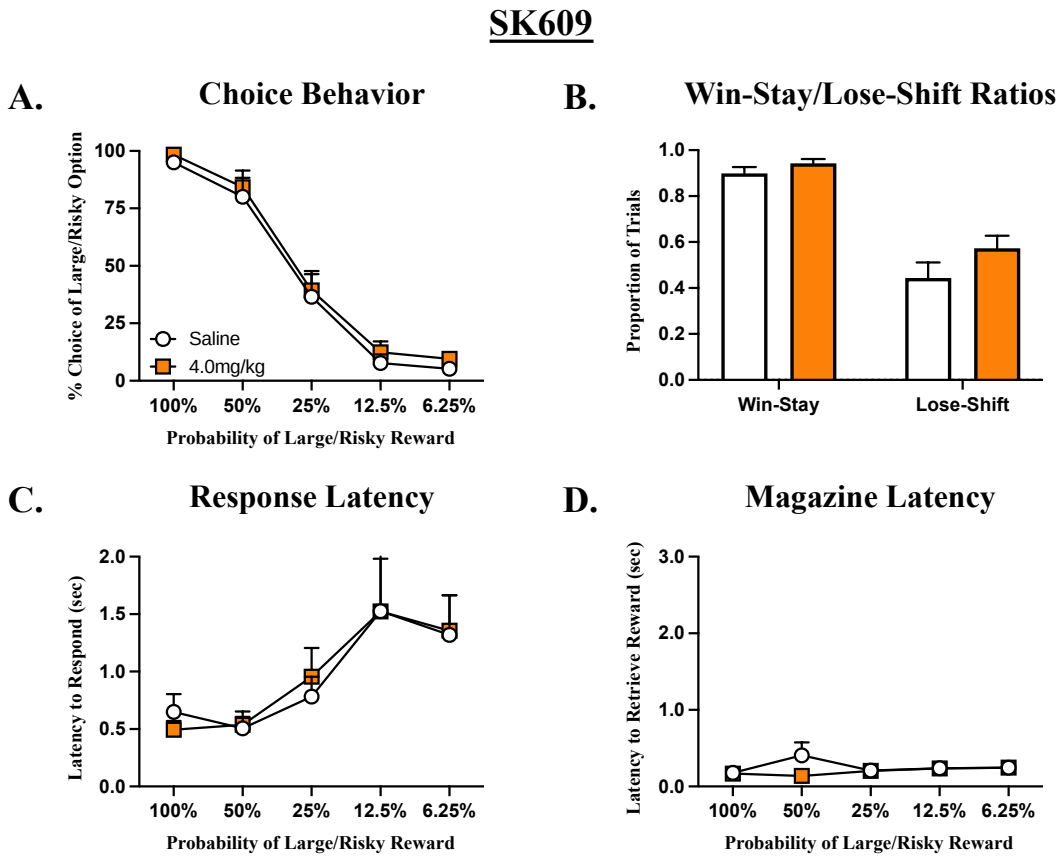


Figure 10. Effects of SK609 on PDT performance. (n = 12) A) Choice Behavior- no specific effects observed. B) Win-Stay/Lose-Shift Ratios- SK609 trended towards a treatment effect at p = 0.0693. C) Response Latency- no specific effects observed. D) Magazine Latency- no specific effects observed. Symbols and bars represent mean ± SEM.

4.5 Discussion

In this set of experiments, we evaluated three agents with cognitive-enhancing properties for their potential to increase risky choice behavior as a side effect. While all three drugs have been shown to improve selected dimensions of PFC-mediated cognitive

function, these data show the effects of SK609 can be dissociated from the psychostimulant class of drugs using the PDT. AMPH and MPH increased risky choice behavior at their cognition-improving doses, whereas SK609 did not. These results highlight the roles of NE transporter blockade and selective D3 activation in pro-cognitive action without side effect liability of increasing risky behavior. The absence of DA transporter blockade and non-selective dopaminergic elevation are beneficial properties of SK609 that differentiate it from traditional pro-cognitive psychostimulants.

Non-stimulant therapies have become a favorable alternative to stimulant treatments because of their minimal abuse potential and fewer side effects. Atomoxetine (ATX), another selective NE reuptake blocker, is a non-stimulant agent used for treating ADHD symptoms (Durell et al., 2013; Michelson et al., 2003; Michelson et al., 2001) and is known for improving rodent performance in cognitive tasks (Cain et al., 2011; Navarra et al., 2008; Robinson et al., 2008). In contrast to AMPH, rats treated with a low dose of ATX (0.3 mg/kg) showed very minimal increases in risky choice behavior within the PDT (Montes et al., 2015) whereas slightly higher doses (1 and 3 mg/kg) did not. While all three doses of ATX are considered to be clinically relevant, it is suggested that the lowest 0.3 dose of ATX may have greater selectivity for the NE transporter compared to the 1 and 3 mg/kg doses, which may also block other monoamine reuptake (Bymaster et al., 2002). Here, we observed that SK609, which also has selectivity for the NE transporter, did not alter choice preference, demonstrating the effectiveness of this drug to improve cognition while limiting risk-taking behavior as a side-effect. Together, these experiments laid the groundwork for testing other pharmacological agents with selective NE transporter and receptor-specific properties.

While it is clear that stimulants and non-stimulants have differing potentials to increase risk-taking behavior, there are also differences between MPH and AMPH. MPH increased risky choice at its cognitive-enhancing dose; however, it did not alter feedback sensitivity. AMPH not only increased risky choice at all doses, but these effects were driven by reduced sensitivity to non-rewarded risky choices, a behavior observed only at the highest dose (8.0 mg/kg) of MPH. These differential effects may be due to the different mechanisms by which MPH and AMPH enhance catecholamine transmission. MPH and AMPH both block the DA and NE transporters; however, AMPH also induces catecholamine release through transporter-mediated reverse transport (Sulzer et al., 2005). In combination, these mechanisms may lead to supranormal increases in catecholamine activity and excessive receptor stimulation, resulting in a secondary effect on feedback sensitivity. It may be that the high 8.0 mg/kg dose of MPH mimics these effects whereas the low 2.0 mg/kg dose of MPH may not be sufficient to alter lose-shift behavior. Nevertheless, these findings indicate that while psychostimulants can promote risky behavior, not all aspects of risk/reward decision making are affected in the same way. MPH may be a more favorable option over AMPH when considering treatment options for improving cognitive performance while limiting side-effect symptoms.

Interestingly, no changes in response or magazine latencies were observed across all treatment conditions. Although AMPH and high doses of MPH decreased lose-shift tendencies, this reduced sensitivity to non-rewarded outcomes does not appear to promote greater or lesser deliberation of subsequent decisions. These lack of changes in response latencies were also surprising given the hyperactivity and inattention that is typically observed with higher doses of these drugs. AMPH abusers have been shown to exhibit

slower deliberation times in comparison to control patients within the Cambridge Gambling task (Rogers, Everitt, et al., 1999). Therefore, it is possible that intermittent dosing of AMPH and MPH is not sufficient to cause significant changes in response latencies but continuous/repeated long-term exposure to stimulant agents can lead to alterations in the rate in which cost/benefit contingencies are effectively evaluated. Given that SK609 did not alter choice preference or feedback sensitivity, it is unsurprising that response and magazine latencies were also unaffected. Taken together, these results suggest that stimulant and non-stimulant agents do not significantly affect the rate at which cost/benefit contingencies are evaluated and the desire to collect one's reward.

4.6 Conclusion

From these validation studies, we successfully produced characteristic PD curves, confirmed our ability to detect changes in PD curves following pharmacological manipulations, and differentiated the effects of stimulant and non-stimulant cognitive-enhancing compounds. Our results highlight a potential side-effect (i.e., increased risky behavior) of stimulant compounds that should be considered when prescribing treatment options for patients with PFC-mediated cognitive dysfunctions. By validating this task according to results obtained in other laboratories, we felt confident using the PDT to evaluate the impact of rmTBI on risk/reward decision making.

Chapter 5

Effects of Repetitive Mild Traumatic Brain Injury on Learning and Performance of the Probabilistic Discounting Task

5.1 Introduction

In patients with traumatic brain injury (TBI), damage to the prefrontal cortex (PFC) can cause difficulty in making deliberate and advantageous choices, resulting in inappropriate decisions and increased risk taking (Bechara et al., 1994; Bechara et al., 1998; Bechara et al., 2000). Impaired decision making is a major concern because of its importance in evaluating the risks and benefits associated with daily decisions. Individuals such as athletes, military personnel, and domestic violence victims are most at risk of sustaining multiple TBIs, the majority of which are classified as mild (mTBI), or concussions. However, these populations have historically been understudied when it comes to evaluating the effects of repetitive (rmTBI) insults on PFC-mediated executive processes such as risk/reward decision making.

Clinical studies often struggle to control for the severity or focal point of injury when examining the cognitive deficits following TBI. The goal of this dissertation was to develop a controlled pre-clinical model of rmTBI to experimentally evaluate the effects of rmTBI on PFC-mediated risk/reward decision making behavior using the probabilistic discounting task (PDT). As described in the **Risk/Reward Decision Making** section of **Chapter 1**, performance in this task relies on intact PFC functioning. Experimental manipulations to neural (St Onge & Floresco, 2010; Stopper et al., 2014) or catecholaminergic (Jenni et al., 2021; St Onge et al., 2011) activity within the PFC have

been shown to alter risk-related decisions in the PDT. Therefore, TBI-induced disruptions to PFC processes that mediate risk/reward decision making should be detectable with this task. Here, we describe initial experiments performed using the PDT in effort to characterize the effects of single versus repetitive mTBI on risk/reward decision making.

5.2 Rationale

A lack of information exists regarding the effects of rmTBI on risk/reward decision making, including whether rmTBI impairs learning and adapting to changing cost/benefit contingencies associated with large, probabilistic rewards. As observed in **Chapter 4**, untreated animals required ~18 days to establish acquisition and stable performance in the PDT. Assuming that TBI hinders acquisition of the PDT, we wanted to see if this results in increased risky choice preference compared to uninjured animals. These mTBI-induced disruptions in task performance may further be potentiated by repeated impacts. As such, the objective here was to evaluate the effects of mTBI on acquisition and performance of the PDT in animals that had no prior pre-training on the task prior to surgeries. *We hypothesized that animals that received rmTBI would take longer to achieve stable criterion performance in the PDT and demonstrate greater risk-taking behavior compared to single and uninjured animals.*

5.3 Methods

5.3.1 Animals

Thirty-one male Long-Evans rats were used in this study. Animals were obtained at 6-7 weeks old/150-200g from Charles River Laboratories and underwent the housing,

acclimation, and food regulation conditions described in **Chapter 3**.

5.3.2 Probabilistic Discounting Task

5.3.2.1 Lever-Pressing Training and PDT Testing. Animals underwent the lever-pressing training protocols described in **Chapter 3**. Animals were then rank ordered based on the total amount of days they took to pass all training programs. Animals who completed all training programs the fastest were assigned a higher rank. If more than one animal passed all training sessions in the same number of days, the rat with the highest number of lever presses was ranked higher. A reverse Latin square method was then used to assign rats to one of three surgical groups: sham (uninjured), single injury (smTBI), or repetitive injury (rmTBI). Forty-eight hours after the final surgery, animals were introduced to the PDT (described in **Chapter 3**) and tested 5 days per week for four weeks.

5.3.3 Surgery

At 9-10 weeks of age, rats underwent the surgical procedures described in **Chapter 3**. Briefly, a 5mm diameter metal impactor tip was positioned on the skull surface along the sagittal suture with the edge of the tip aligned with bregma. The tip was then electronically driven at a velocity of 5.5m/s and a depth of 2.5mm below the surface point of contact with a dwell time of 100ms. rmTBI rats received an impact on all 3 surgery days whereas smTBI rats received sham surgeries on the first 2 surgery days and an impact on the 3rd day. Sham rats underwent the same surgical procedures but were not impacted on any day.

5.3.4 Statistical Analysis

All data analysis was performed using GraphPad Prism software (GraphPad Software, San Diego CA). Righting reflex times were measured using a two-way repeated measures ANOVA with surgery day (day 1, day 2, and day 3) as the within-subjects factor and injury condition (sham, smTBI, and rmTBI) as the between-subjects factor. For post-surgery PDT behavior, choice behavior and response latencies were analyzed using two-way repeated-measures ANOVAs with trial block (100%, 50%, 25%, 12.5%, and 6.25%) as the within-subjects factor and injury condition (sham, smTBI, and rmTBI) as the between-subjects factor. Win-Stay/Lost-Shift behavior was also analyzed using two-way repeated-measures ANOVAs with feedback (win-stay and lose-shift) as the within-subjects factors and injury condition (sham, smTBI, and rmTBI) as the between-subjects factor. Magazine latencies were analyzed using two-way mixed-effects ANOVAs with trial block (100%, 50%, 25%, 12.5%, and 6.25%) as the within-subjects factor and injury condition (sham, smTBI, and rmTBI) as the between-subjects factor. Dunnett's multiple comparisons tests, when appropriate, were used to compare individual differences when overall significance was found. For all results, statistical significance was determined by a p value < 0.05.

5.4 Experimental Results

5.4.1 Acute Response to Injury

rmTBI resulted in longer righting reflex times compared to those of sham animals (**Table 2**). Analysis of righting reflex data failed to reveal a main effect of day [$F(1.695, 47.46) = 2.544$, $p = 0.0974$], but did reveal a significant main effect of injury [$F(2, 28) =$

3.519, p = 0.0433] as well as a significant day x injury interaction [F (4, 56) = 5.321, p = 0.0011]. Dunnett's multiple comparisons analysis revealed that rmTBI rats demonstrated a trend towards longer righting reflex times compared to sham rats on day 2 (p = 0.0552).

Table 2

Righting Reflex Times of Non-Pretrained Animals (2.5mm Impact Depth)

Injury Condition	N	Righting Reflex (sec)		
		Surgery Day 1	Surgery Day 2	Surgery Day 3
sham	11	322.5 ± 52.9	211.6 ± 42.7	272.0 ± 43.2
smTBI	10	217.7 ± 31.1	187.7 ± 28.4	341.2 ± 45.8
rmTBI	10	467.4 ± 50.5	400.1 ± 66.6^	274.4 ± 62.4

Note. Average righting reflex times (seconds) of sham, single (smTBI), and repetitive (rmTBI) injury groups across all three surgery days. On surgery day 2, rmTBI males demonstrated a trend towards longer righting reflex times compared to sham animals. Values represent mean ± SEM. ^ denotes $p < 0.1$ from shams analyzed with Dunnett's multiple comparisons tests.

5.4.2 Effects of mTBI on Task Acquisition

As described in the **PDT Training and Testing** section of **Chapter 3**, the requirements to achieve baseline criteria included choosing the risky lever in >80% of trials in the 100% block and maintaining stable patterns of choice for 3 consecutive sessions. This involved analyzing 3 consecutive sessions with a repeated measures ANOVA, where rats had to demonstrate a significant main effect of block ($p < 0.05$), but not a main effect of day or day x block interaction ($p > 0.1$). Following surgery, smTBI and rmTBI groups required 16 days of task performance before stable performance was achieved whereas sham animals required 20 days (**Fig. 11**).

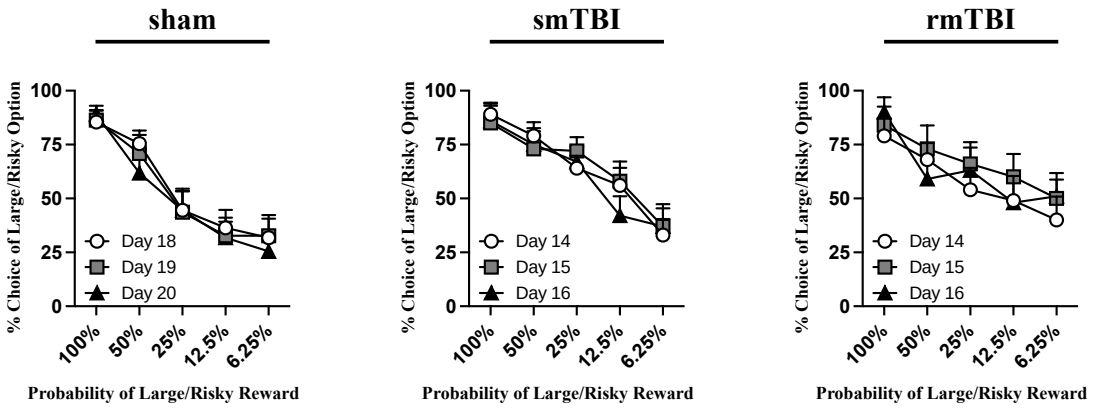


Figure 11. Days required for non-pretrained animals to achieve stable performance in the PDT**.** Sham animals achieved stable performance in 20 days post-final surgery whereas single and repetitive injured animals required only 16 days. Symbols represent mean ± SEM.

5.4.3 Effects of mTBI on Choice Behavior

Across all 4 weeks post-final surgery (**Fig. 12**), analysis of choice data revealed a significant main effect of block [F (3.198, 89.55) = 9.743, $p < 0.0001$; F (2.875, 80.50) = 7.647, $p = 0.0002$; F (2.568, 71.90) = 25.82, $p < 0.0001$; F (2.875, 80.50) = 26.71, $p < 0.0001$, respectively]. However, there was no main effect of injury [F (2, 28) = 0.3500, $p = 0.7077$; F (2, 28) = 0.6168, $p = 0.5468$; F (2, 28) = 0.6090, $p = 0.5509$; F (2, 28) = 0.06919, $p = 0.9333$, respectively] or a block x injury interaction [F (8, 112) = 1.393, $p = 0.2074$; F (8, 112) = 0.4488, $p = 0.8891$; F (8, 112) = 0.4487, $p = 0.8891$; F (8, 112) = 1.499, $p = 0.1653$, respectively] in weeks 1-4.

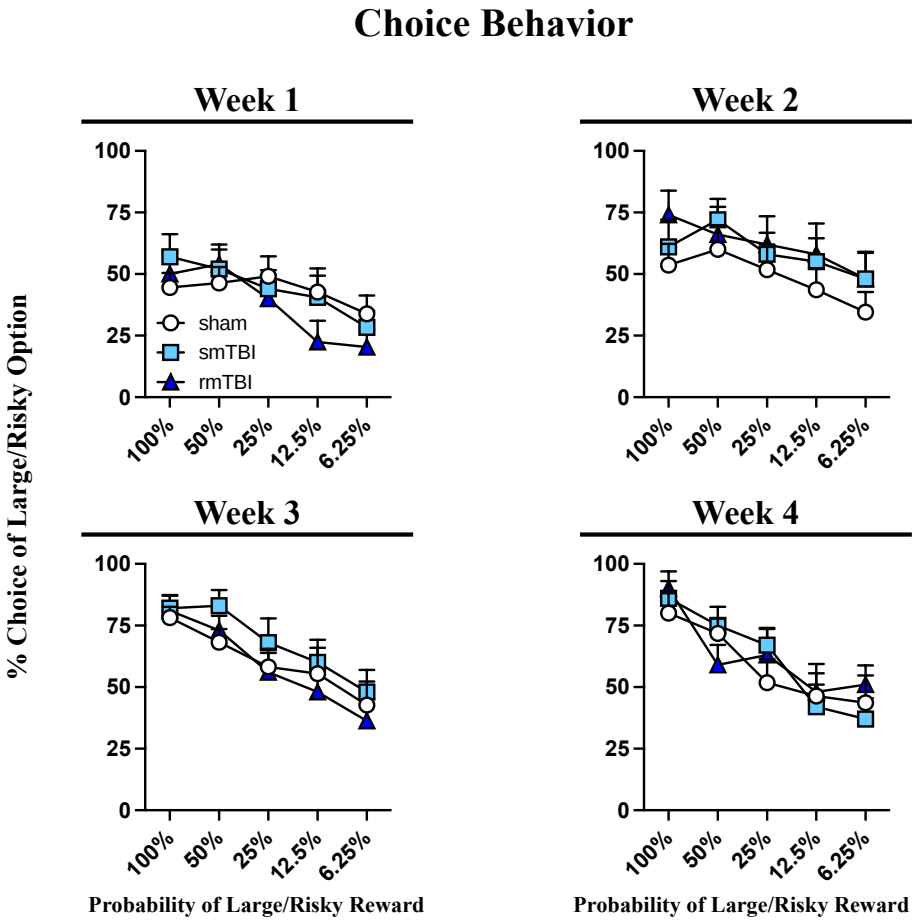


Figure 12. Post-surgery choice performance in non-pretrained animals. Choice performance is illustrated across four weeks post-final surgery. No differences in choice behavior were found between sham, single (smTBI), or repetitive (rmTBI) injury groups in weeks 1-4. Symbols represent mean ± SEM.

5.4.4 Effects of mTBI on Win-Stay/Lose-Shift Behavior

In weeks 1 and 2 post-final surgery (**Fig. 13**), analysis of win-stay/lose-shift data failed to reveal a significant main effect of feedback [F (1, 28) = 1.994, p = 0.1689; F (1, 28) = 1.753, p = 0.1962, respectively], a main effect of injury [F (2, 28) = 0.03072, p = 0.9698; F (2, 28) = 0.3271, p = 0.7237, respectively] or a feedback x injury interaction [F (2, 28) = 0.08755, p = 0.9164; F (2, 28) = 0.4822, p = 0.6225, respectively].

In weeks 3 and 4 post-final surgery, analysis of win-stay/lose-shift data revealed a significant main effect of feedback [F (1, 28) = 22.73, p < 0.0001; F (1, 28) = 25.59, p < 0.0001, respectively] but did not yield a main effect of injury [F (2, 28) = 0.2351, p = 0.7920; F (2, 28) = 0.07225, p = 0.9305, respectively] or a feedback x injury interaction [F (2, 28) = 0.3495, p = 0.7081; F (2, 28) = 0.05457, p = 0.9470, respectively].

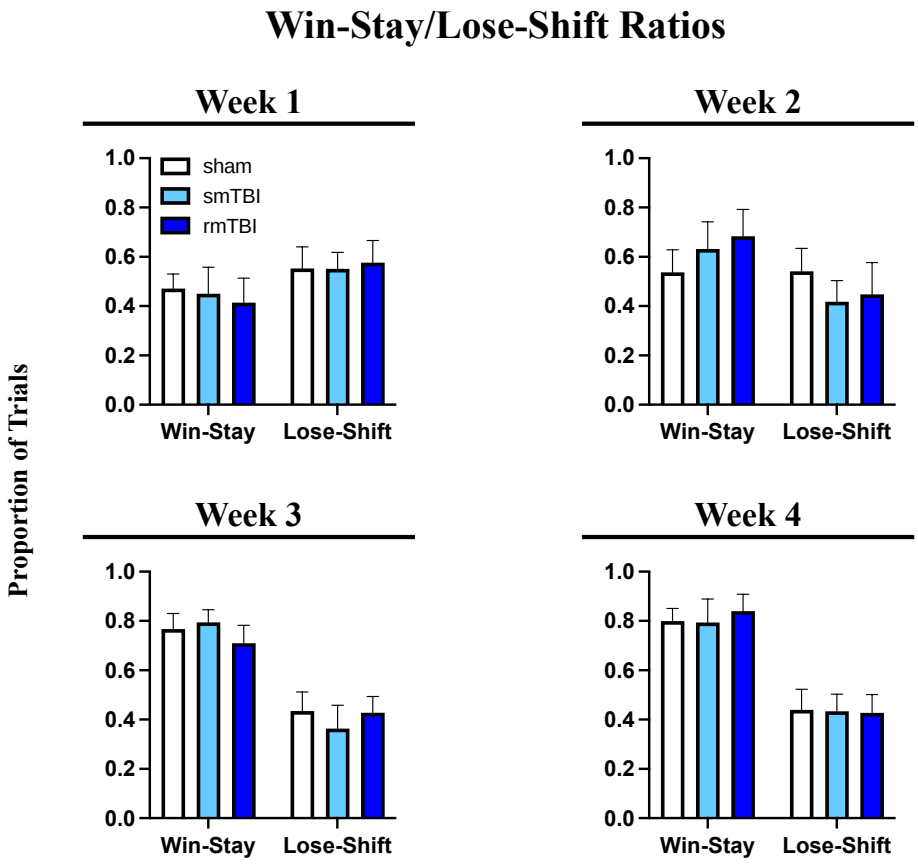


Figure 13. Post-surgery win-stay/lose-shift performance in non-pretrained animals. Win-Stay/Lose-Shift ratios are illustrated across four weeks post-final surgery. No differences in win-stay/lose-shift behavior were found between sham, single (smTBI), or repetitive (rmTBI) injury groups in weeks 1-4. Bars represent mean ± SEM.

5.4.5 Effects of mTBI on Response and Magazine Latencies

Analysis of response latency data (**Fig. 14A**) revealed a significant main effect of block in week 1 post-final surgery [F (2.632, 73.70) = 4.296, p = 0.0101] but not in weeks 2-4 [F (2.799, 78.38) = 1.490, p = 0.2256; F (2.842, 79.58) = 0.3444, p = 0.7826; F (1.833, 51.32) = 1.887, p = 0.1649, respectively]. Across all 4 weeks post-final surgery, analysis of response latency data failed to reveal a significant main effect of injury [F (2, 28) = 0.7400, p = 0.4862; F (2, 28) = 1.138, p = 0.3350; F (2, 28) = 1.431, p = 0.2559; F (2, 28) = 0.5649, p = 0.5748, respectively] or block x injury interaction [F (8, 112) = 0.5715, p = 0.7983; F (8, 112) = 0.4751, p = 0.8715; F (8, 112) = 0.6913, p = 0.6984; F (8, 112) = 0.8183, p = 0.5880, respectively].

Analysis of magazine latency data (**Fig. 14B**) failed to reveal a significant main effect of block in weeks 1 and 2 post-final surgery [F (2.255, 62.57) = 1.206, p = 0.3094; F (2.191, 75.61) = 0.7300, p = 0.4970, respectively] but did reveal a significant main effect of block in weeks 3 and 4 [F (2.230, 61.87) = 3.297, p = 0.0386; F (1.680, 57.96) = 4.803, p = 0.0161, respectively]. In weeks 1-3 post-final surgery, analysis of magazine latency data failed to reveal a significant main effect of injury [F (2, 28) = 0.2726, p = 0.7634; F (2, 138) = 0.05466, p = 0.9468; F (2, 28) = 1.199, p = 0.3165, respectively] or a block x injury interaction [F (8, 111) = 0.9954, p = 0.4437; F (8, 138) = 1.144, p = 0.3379; F (8, 111) = 0.7176, p = 0.6756, respectively]. However, in week 4 post-final surgery, analysis of magazine latency data did reveal a significant main effect of injury [F (2, 138) = 5.390, p = 0.0056] as well as a block x injury interaction [F (8, 138) = 3.773, p = 0.0005]. Dunnett's multiple comparisons analysis determined that smTBI rats demonstrated a trend

towards increased magazine latency ($p < 0.1$) in the 50% and 25% blocks in comparison to sham rats.

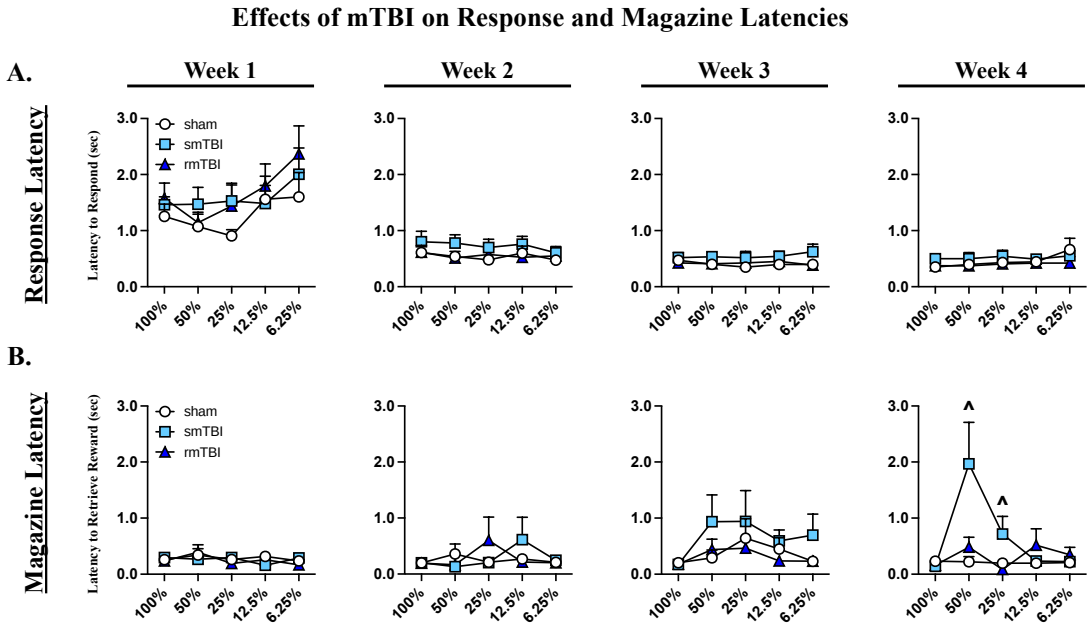


Figure 14. Post-surgery latency performance in non-pretrained animals. Response and magazine latencies are illustrated across four weeks post-final surgery. A) Response Latency- no differences in response latencies were found between sham, single (smTBI), or repetitive (rmTBI) injury groups in weeks 1-4. B) Magazine Latency- no differences in magazine latencies were found between injury groups in week 1-3. In week 4, smTBI resulted in a trend towards increased magazine latencies in the 50% and 25% blocks. ^ on graph denotes $p < 0.1$ versus sham analyzed by Dunnett's multiple comparisons tests. Symbols represent mean ± SEM.

5.5 Discussion

This study investigated the effects of mTBI on risk/reward decision making using the PDT. First, we assessed whether mTBI impaired the rate at which animals learned the cost/benefit contingencies associated with the PDT to establish consistent patterns of choice. Single and repetitive injured animals took 16 days to acquire the PDT, whereas the

sham group took 20 days. Although it appears that injured rats achieved stable performance faster than sham animals, it must be noted that one sham rat demonstrated slower acquisition of the PDT, leading to the sham group not meeting criteria. As a group, sham animals met all statistical criteria for maintaining stable patterns of choice by day 16; however, the sham group only chose the risky lever approximately 78% of trials instead of >80% due to the low amount of risky choices made by one rat. By day 20, this animal was performing similarly to the rest of the group, resulting in shams meeting all baseline criteria. Thus, we cannot definitively conclude that mTBI reduces the amount of days needed for rats to achieve acquisition of the PDT.

When performance in the PDT was evaluated across all four testing weeks, no significant differences between injury groups were observed, nor were any changes detected in win-stay or lose-shift behavior. Furthermore, we did not observe any biologically relevant effects of mTBI on response or magazine latencies, indicating that information processing and motivation to collect rewards were not affected. Although these findings initially suggest that mTBI does not alter risk/reward decision making processes as assessed with the PDT, these results may be a reflection of our experimental design. As observed in previous laboratories (St Onge & Floresco, 2009) and in our validation study (**see Chapter 4 for review**), animals require approximately two and a half weeks to learn and acquire criterion performance of the PDT. This, combined with the possibility that the effects of our injury model may have been too mild or transient, may have prevented us from detecting potential TBI-induced changes in risky choice behavior. For this reason, we did not continue with these experiments using female rodents.

In order to improve upon this developing model for assessing the effects of mTBI on risk/reward decision making using the PDT, we sought to redesign our experimental framework by first requiring animals to establish stable choice patterns prior to mTBI surgery. This experimental design was utilized in the pharmacological experiments conducted in **Chapter 4**; however, previous studies had already established a framework for evaluating pharmacological agents in the PDT (Floresco & Whelan, 2009; Montes et al., 2015; St Onge et al., 2010; St Onge & Floresco, 2009; Stopper et al., 2013). Since the PDT had not yet been used to evaluate the effects of mTBI, we first wanted to assess whether mTBI impaired the rate of learning of this task. As shown in this Chapter, we were unable to discern whether mTBI affected risky choice; however, these results guided us to improve our approach for potentially detecting changes in risk/reward decision making using the PDT.

5.6 Conclusion

The experiments conducted in this Chapter revealed no differences in risky choice following mTBI when animals sustained injuries prior to PDT testing. Due to the amount of time needed to learn and establish stable performance in the PDT combined with potentially transient effects of mTBI, we suspect we may have missed the window for detecting possible injury-induced changes in risky choice behavior. As such, our experimental design was modified for subsequent Chapters to allow animals to first train and establish stable choice patterns in the PDT before receiving sham or mTBI surgeries. Animals then returned to the PDT for testing to better evaluate the effects of mTBI on risk/reward decision making.

Chapter 6

Characterizing the Effects of Repetitive Mild Traumatic Brain Injury on Risk/Reward Decision Making

Disclaimer: Portions of this chapter were adapted from: Knapp CP, Papadopoulos E, Loweth JA, et al. Perturbations in risk/reward decision making and frontal cortical catecholamine regulation induced by mild traumatic brain injury. *Behav Brain Res*. 467:115002. doi:10.1016/j.bbr.2024.115002

6.1 Introduction

In the preceding Chapter, we were not able to detect meaningful differences in acquisition of baseline criteria or risky choice preference during probabilistic discounting task (PDT) performance following mild traumatic brain injury (mTBI) in animals that received no pre-surgery pretraining. We speculated that our injury model may have produced effects which were too mild or transient to be effectively detected and differentiated from those of general task acquisition. Thus, we modified our experimental model to allow animals to first establish stable choice patterns in the PDT before receiving sham or mTBI surgeries. In the present Chapter, we executed this updated strategy to assess the effects of repetitive mTBI (rmTBI) on risk/reward decision making.

6.2 Rationale

Mild TBIs are often referred to as a silent epidemic because they frequently go unrecognized, unreported, and untreated. While the effects of single injuries are often

transient, evidence suggests that repetitive mild injuries can result in severe and long-lasting cognitive impairments (Avedesian et al., 2021; Collins et al., 1999; De Beaumont et al., 2007; Rosenbaum & Lipton, 2012; Wall et al., 2006). The prefrontal cortex (PFC), which is most frequently affected following TBI events (Bigler, 2001; McAllister, 2011), mediates complex cognitive processes that regulate decision making and action in situations that involve uncertain risk/reward outcomes. As discussed in **Chapter 1**, the Iowa Gambling Task (IGT) (Bechara et al., 1994) has primarily been used in clinical assessments of risk/reward decision making in TBI patients (see the **Iowa Gambling Task** section of **Chapter 1** for review). Initial studies established that patients who have experienced TBI, especially with damage encroaching on the PFC, displayed suboptimal decision profiles, resulting in greater loss of money on the IGT (Bechara et al., 1994; Bechara et al., 1998; Bechara et al., 2000). Although clinical studies have consistently reported increased risk-taking behavior in single TBI cases (Bechara et al., 1994; Bechara et al., 1998; Bechara et al., 2000; Levine et al., 2005; Manes et al., 2002; Rogers, Everitt, et al., 1999), the effects of repetitive mild injuries have not been explored. One limitation of clinical studies on how cognitive functions may be affected by repetitive TBI is that it is difficult to control for the severity, timing, and number of injuries that a TBI patient suffers. Moreover, much of the current TBI research does not include a proportionate number of men and women in their assessments, despite numerous reports of sex differences in TBI susceptibility rates, post-injury symptoms, and recovery patterns (see the **Sex Differences in Traumatic Brain Injury** section of **Chapter 1** for review). Post-TBI assessments of decision making often group men and women together (Bechara et al., 1994; Bechara et al., 1998; Bechara et al., 2000; Manes et al., 2002; Rogers, Everitt, et al.,

1999), making it difficult to differentiate potential sex differences in risk/reward decision making following injury. These deficiencies have underscored the need for the development of pre-clinical models that can appropriately access, reveal, and differentiate potential sex-specific deficits in PFC-mediated cognitive processes following TBI. As such, the focus of **Chapter 6** was to investigate how rmTBI affects risk/reward decision making behavior and whether these outcomes can be differentiated by sex. *We hypothesized that rmTBI produces greater increases in risk-taking behavior compared to single and uninjured animals, and that these effects are more severe and longer-lasting in females compared to males.*

6.3 Methods

6.3.1 Animals

Thirty-one male and thirty female Long-Evans rats were used in this study. Animals were obtained at 3-4 weeks old/50-75g from Charles River Laboratories and underwent the housing, acclimation, and food regulation conditions described in **Chapter 3**.

6.3.2 Probabilistic Discounting Task and Surgical Procedures

Animals underwent the lever-pressing and PDT training protocols described in **Chapter 3**. Rats required ~17 days of training before stable criterion performance was achieved. Animals were then rank ordered based on the average ratio of large/risky lever presses over successful free-choice trials across the last three training days. Animals with the highest ratio were assigned a higher rank. A reverse Latin square method was then used to assign rats to one of three surgical groups: sham (uninjured), single injury (smTBI), or

repetitive injury (rmTBI). This method was used to limit bias when forming surgical groups by having equal amounts of risk-prone and risk-adverse rats in each group. Animals (9-10 weeks of age) then underwent the surgical procedures described in **Chapter 3**. Forty-eight hours after the final surgery, animals were reintroduced to the PDT and tested 5 days per week for four weeks to assess changes in risk/reward decision making.

6.3.3 Statistical Analysis

Analysis of righting reflex and win-stay/lose-shift data was performed using GraphPad Prism software (GraphPad Software, San Diego CA). Analysis of choice behavior as well as response and magazine latencies were performed using SPSS software (IBM, SPSS Inc.) as well as GraphPad Prism software. Male and female righting reflex data were analyzed separately using two-way repeated measures ANOVAs with surgery day (day 1, day 2, and day 3) as the within-subjects factor and injury condition (sham, smTBI, and rmTBI) as the between-subjects factor. For post-surgery PDT behavior, choice behavior as well as response and magazine latency data were averaged across 3 consecutive sessions, respectively, and analyzed using three-way mixed-design ANOVAs with trial block (100%, 50%, 25%, 12.5%, and 6.25%) as the within-subjects factor and injury condition (sham, smTBI, and rmTBI) and sex (male and female) as the between-subjects factors. Additional within-subjects analyses were performed for choice behavior and magazine latency data for each surgical group using two-way repeated measures ANOVAs with trial block (100%, 50%, 25%, 12.5%, and 6.25%) and phase (pre-surgery and post-surgery) as the within-subjects factors. The effect of trial block was always significant ($p < 0.05$) for choice behavior and response latencies, but not for magazine latencies ($p >$

0.05), and will not be discussed further. Win-stay/lose-shift data were computed across 3 consecutive sessions and analyzed using three-way mixed-design ANOVAs with feedback (win-stay and lose-shift) as the within-subjects factor and injury condition (sham, smTBI, and rmTBI) and sex (male and female) as the between-subjects factors. Additional within-subjects analyses were performed for win-stay/lose-shift data for each surgical group using two-way repeated measures ANOVAs with feedback (win-stay and lose shift) and phase (pre-surgery and post-surgery) as the within-subjects factors. Dunnett's multiple comparisons tests, when appropriate, were used to compare individual differences when overall significance was found. For all results, statistical significance was determined by a p value < 0.05.

6.4 Experimental Results

6.4.1 Acute Response to Injury

Immediately following sham injury or mTBI, the latency to regain righting reflex was recorded. For males (**Table 3**), analysis of righting reflex data failed to reveal a main effect of day [$F (1.783, 49.92) = 3.117$, $p = 0.0585$], injury [$F (2, 28) = 1.432$, $p = 0.2557$], or a day x injury interaction [$F (4, 56) = 1.469$, $p = 0.2238$].

mTBI in females resulted in longer righting reflex times compared to sham animals across all three surgical days (**Table 3**). Analysis of righting reflex data revealed significant main effects of both day [$F (1.995, 53.86) = 12.97$, $p < 0.0001$] and injury [$F (2, 27) = 12.00$, $p = 0.0002$] as well as a significant day x injury interaction [$F (4, 54) = 6.146$, $p = 0.0004$]. Dunnett's multiple comparisons analysis revealed rmTBI rats demonstrated longer righting reflex times compared to sham rats on days 1 ($p = 0.0038$) and 2 ($p =$

0.0057) whereas smTBI rats demonstrated a trend towards longer righting reflex times on day 3 ($p = 0.0634$).

Table 3

Righting Reflex Times of Pretrained Animals (2.5mm Impact Depth)

Sex	Injury Condition	N	Righting Reflex (sec)		
			Surgery Day 1	Surgery Day 2	Surgery Day 3
Male	sham	11	341.5 ± 65.6	255.1 ± 35.3	266.1 ± 27.6
Female	sham	11	296.5 ± 29.1	177.1 ± 21.3	210.2 ± 24.3
Male	smTBI	10	310.5 ± 43.2	283.4 ± 43.6	363.5 ± 55.0
Female	smTBI	9	298.9 ± 43.3	202.0 ± 25.5	313.6 ± 37.1^
Male	rmTBI	10	458.9 ± 43.4	325.4 ± 41.1	311.7 ± 57.1
Female	rmTBI	10	537.9 ± 56.3*	374.7 ± 49.3*	224.4 ± 33.3

Note. Average righting reflex times (seconds) of male and female sham, single (smTBI), and repetitive (rmTBI) injury groups across all three surgery days. On surgery days 1 and 2, rmTBI females exhibited longer righting reflex times compared to sham animals whereas smTBI females demonstrated a trend towards longer righting reflex times on surgery day 3. No differences in righting reflex times were observed in males. Values represent mean ± SEM. * denotes $p < 0.05$ and ^ denotes $p < 0.1$ from shams analyzed with Dunnett's multiple comparisons tests.

6.4.2 Effects of mTBI on Choice Behavior

In the first week post-final surgery (**Fig. 15**), analysis of choice data revealed a significant main effect of injury [$F (2, 55) = 3.582$, $p = 0.034$] with no injury x block, or three-way interaction with the sex factors (all $ps > 0.05$). There were also no main effects of sex [$F (1, 55) = 2.424$, $p = 0.125$] or injury x sex interaction [$F (2, 55) = 0.303$, $p = 0.740$]. The main effect of injury reflected the observation that TBI increased risky choice during Week 1 of testing relative to the sham group. This was confirmed with Dunnett's multiple comparisons analysis, that revealed smTBI rats displayed a significant increase in

risky choice ($p = 0.035$) in comparison to sham rats, and a trend towards a significant increase in risky choice following rmTBI ($p = 0.073$). Although we did not observe significant interactions with the sex factor, visual inspection of the data plotted separately for each sex (**Fig. 15A, top left inset**) suggest that the increase in risky choice was more robust in the female injury groups in the first week post-final injury.

In comparison to the effects observed in the first week following TBI, analysis of choice data from weeks 2-4 post injury failed to reveal a significant main effect of injury [$F(2, 55) = 1.984$, $p = 0.147$; $F(2, 55) = 0.856$, $p = 0.431$; $F(2, 55) = 1.703$, $p = 0.192$, respectively], sex [$F(1, 55) = 0.170$, $p = 0.682$; $F(1, 55) = 0.952$, $p = 0.333$; $F(1, 55) = 0.667$, $p = 0.418$, respectively] or injury x sex interaction [$F(2, 55) = 0.213$, $p = 0.809$; $F(2, 55) = 0.254$, $p = 0.777$; $F(2, 55) = 0.177$, $p = 0.838$, respectively]. Likewise, these analyses failed any other interactions of block x injury, block x sex or block x injury x sex ($p > 0.05$) in weeks 2-4. Collectively, these data indicate that TBI increases risky choice during probabilistic discounting, but these effects dissipate after extended re-training.

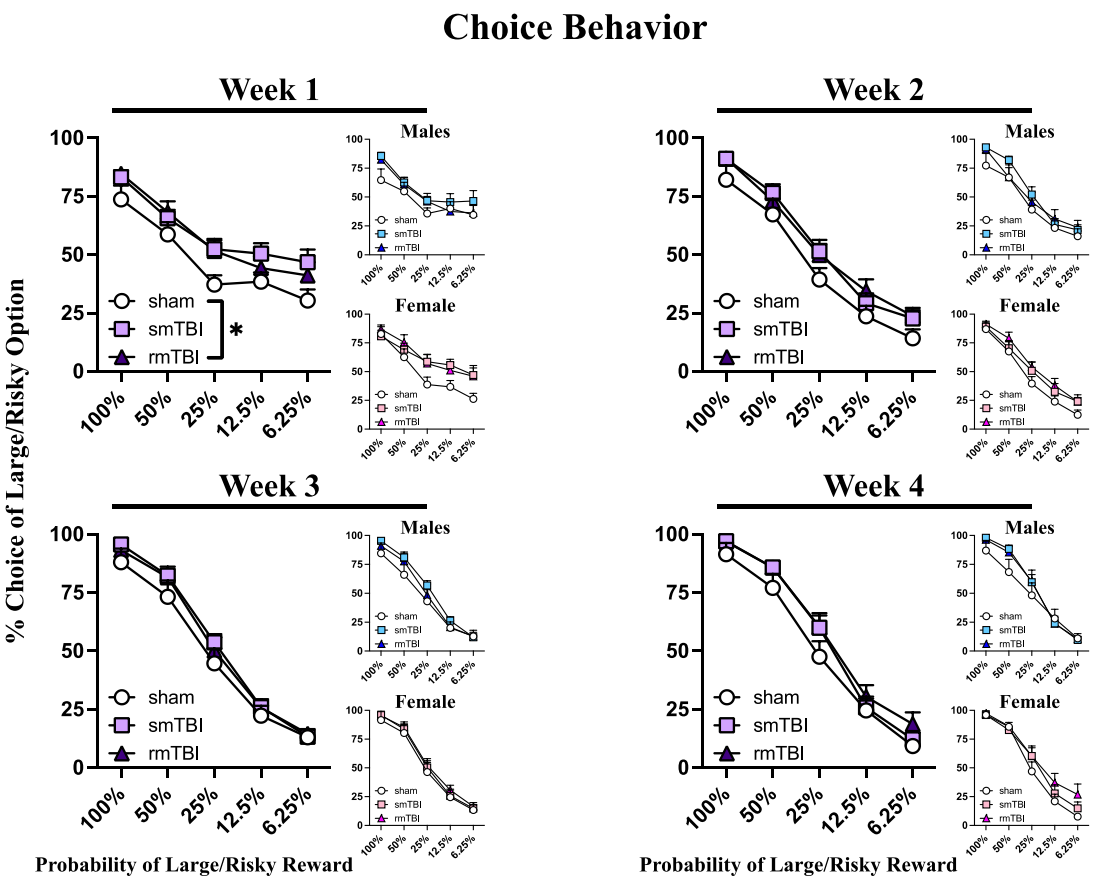


Figure 15. Post-surgery choice performance between-subjects (2.5mm impact depth). Choice behavior across four weeks post-final surgery. Line graphs represent percent choice of the large/risky option across five trial blocks. In week 1, a main effect of injury was observed when males and females were combined for analysis (purple graph). No differences in choice behavior were found between sham, single (smTBI), or repetitive (rmTBI) injury groups in weeks 2-4. Insets display choice behavior of males (blue) and females (pink) separately. No significant differences in choice behavior were found between sham, smTBI, or rmTBI groups for either males or females separately in weeks 1-4, although increased risky choice preference in injured females is clearly noted upon visual inspection within the first week post-final injury. Symbols represent mean ± SEM. * denotes $p < 0.05$ main effect of injury analyzed with three-way ANOVA.

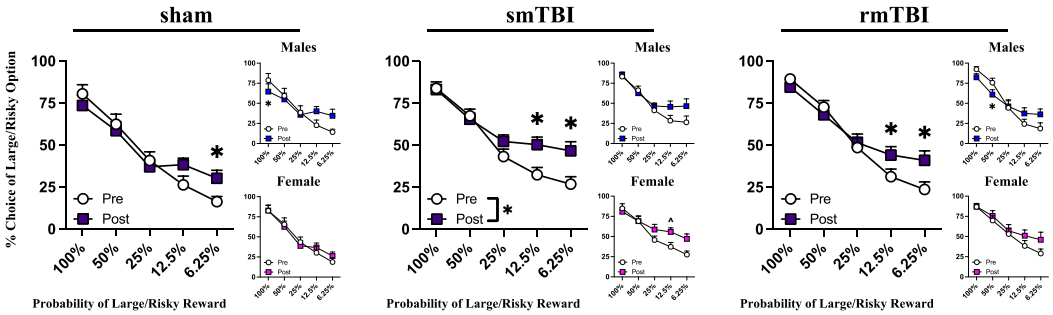
To further confirm the effects observed post-final injury, within-subjects analyses were performed for sham, smTBI, and rmTBI groups, respectively, within each testing week (**Fig. 16**). In the first week post-final surgery, analysis of choice data failed to reveal

a significant main effect of phase in the sham group [F (1.000, 21.00) = 0.8657, p = 0.3627], but did reveal a significant main effect of phase in the smTBI group [F (1.000, 18.00) = 6.077, p = 0.0240] and a trend towards a significant main effect of phase in the rmTBI group [F (1.000, 19.00) = 3.441, p = 0.0792]. These results support those described above in demonstrating that mTBI has increased risky choice preference during the first week following injury. A significant block x phase interaction was additionally observed within sham, smTBI, and rmTBI groups [F (3.075, 64.57) = 6.755, p = 0.0004; F (2.517, 45.31) = 6.186, p = 0.0022; F (2.523, 47.94) = 8.254, p = 0.0003, respectively]. Dunnett's multiple comparisons analysis revealed that sham rats displayed a significant increase in risky choice (p = 0.0266) in the 6.25% block in comparison to their pre-surgery performance, whereas smTBI and rmTBI rats displayed significant increases in risky choice in both the 12.5% (p < 0.05 for both groups) and 6.25% (p < 0.05 for both groups) blocks.

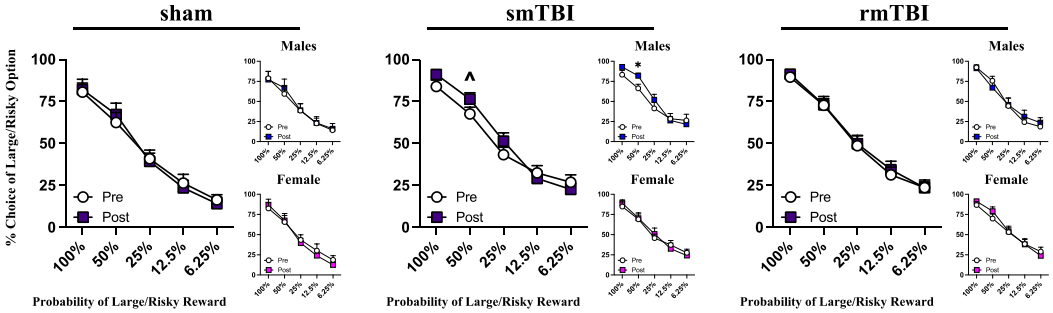
In comparison to the effects observed in the first week following TBI, analysis of choice data in weeks 2-4 post injury failed to reveal a significant main effect of phase in sham [F (1.000, 21.00) = 0.001065, p = 0.9743; F (1.000, 21.00) = 0.9539, p = 0.3399; F (1.000, 21.00) = 1.737, p = 0.2017, respectively], smTBI [F (1.000, 18.00) = 1.494, p = 0.2374; F (1.000, 18.00) = 0.7363, p = 0.4021; F (1.000, 18.00) = 1.455, p = 0.2434, respectively] and rmTBI groups [F (1.000, 19.00) = 0.1287, p = 0.7237; F (1.000, 19.00) = 0.003117, p = 0.9561; F (1.000, 19.00) = 1.306, p = 0.2674, respectively]. No significant block x phase interactions were observed in the sham or rmTBI groups in week 2 post-final injury [F (2.753, 57.82) = 1.065, p = 0.3674; F (3.475, 66.02) = 0.1451, p = 0.9505, respectively], but a significant block x phase interaction was observed in the smTBI group [F (2.705, 48.68) = 4.287, p = 0.0113]. Dunnett's multiple comparisons analysis revealed

that smTBI rats displayed a slight trend towards increased risky choice in the 50% block ($p < 0.0753$); however, visual inspection of the data suggests that these effects are most likely the result of animals returning to and exceeding pre-surgical baseline performances. As such, by weeks 3 and 4, all surgery groups were now demonstrating significant interactions of block x phase ($p < 0.05$). Taken together, these data further confirm that mTBI transiently increases risky choice during probabilistic discounting, but that these effects resolve after extended re-training.

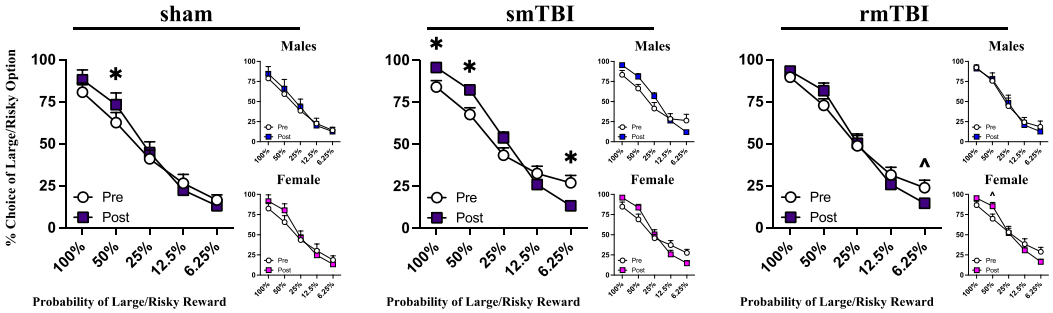
Choice Behavior: Week 1 Post-Surgery



Choice Behavior: Week 2 Post-Surgery



Choice Behavior: Week 3 Post-Surgery



Choice Behavior: Week 4 Post-Surgery

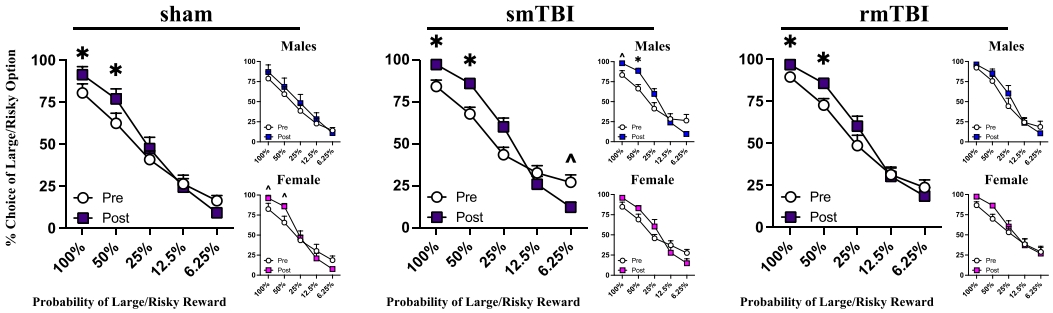


Figure 16. Post-surgery choice performance within-subjects (2.5mm impact depth). Comparison of pre- versus post-surgery choice behavior during each testing week in sham, single (smTBI), and repetitive (rmTBI) injury groups. Line graphs represent percent choice of the large/risky option across five trial blocks. Insets display choice behavior of males (blue) and females (pink) separately. In week 1, a main effect of phase was observed in the smTBI group when males and females were combined for analysis (purple graph). Upon evaluating males and females separately, alterations in choice preference were observed across all surgical groups; however, clear increases in risky choice preference were observed in the low probability blocks. In weeks 2-4, all surgery groups were performing similarly to, or in some cases, exceeding their pre-surgery performances. Symbols represent mean ± SEM. On legend, * denotes $p < 0.05$ main effect of phase analyzed with two-way ANOVA. On graphs, * denotes $p < 0.05$ and ^ denotes $p < 0.1$ from pre-surgery performance analyzed with Dunnett's

6.4.3 Effects of mTBI on Win-Stay/Lose-Shift Behavior

Separate analyses compared how TBI affected win-stay/lose-shift behavior. Across weeks 1-4 of testing (**Table 4**), analysis of win-stay/lose-shift data revealed a significant main effect of feedback ($p < 0.05$), reflecting a difference in the proportion of win-stay and lose-shift tendencies made. However, these analyses did not yield a main effect of injury [$F(2, 55) = 0.1624$, $p = 0.8505$; $F(2, 55) = 0.1612$, $p = 0.8515$; $F(2, 55) = 0.1800$, $p = 0.8357$; $F(2, 55) = 0.08664$, $p = 0.9171$, respectively], sex [$F(1, 55) = 0.5165$, $p = 0.4754$; $F(1, 55) = 0.2277$, $p = 0.6351$; $F(1, 55) = 0.3930$, $p = 0.5333$; $F(1, 55) = 0.02500$, $p = 0.8749$, respectively], or injury x sex interaction [$F(2, 55) = 3.128$, $p = 0.0517$; $F(2, 55) = 1.579$, $p = 0.2154$; $F(2, 55) = 0.7468$, $p = 0.4786$; $F(2, 55) = 0.02481$, $p = 0.9755$, respectively]. Additionally, there were no other interactions of feedback x injury, feedback x sex, and feedback x injury x sex ($p > 0.05$) in weeks 1-4.

When win-stay/lose-shift behavior of sham, smTBI, and rmTBI groups were compared to pre-surgery baselines, respectively, all groups demonstrated significant main effects of feedback ($p < 0.05$) across all 4 weeks, agreeing with the results reported above.

Across weeks 1-4, analysis of win-stay/lose-shift data failed to reveal a significant main effect of phase in both sham and rmTBI groups ($p < 0.05$), whereas a significant main effect of phase [$F (1.000, 18.00) = 5.956$, $p = 0.0252$] was observed in the smTBI group during week 1 only. No additional main effects of phase in smTBI animals were observed across weeks 2-4 ($p < 0.05$). No significant feedback x phase interactions in sham [$F (1.000, 21.00) = 0.08871$, $p = 0.7687$], smTBI [$F (1.000, 18.00) = 2.210$, $p = 0.1544$], or rmTBI groups [$F (1.000, 19.00) = 0.01229$, $p = 0.9129$] were observed in week 1. In week 2, only the smTBI group demonstrated a significant feedback x phase interaction [$F (1.000, 18.00) = 8.058$, $p = 0.0109$] whereas in week 3 post-final surgery, both the sham and smTBI groups were demonstrating significant feedback x phase interactions [$F (1.000, 21.00) = 13.48$, $p = 0.0014$; $F (1.000, 18.00) = 8.659$, $p = 0.0087$, respectively]. By week 4, all surgery groups were now demonstrating significant interactions of feedback x phase ($F (1.000, 21.00) = 12.22$, $p = 0.0022$; $F (1.000, 18.00) = 35.88$, $p < 0.0001$; $F (1.000, 19.00) = 6.683$, $p = 0.0181$, respectively) (data not shown). In the same manner as our choice behavior data, we attribute these effects on win-stay/lose-shift behavior to animals improving on the PDT due to extended re-training.

Table 4

Effects of 2.5mm mTBI on Win-Stay/Lose-Shift Performance

	Win-Stay			Lose-Shift		
	Combined	Male	Female	Combined	Male	Female
Week 1						
sham	0.72 ± 0.05	0.64 ± 0.08	0.80 ± 0.04	0.52 ± 0.04	0.53 ± 0.05	0.51 ± 0.05
smTBI	0.78 ± 0.03	0.80 ± 0.03	0.75 ± 0.04	0.44 ± 0.03	0.46 ± 0.04	0.41 ± 0.05
rmTBI	0.79 ± 0.03	0.75 ± 0.05	0.84 ± 0.04	0.44 ± 0.04	0.47 ± 0.05	0.41 ± 0.07
Week 2						
sham	0.79 ± 0.06	0.74 ± 0.09	0.83 ± 0.06	0.55 ± 0.05	0.53 ± 0.08	0.56 ± 0.05
smTBI	0.86 ± 0.03	0.89 ± 0.02	0.82 ± 0.05	0.49 ± 0.03	0.49 ± 0.05	0.49 ± 0.04
rmTBI	0.86 ± 0.02	0.83 ± 0.04	0.89 ± 0.03	0.50 ± 0.04	0.53 ± 0.08	0.48 ± 0.04
Week 3						
sham	0.85 ± 0.05	0.80 ± 0.09	0.90 ± 0.06	0.49 ± 0.05	0.56 ± 0.08	0.42 ± 0.04
smTBI	0.89 ± 0.02	0.88 ± 0.03	0.90 ± 0.03	0.42 ± 0.02	0.41 ± 0.04	0.43 ± 0.03
rmTBI	0.89 ± 0.02	0.87 ± 0.04	0.91 ± 0.01	0.43 ± 0.04	0.49 ± 0.05	0.38 ± 0.05
Week 4						
sham	0.88 ± 0.04	0.82 ± 0.09	0.94 ± 0.01	0.42 ± 0.05	0.48 ± 0.09	0.37 ± 0.04
smTBI	0.95 ± 0.02	0.95 ± 0.03	0.94 ± 0.02	0.35 ± 0.02	0.35 ± 0.03	0.36 ± 0.04
rmTBI	0.92 ± 0.01	0.91 ± 0.02	0.93 ± 0.02	0.36 ± 0.04	0.37 ± 0.06	0.36 ± 0.04

Note. Win-Stay/Lose-Shift ratios across four weeks post-final surgery. No differences in win-stay or lose-shift behavior were found between or within sham, single (smTBI), or repetitive (rmTBI) injury groups in weeks 1-4 post-surgery. Values represent mean ± SEM.

6.4.4 Effects of mTBI on Response Latency

Separate analyses compared how TBI affected response latencies. Across weeks 1-4 of testing (**Fig. 17**), analysis of response latency revealed a significant main effect of sex [F (1, 55) = 4.008, p = 0.050; F (1, 55) = 9.535, p = 0.003; F (1, 55) = 8.993, p = 0.004; F (1, 55) = 8.561, p = 0.005, respectively], reflecting that females were generally slower to make choices compared to males. However, these analyses did not yield a main effect of injury [F (2, 55) = 0.088, p = 0.915; F (2, 55) = 0.413, p = 0.664; F (2, 55) = 0.861, p =

0.428; F (2, 55) = 0.888, p = 0.417, respectively] or injury x sex interaction [F (2, 55) = 1.595, p = 0.212; F (2, 55) = 1.516, p = 0.229; F (2, 55) = 0.830, p = 0.441; F (2, 55) = 0.245, p = 0.783, respectively]. There were no other interactions of block x injury, block x sex, and block x injury x sex (p > 0.05) in weeks 1-4.

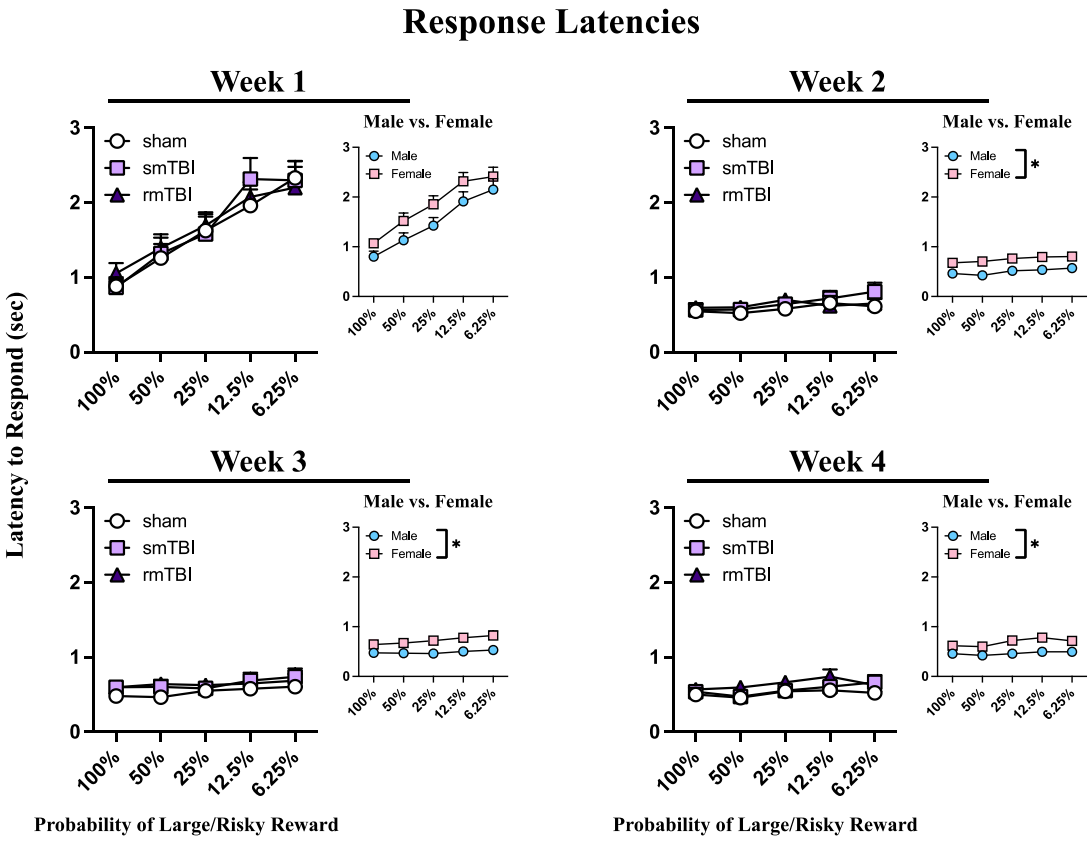


Figure 17. Post-surgery response latency performance between-subjects (2.5mm impact depth). Response latencies across four weeks post-final surgery. Line graphs choice response latencies across five trial blocks. No differences in response latencies were found between sham, single (smTBI), or repetitive (rmTBI) injury groups in weeks 1-4 post-surgery. Insets display overall male vs. female response latencies across all trial blocks. Across all 4 weeks, females demonstrated slower response latencies compared to males. Symbols represent mean ± SEM. * denotes p < 0.05.

Since we found clear differences in response latencies between males and females, we wanted to see whether there were differences in latencies to choose different options. Subsequent three-way ANOVA analyses were therefore conducted to compare response latencies of trials that resulted in a risky choice versus a certain one (**Fig. 18**). Across weeks 1-4 of testing, analysis of response latency revealed a significant main effect of sex [$F(1, 55) = 4.600$, $p = 0.0364$; $F(1, 55) = 7.752$, $p = 0.0073$; $F(1, 55) = 5.119$, $p = 0.0276$; $F(1, 55) = 8.500$, $p = 0.0051$, respectively], reflecting, again, that females were slower to make choices compared to males. However, these analyses did not yield a main effect of choice type [$F(1, 55) = 0.6358$, $p = 0.4287$; $F(1, 55) = 1.388$, $p = 0.2438$; $F(1, 54) = 2.152$, $p = 0.1482$; $F(1, 55) = 3.731$, $p = 0.0586$, respectively], injury [$F(2, 55) = 0.2468$, $p = 0.7822$; $F(2, 55) = 0.1290$, $p = 0.8792$; $F(2, 55) = 1.391$, $p = 0.2576$; $F(2, 55) = 1.409$, $p = 0.2531$, respectively] or injury x sex interaction [$F(2, 55) = 1.303$, $p = 0.2800$; $F(2, 55) = 1.641$, $p = 0.2036$; $F(2, 55) = 0.9807$, $p = 0.3815$; $F(2, 55) = 0.9509$, $p = 0.3926$, respectively]. There were no other interactions of choice x injury, choice x sex, and choice x injury x sex ($p > 0.05$) in weeks 1-4.

When the latencies of risky and certain choices were assessed separately, males, regardless of injury condition, showed no differences in response latencies of trials that ended in a risky [$F(2, 28) = 1.091$, $p = 0.3497$; $F(2, 28) = 1.495$, $p = 0.2417$, respectively) or certain [$F(2, 28) = 0.05336$, $p = 0.9481$; $F(2,28) = 0.1916$, $p = 0.8267$, respectively] choice in weeks 1 and 2 post-final injury (**Fig. 18A**). However, in week 3, there was a strong trend towards increased latencies in trials that ended in risky choices ($F(2, 27) = 3.194$, $p = 0.0569$), and by week 4, this effect of mTBI was significant ($F(2, 28) = 3.368$, $p = 0.0489$). Dunnett's multiple comparisons analysis revealed that rmTBI rats were slower

to make riskier choices ($p = 0.0297$) compared to sham animals. No significant differences in response latencies between male injury groups were observed during trials that ended in certain ($p > 0.1$) choices in weeks 3 and 4. In comparison, there were no significant differences between female injury groups in response latencies of trials that ended in either a risky or certain (all p values > 0.1) choice across weeks 1-4 (**Fig. 18B**).

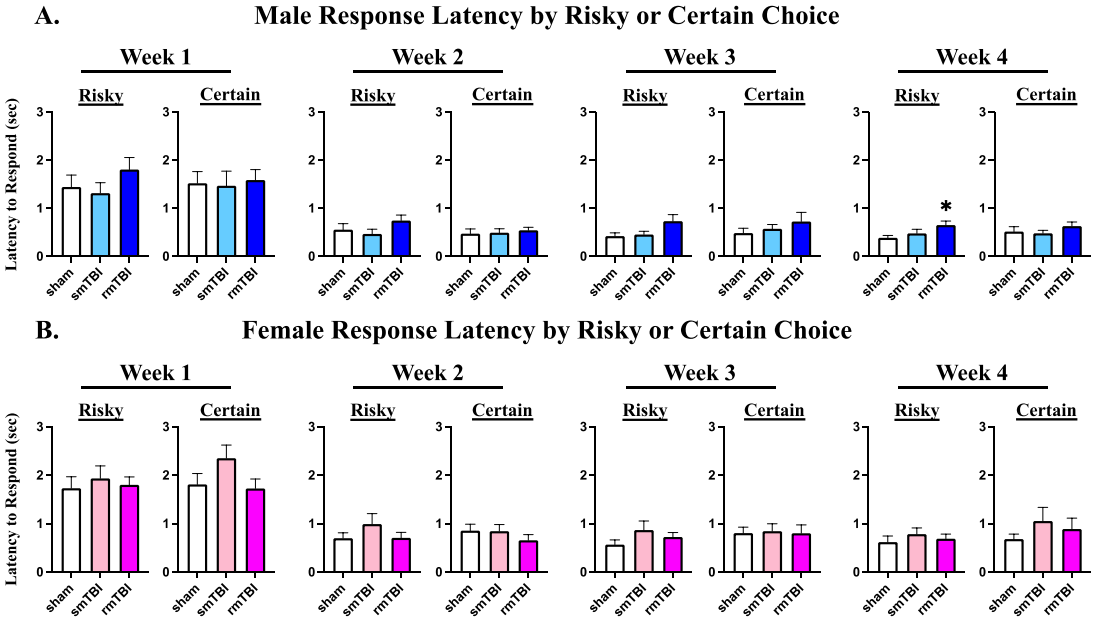


Figure 18. Response latency by risky or certain choice (2.5mm impact depth). Response latencies separated by choice type across four weeks post-final surgery. Bar graphs represent the averaged total percentage of response latencies for trials that ended in either a risky or certain choice across all trial blocks. **A)** Males: No differences in response latencies were found between sham, single (smTBI), or repetitive (rmTBI) injury groups in weeks 1-2 post-final surgery. In week 3, rmTBI tended to increase response latency in risky choice trials, and this effect reached significance in week 4. **B)** Females: No differences in response latencies were found between sham, smTBI, or rmTBI injury groups in in weeks 1-4. Bars represent mean ± SEM. * denotes $p < 0.05$ from shams analyzed with Dunnett's multiple comparisons tests.

6.4.5 Effects of mTBI on Magazine Latency

Across weeks 1-4 of testing (**Table 5**), analysis of magazine latency data failed to reveal a main effect of injury [$F(2, 54) = 0.032$, $p = 0.969$; $F(2, 55) = 0.067$, $p = 0.935$; $F(2, 55) = 1.450$, $p = 0.243$; $F(2, 55) = 1.861$, $p = 0.165$, respectively], sex [$F(1, 54) = 0.131$, $p = 0.718$; $F(1, 55) = 0.079$, $p = 0.779$; $F(1, 55) = 0.351$, $p = 0.556$; $F(1, 55) = 0.130$, $p = 0.720$, respectively], or injury x sex interaction [$F(2, 54) = 0.368$, $p = 0.694$; $F(2, 55) = 0.332$, $p = 0.719$; $F(2, 55) = 0.166$, $p = 0.847$; $F(2, 55) = 0.478$, $p = 0.623$, respectively]. Additionally, there were no other interactions of block x injury, block x sex, and block x injury x sex ($p > 0.05$) in weeks 1-4.

When magazine latencies of sham, smTBI, and rmTBI groups were compared to pre-surgery baselines, respectively, all groups demonstrated a significant main effect of phase during week 1 [$F(1.000, 21.00) = 6.505$, $p = 0.0186$; $F(1.000, 18.00) = 9.687$, $p = 0.0060$; $F(1.000, 19.00) = 9.460$, $p = 0.0062$, respectively] but no block x phase interaction [$F(2.029, 42.62) = 0.5391$, $p = 0.5898$; $F(2.296, 40.76) = 0.9428$, $p = 0.4088$; $F(2.037, 38.71) = 0.7170$, $p = 0.4970$, respectively]. Additional significant main effects of phase were observed in smTBI animals at weeks 3 and 4 [$F(1.000, 18.00) = 14.60$, $p = 0.0013$; $F(1.000, 18.00) = 25.30$, $p < 0.0001$, respectively], but we conclude that these effects were not biologically relevant. Additionally, no block x phase interactions ($p > 0.05$) were observed within all surgery groups, respectively, across weeks 2-4 (data not shown). Collectively, these results indicate there are no changes in motivation to collect rewards following mTBI.

Table 5

Effects of 2.5mm mTBI on Magazine Latency

	Magazine Latency		
	Combined	Male	Female
Week 1			
sham	0.35 ± 0.03	0.36 ± 0.06	0.35 ± 0.03
smTBI	0.37 ± 0.03	0.32 ± 0.03	0.42 ± 0.06
rmTBI	0.35 ± 0.02	0.35 ± 0.03	0.35 ± 0.03
Week 2			
sham	0.30 ± 0.03	0.32 ± 0.06	0.28 ± 0.02
smTBI	0.29 ± 0.01	0.28 ± 0.02	0.29 ± 0.02
rmTBI	0.30 ± 0.02	0.29 ± 0.02	0.30 ± 0.03
Week 3			
sham	0.29 ± 0.03	0.31 ± 0.05	0.28 ± 0.02
smTBI	0.26 ± 0.01	0.26 ± 0.02	0.26 ± 0.02
rmTBI	0.32 ± 0.03	0.33 ± 0.04	0.31 ± 0.03
Week 4			
sham	0.27 ± 0.03	0.27 ± 0.05	0.26 ± 0.02
smTBI	0.25 ± 0.01	0.25 ± 0.02	0.24 ± 0.02
rmTBI	0.31 ± 0.03	0.29 ± 0.03	0.34 ± 0.06

Note. Magazine Latencies across four weeks post-final surgery. No differences in the latency to retrieve rewards were found between or within sham, single (smTBI), or repetitive (rmTBI) injury groups in weeks 1-4 post-surgery. Values represent mean ± SEM.

6.5 Discussion

Using a rodent assay of probabilistic discounting, the present study evaluated the effects of rmTBI on risk/reward decision making. During the first week following injury, mild TBI increased risky choice, and this effect achieved statistical significance in smTBI animals. Upon further inspection, it appeared that these increases were more pronounced

in the female injury groups. However, we did not observe changes in win-stay/lose-shift behavior during Week 1, suggesting that this increase in risk preference was not driven by altered sensitivity to rewarded or non-rewarded risky choices. Additionally, with extended testing, these effects resolved by week 2 post-injury indicating that the effects of mTBI on choice behavior are transient. While the effects of single injury align with previous TBI research, this study offers new insights into the effects of repetitive injury on risk/reward decision making. rmTBI does in fact disrupt adjustments in choice biases in response to changes in the relative risk of not obtaining rewards, but to a slightly lesser degree than a single impact.

Choice behavior on this assay is guided in part by the mPFC, which exhibits robust neural activity during periods of anticipation and subsequent interpretation of risk-related outcomes (Euston et al., 2012; Fellows, 2007; Fukui et al., 2005). Following decisions, the mPFC updates value representations based on changes in reward probabilities to facilitate more efficient choices (Bercovici et al., 2023; St Onge et al., 2011; St Onge & Floresco, 2010). Inactivation of the PFC increases risky choices on the PDT (St Onge & Floresco, 2010) when reward probabilities decrease over a session, and also increases risk taking on other tasks (Paine et al., 2013; Zeeb et al., 2015). The medial OFC (mOFC) has also been shown to exert influence over choice behavior through assessing reward values (Adinoff et al., 2006; Elliott et al., 2000; O'Doherty et al., 2001; Rushworth et al., 2011; Sescousse et al., 2010; van den Bos et al., 2013) and mitigating the impact that large/probabilistic rewards exert on subsequent decisions (Jenni et al., 2021; Stopper et al., 2014). Similar to the mPFC, inactivation of the mOFC in rodents leads to increased risky choice in the PDT (Stopper et al., 2014). During a probabilistic reversal learning task, disruption of mOFC

activity impairs the ability to incorporate information from previous outcomes to guide subsequent actions (Dalton et al., 2016). TBI patients with both mPFC and mOFC damage demonstrate difficulty learning from previous mistakes, insensitivity to future consequence of risk-related choices, and overall increased risky behavior (Bechara et al., 1994; Bechara et al., 1998; Bechara et al., 2000). Based on these previous observations, it is likely that our findings of increased risky choice induced by mTBI are related to disruptions in mPFC and mOFC operations.

When choice latencies were examined, we found that males were generally quicker to make choices than females in the PDT regardless of injury condition. Latencies to retrieve rewards, however, were not different between males and females, indicating these differences in choice latencies were not due to differences in motivation levels. A more detailed analysis of choice latencies partitioned by whether rats made risky or certain choices, revealed a delayed effect of mTBI in males that approached statistical significance at week 3, and was fully apparent by week 4 post-final injury. Here, males that received repetitive injury showed increased hesitation to make risky choices. A previous study found that inactivation of the lateral OFC (lOFC) in males led to increased choice latencies in the PDT (St Onge & Floresco, 2010), suggesting that these effects of rmTBI in males may be lOFC-related. Notably, patients with OFC damage have also demonstrated longer deliberation times in risk/reward decision making tasks (Manes et al., 2002; Rogers, Everitt, et al., 1999). These longer response times have been attributed to TBI patients having difficulty resolving competing options associated with uncertain or probabilistic outcomes (Rogers, Everitt, et al., 1999; Salmond et al., 2005). As a result, these patients tend to make more disadvantageous choices when compared to non-injured individuals.

Although these studies have focused primarily on single TBI cases, long-term deficits in information processing speed have been documented following repetitive TBIs (Collins et al., 1999; Rosenbaum & Lipton, 2012). Our results are consistent with these studies; however, our data indicates that these effects on response times do not occur immediately after injury, but rather develop over time. This finding is significant given that clinical studies often struggle with determining the onset of individual TBI-induced effects. Currently, we can only report that these longer choice latencies appear to manifest by week 4 after injury. Further research will be necessary to determine the full duration of these effects.

Interestingly, females appeared to be relatively impervious to injury-induced changes in choice latencies. It is known that males and females process reward-related information differently and use different strategies when making probabilistic-based decisions (Orsini & Setlow, 2017; van den Bos et al., 2013; van den Bos et al., 2012). These differences in information processing can be attributed to sex differences in OFC activity during risk/reward decision making. Neuroimaging of the OFC during performance on the IGT found that males exhibit greater lOFC activity during task performance compared to females, while females exhibit mOFC-related activity during risk/reward decision making (Bolla et al., 2004). This would suggest that damage to the OFC may have a greater impact on lOFC-related processes, such as processing speed, in males than in females. Accordingly, one study that examined longitudinal patterns of decision making following pediatric TBI reported that injured males had difficulty processing information necessary to determine whether risks should be pursued, resulting in disadvantageous decisions, whereas females were still capable of making appropriate risk estimations (Schmidt et al.,

2012). Although these observations may explain why we did not observe changes in female choice latencies as a result of rmTBI, it is also possible that we are experiencing a ceiling effect with our female groups, making it difficult to detect potential changes in choice latencies. Future investigations are therefore needed to fully understand the sex-specific mechanisms underlying these differences following rmTBI.

6.6 Conclusion

Overall, we report that mTBI produces transient increases in risk preferences, which appear to be more heavily influenced by our female injury groups. While the effects of rmTBI are not as robust as those of a single impact, repetitive injury does appear to disrupt adjustments in choice biases in response to changes in the value of large/risky rewards. Additionally, we found that males are more likely to experience delayed disruptions in cost/benefit evaluations, resulting in longer deliberation periods when making risky choices.

Chapter 7

Developing of a Stronger Model of Repetitive Mild Traumatic Brain Injury: Increasing Injury Depth

7.1 Introduction

In **Chapter 6**, we observed transient increases in risky choice preference following mild traumatic brain injury (mTBI). These effects were driven mainly by single injury (smTBI), whereas the effects of repetitive mTBI (rmTBI) were just shy of significance. Additionally, females appeared to contribute more towards this increase in risky choice preference compared to males. Although these results initially suggested that males are less likely to experience changes in risk preference following repetitive mild head injuries, it is also possible that our current injury parameters in our male subjects might be just sub-threshold for producing deficits in choice preference in PDT. Thus, the goal of this Chapter was to characterize a stronger model of mTBI that we believe would allow detection of potential changes in choice behavior in male rats and possibly amplify the effects observed in females.

7.2 Rationale

Within the controlled cortical impact (CCI) model, injury severity is controlled by different injury parameters that include depth, velocity, dwell time, and even the size of the tip used for delivering the CCI injury. Tip diameter and depth have been described as the primary contributors of injury severity (Osier & Dixon, 2016). During initial characterization of the neurological and histological effects of mild, moderate, and severe

open-skull CCI, velocity was maintained at 6 m/s; however, depth of injury was progressively increased from 1mm (mild) to 2mm (moderate) to 3mm (severe) (Dixon et al., 1991). Unfortunately, studies to characterize experimental parameters to define injury severity using a closed-skull CCI have not been reported. Therefore, we aimed to increase our depth parameter to produce a stronger impact while still maintaining a mild-level injury. In **Chapters 5** and **6** of this dissertation, an injury depth of 2.5mm was used. For the experiments described in this Chapter, depths of 3.0mm and 3.5mm were selected. In order to compare these increased depths to the current 2.5mm depth model and ensure mild severity was maintained, several measures were assessed following injury including survival rate of animals post-surgery, adverse weight gain or loss, righting reflex times, and superficial skull damage. Frequent death from impact as well as dramatic shifts in weight gain or loss would indicate that one or both of these increased depths are producing injuries beyond a mild level. However, to ensure that there is increased damage from these injuries, righting reflex and superficial damage to the skull were assessed. As mentioned in **Chapter 3**, righting reflex is defined as the time it takes a rat to turn over from a supine position. A previous study determined that physiological responses such as righting time immediately after injury can serve as predictive markers of behavioral and histological deficits (Grin'kina et al., 2016). As such, longer righting reflexes would be indicative of a stronger impact. Damage to the skull as the result of closed head-CCI (CH-CCI) can result in the presence of fractures and hematoma. As described in **Chapter 1**, skull fracturing and intracranial hematomas are classified as primary injuries and minimal fracturing and hematoma are often observed with our 2.5mm depth model. Increased fracturing and hematoma presence following the 3.0 and 3.5mm depths would indicate that these depths

are producing mTBIs of increased force. Taken together, the objective of **Chapter 7** was to evaluate the effects of rmTBI of increased depths on survival rate, weight gain/loss, righting reflex, and superficial skull damage. *We hypothesized that animals that received rmTBI of deeper impact depths would survive their surgeries and not experience dramatic shifts in weight gain or loss, but also demonstrate longer righting reflex times and increased superficial skull damage than animals that sustained rmTBI with our current 2.5mm depth model.*

7.3 Methods

7.3.1 Animals

Surgery data from nineteen male and twenty-nine female Long-Evans rats that received either sham or rmTBI surgery at a 2.5mm depth from **Chapter 6** were used for this analysis. An additional seven male and eight female Long-Evans rats were incorporated into this study. These animals were obtained at 5-6 weeks old/100-125g from either Charles River or Envigo Laboratories and underwent the housing, acclimation, and food regulation conditions described in **Chapter 3**.

7.3.2 Surgery

At 9-10 weeks of age, rats were assigned to one of two surgical groups: rmTBI at a 3.0mm depth or rmTBI at a 3.5mm depth. Both groups underwent the same surgical procedures as described in **Chapter 3**, but the depth of impact below the surface point of contact was either 3.0mm or 3.5mm. The velocity of 5.5m/s and dwell time of 100ms

remained constant. Following injury, righting reflex times were recorded and the skull was evaluated for the presence of fracturing, indentation, and hematoma.

7.3.3 Statistical Analysis

All data analysis was performed using GraphPad Prism software (GraphPad Software, San Diego CA). Weight gain/loss and righting reflex times were analyzed using a two-way repeated measures ANOVA with surgery day (day 1, day 2, and day 3) as the within-subjects factor and injury condition (sham, 2.5mm, 3.0mm, and 3.5mm) as the between-subjects factor. An additional one-way ANOVA was used to analyze group differences (sham, 2.5mm, 3.0mm, and 3.5mm) on surgery day 3 only. The degree of visual observations of superficial damage to the skull across injury depths, including fracturing, skull indentations, and hematomas were assessed on surgery day 3 and plotted on individual pie charts. Due to the small sample sizes of male and female 3.0mm and 3.5mm depth groups, males and females were combined for all analyses. Dunnett's multiple comparisons tests, when appropriate, were used to compare individual differences when overall significance was found. For all results, statistical significance was determined by a p value < 0.05.

7.4 Experimental Results

7.4.1 Effects of Increasing Injury Depths on Survivability and Weight Change

Following injury, we observed a 100% survival rate among rats that received both the 3.0 and 3.5mm depths. Additionally, when the weights of animals were assessed on each surgery day (**Table 6**), ANOVA analysis revealed a significant main effect of day [F

(1.448, 85.41) = 265.3, p < 0.0001], but not a main effect of injury [F (3, 59) = 0.4362, p = 0.7279] nor a day x injury interaction [F (6, 118) = 0.5131, p = 0.7974], indicating that there was no significant weight gain or loss between surgery days in all injury groups.

Given that behavioral testing begins 48 hours following the final surgery, a subsequent ordinary one-way ANOVA was conducted to compare the weights of all surgery groups at the 48-hour timepoint (**Fig. 19**). No differences in weights between surgery groups were observed 48 hours post-final surgery [F (3, 59) = 0.3331, p = 0.8014], further confirming that the 3.0 and 3.5mm depth do not significantly alter weight gain/loss.

Table 6

Average Weights of Animals across Various Impact Depths

Injury Condition	N	Weight (g)		
		Surgery Day 1	Surgery Day 2	Surgery Day 3
sham	25	182.7 ± 3.8	201.0 ± 3.9	215.7 ± 4.9
2.5mm	23	183.0 ± 4.6	200.4 ± 5.0	216.3 ± 5.6
3.0mm	6	190.7 ± 6.3	212.0 ± 11.2	226.0 ± 13.0
3.5mm	9	191.4 ± 7.8	205.1 ± 9.0	220.9 ± 9.6

Note. Average weights (grams) of sham, 2.5mm, 3.0mm, and 3.5mm injury depth groups on all three surgery days. No significant differences in the amount of weight gained or lost were observed between surgery groups on any surgery day. Values represent mean ± SEM.

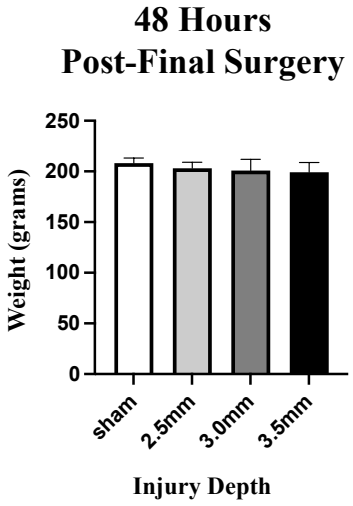


Figure 19. Comparison of post-surgery weight across various impact depths. Average weights of rats 48 hours post-final surgery. No differences in weights were observed between injury groups 48 hours after the final surgery. Bars represent mean ± SEM.

7.4.2 Effects of Increasing Injury Depths on Righting Reflex Times

Immediately following sham injury or mTBI, the latency to regain righting reflex was recorded. First, we compared righting reflex times between each surgery day. Analysis of righting reflex data (**Table 7**) revealed a significant main effect of both day [$F(1.860, 109.8) = 20.02$, $p < 0.0001$] and injury [$F(3, 59) = 7.074$, $p = 0.004$] as well as a significant day x injury interaction [$F(6, 118) = 3.188$, $p = 0.0062$]. Dunnett's multiple comparisons analysis revealed that rats that received sham or rmTBI surgeries at the 2.5mm and 3.0mm injury depths demonstrated shorter righting reflex times on surgery days 2 ($p = 0.0605$, $p = 0.0048$, and $p = 0.0259$, respectively) and 3 ($p = 0.0227$, $p < 0.001$, and $p = 0.0224$, respectively) compared to day 1, respectively. Rats that received the 3.5mm injury depth

did not display any significant changes in righting reflex times across all surgery days ($p > 0.1$).

When we compared the righting reflex times of all groups to each other on surgery day 3 (**Fig. 20**), there was a significant effect of injury on righting reflex times [$F(3, 59) = 3.050$, $p = 0.0355$]. Dunnett's multiple comparisons analysis determined that rats that received the 3.5mm depth injury displayed significantly longer righting reflex times ($p = 0.0198$) compared to sham rats, indicative of a stronger impact.

Table 7

Righting Reflex Times across Various Impact Depths

Injury Condition	N	Righting Reflex (sec)		
		Surgery Day 1	Surgery Day 2	Surgery Day 3
sham	25	330.1 ± 39.5	237.3 ± 25.7^	222.8 ± 17.6*
2.5mm	23	544.3 ± 35.8	375.3 ± 35.2*	235.7 ± 30.5*
3.0mm	6	485.8 ± 25.2	308.2 ± 64.1*	301.2 ± 69.8*
3.5mm	9	443.2 ± 42.8	397.2 ± 21.3	365.4 ± 54.0

Note. Average righting reflex times (seconds) of sham, 2.5mm, 3.0mm, and 3.5mm injury depth groups across all three surgery days. Rats that received sham or rmTBI surgeries at the 2.5mm and 3.0mm injury depths demonstrated shorter righting reflex times on surgery days 2 and 3 compared to those of surgery day 1, respectively. No significant differences in righting reflex times across all surgery days were observed in rats that received rmTBI surgeries at the 3.5 mm depth. Values represent mean ± SEM. * denotes $p < 0.05$ and ^ denotes $p < 0.1$ from surgery day 1 analyzed with Dunnett's multiple comparisons tests.

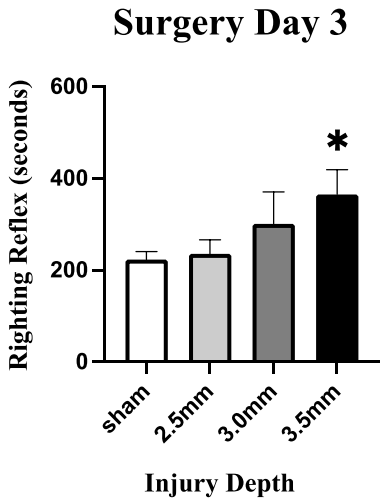


Figure 20. Comparison of righting reflex times across various impact depths. Righting reflex times between surgery groups on surgery day 3. Rats that received rmTBI at a 3.5 mm depth displayed longer righting reflex times in comparison to sham rats. Bars represent mean ± SEM. * denotes $p < 0.05$ from sham analyzed with Dunnett's multiple comparisons tests.

7.4.3 Effects of Increasing Injury Depths on Skull Fracturing and Indentation

Skull fractures were defined as breaks in the skull and have been observed previously in the CH-CCI model (Huh et al., 2008; Lengel et al., 2022; McCorkle et al., 2022). Based on the extent of breakage observed, skull fractures were classified as minimal, intermediate, or significant. Short linear fractures at the site of impact were defined as minimal, whereas longer and crescent-shaped fractures were characterized as being moderate. Multiple fractures along with circular fractures caused by the rounded metal impactor tip were considered significant. Skull fracturing was assessed on surgery

day 3 (**Fig. 21**). 96% of animals that received rmTBI at the 2.5mm injury depth had minimal skull fracturing. As injury depth increased, we began to observe more intermediate and significant skull fracturing. The 3.5mm depth resulted in a broader range of fracturing than either the 2.5mm and 3.0mm depths, indicating that this injury depth delivered the strongest injury.

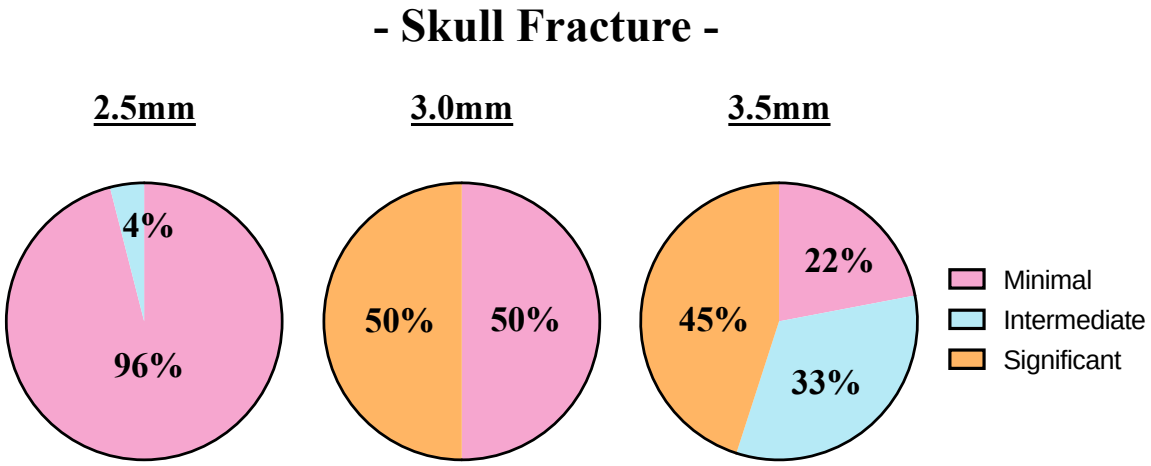


Figure 21. Comparison of skull fracture severity across various impact depths. Skull fracturing on surgery day 3. Rats that received an injury depth of 3.5mm displayed a broader range of fracturing in comparison to rats that received an injury depth of 2.5mm and 3.0mm.

Following rmTBI, occasional indentation of the skull was observed. Skull indentation was defined as an area of skull that was depressed inward due to an impact. When skull indentations were examined on surgery day 3 (**Fig. 22**), 96% of animals that received a 2.5mm injury depth had no skull indentations. As injury depth increased, we began to observe more frequent indentions. Approximately 50% of rats that received either

the 3.0mm or 3.5mm injury depth experienced skull indentations, indicative of a stronger injury.

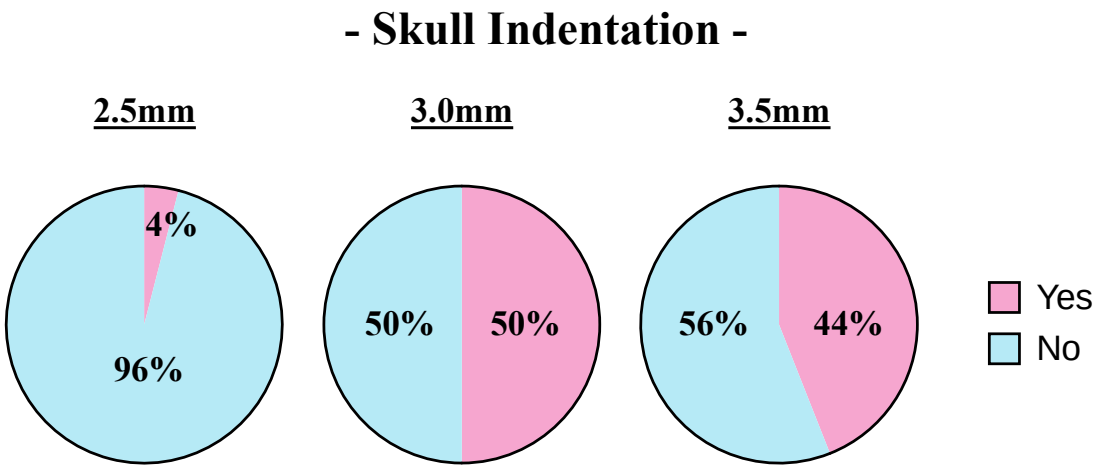


Figure 22. Comparison of skull indentation severity across various impact depths. Skull indentation on surgery day 3. Rats that received an injury depth of 3.0mm or 3.5mm displayed more skull indentation in comparison to rats that received an injury depth of 2.5mm.

7.4.4 Effects of Increasing Injury Depths on Hematoma Severity

Hematomas were identified when blood collection was visible under the skull and were classified as either mild, moderate, or severe (Huh et al., 2008). Small discoloration under the skull at the impact site was considered mild, whereas hematomas that were larger in size at or around the impact site were considered moderate. Hematomas that were present in multiples regions adjacent to the impact site in addition to the injured area were considered to be severe. When the presence of hematomas was examined on surgery day 3 (**Fig. 23**), 78% of rats that received a 2.5mm injury depth had minimal hematomas. As injury depth increased, we began to observe a broader range of hematoma severity. 83% of

rats who received the 3.0mm depth impact had hematomas of intermediate severity. At 3.5mm, we began to observe significant hematoma presence.

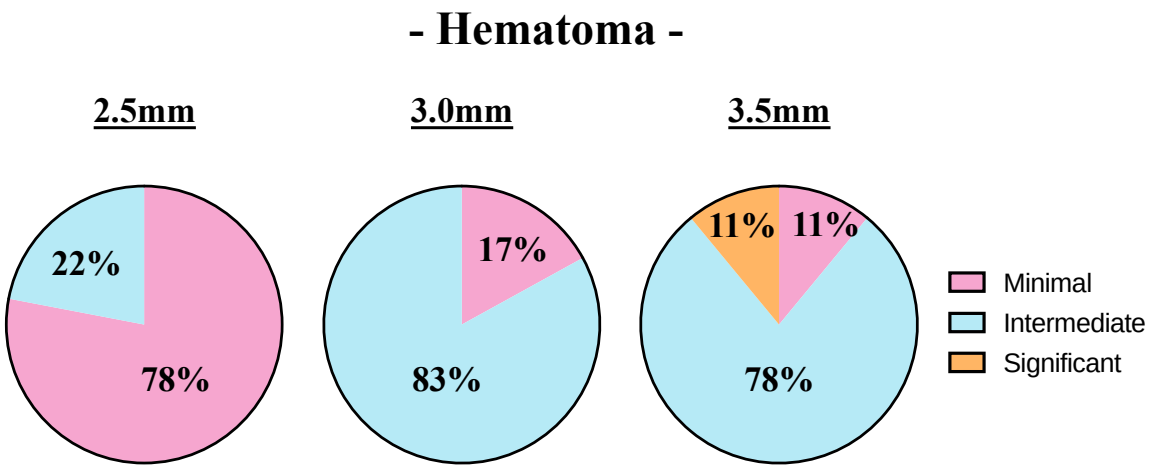


Figure 23. Comparison of hematoma severity across various impact depths. Hematoma severity on surgery day 3. Rats that received an injury depth of 3.5mm displayed a broader range of hematoma in comparison to rats that received an injury depth of 2.5mm and 3.0mm.

7.5 Discussion

The goal of **Chapter 7** was to develop a stronger model of rmTBI that would allow potential detection of changes in choice preference in male rats and amplify those observed in females. Injury depths of 3.0 and 3.5mm were evaluated and compared to the current 2.5mm depth model. Following rmTBI, we observed a 100% survival rate among all injury groups with no adverse effects on weight gain or loss. When the latency to regain righting reflex was assessed, we observed shorter righting reflex times on the second and third surgery days in groups that received rmTBI at depths of 2.5 and 3.0mm. Decreasing righting reflex times across multiple surgery days despite the force of impact remaining constant has been a phenomenon observed in previous studies of repeated mTBI (Briggs

et al., 2016; Hiskens et al., 2021). These studies have attributed this phenomenon to central nervous system adaptation and initiation of neuroprotective pathways in response to repeated head impacts. Intrestingly, the righting reflex times associated with the 3.5mm depth were not significantly different across all surgery days, suggesting that these injuries may have exceeded this threshold for adaptive responses to occur.

It must be noted that sham animals also demonstrated reduced righting reflex times following surgery day 1. While these findings were initially surprising, we believe that these effects were anesthesia-related. The duration of anesthesia exposure can influence the duration of righting reflex times (Berman et al., 2023). During surgeries, animals are briefly exposed to anesthesia in order to expose the skull for sham injury or mTBI. These observed reductions in righting reflex times may be partially caused by repeated exposure to anesthetics across all surgeries. Nevertheless, when righting reflexes were compared between all groups on surgery day 3, statistical analysis confirmed that the overall changes in righting reflex times were directly related to the injuries, themselves. Moreover, it was determined that the 3.5mm depth resulted in the longest righting reflex times, further indicating that this impact depth produces a stronger injury compared to the 2.5 and 3.0mm depths.

Lastly, we evaluated and compared the degree of visual observations of superficial damage to the skull that included fractures, skull indentations, and hematomas. The 3.5mm depth resulted in a broader range of skull fracturing than either the 2.5 or 3.0mm depths. When skull indentations were examined, rats that received either the 3.0 or 3.5mm depth impacts displayed more skull indentations than rats who received the 2.5mm injury depth. Furthermore, the 3.5mm depth resulted in a broader range of hematoma severity. Together,

we believe we have demonstrated that increasing our injury depth to 3.5mm produces a stronger injury affect while preserving comparable visual observation of superficial damage within the realm of mild injury.

7.6 Conclusion

Overall, rats that received a 3.5mm injury depth exhibited greater evidence of injury but still in the range that can be associated with mTBI. From these findings, we believe that this deeper impact depth may reveal previously sub-threshold effects in male rats and amplify those observed in females. As such, the experiments reported in **Chapter 8** describe how rmTBI, at a depth of 3.5mm, affects performance on the PDT in male and female animals.

Chapter 8

Evaluating the Effects of Repetitive Mild Traumatic Brain Injury of Increased Depth on Risk/Reward Decision Making

8.1 Introduction

As discussed in **Chapter 1**, the Glasgow Coma Scale (GCS) (Teasdale & Jennett, 1974) classifies traumatic brain injuries (TBI) as mild (mTBI), moderate, or severe based on the severity of the symptoms exhibited by each patient (see the **Epidemiology, Classification, and Pathophysiology** section of **Chapter 1** for review). Many TBIs are classified as mild, but not all mTBI patients receive the same GCS score. Depending on the magnitude of impact forces, the severity of mTBI can vary, resulting in variability in post-injury symptoms experienced. However, it is unclear whether different degrees of mTBI result in the same level of cognitive dysfunction or whether deficits are linearly related to injury intensity. In regard to risk/reward decision making, we observed mild and transient effects of injury in the probabilistic discounting task (PDT) using a model that produced impacts at a 2.5mm depth. In the preceding Chapter, we modified this model to deliver a stronger form of injury while still maintaining mild severity. In the present Chapter, our goal was to determine if mTBIs of increased strength amplify the effects reported in **Chapter 6** or if they establish new profiles of risk preference.

8.2 Rationale

We established that mTBI increases risky choice in the PDT with injured females seemingly driving these effects. We sought to determine whether these effects would be

amplified following a stronger mTBI. If we observe greater increases in female risky choice behavior following the 3.5mm depth than those observed at the 2.5mm depth, this would reinforce our suspicions from **Chapter 6** regarding female susceptibility to changes in risk/reward decision making behavior following mTBI. If we observe no further differences in risky choice preference following increased depth, this may indicate that we have surpassed the threshold for producing the maximum extent of behavioral effects associated with a mild injury on risk/reward decision making.

Increased risky choice behavior in males would confirm our speculation that our previous injury parameters were sub-threshold for producing deficits in the PDT in male subjects. These results would also confirm the overall assertion that rmTBI increases risk/reward decision making behavior. There is also the possibility of observing no changes in male risky choice behavior with this increased depth. No behavioral changes after two different degrees of mild injury may indicate that mTBI does not affect risk/reward decision making behavior in males. Although this would be an unexpected outcome, these findings would underscore the significance of sex differences in susceptibility to changes in risk/reward decision making following rmTBI. Additionally, these findings would further emphasize the importance of incorporating male and female subjects in studies aimed at assessing TBI-induced changes in complex domains of cognitive processing. As such, our objective here was to determine if a stronger form of rmTBI amplifies the effects observed in females and enables detection of increased risky choice behavior in males**.** *We hypothesized that rmTBI at 3.5mm impact depth exacerbates risky choice preference in females and increases risky choice preference in males.*

8.3 Methods

8.3.1 Animals

Forty-four male and thirty-nine female Long-Evans rats were used in this study. Animals were obtained at 3-4 weeks old/50-75g from Envigo Laboratories and underwent the housing, acclimation, and food regulation conditions described in **Chapter 3**.

8.3.2 Probabilistic Discounting Task and Surgical Procedures

Animals underwent the lever-pressing and PDT training protocols described in **Chapter 3**. Rats required ~19 days of training before stable criterion performance was achieved. As described in **Chapter 6**, animals were rank ordered based on the average ratio of large/risky lever presses over successful free-choice trials across the last three training days. Animals with the highest ratio were assigned a higher rank. A reverse Latin square method was then used to assign rats to one of three surgical groups: sham (uninjured), single injury (smTBI), or repetitive injury (rmTBI). Animals (9-10 weeks of age) then underwent the surgical procedures described in **Chapter 3** using the 3.5mm depth model. Forty-eight hours after the final surgery, animals were reintroduced to the PDT and tested 5 days per week for four weeks to assess changes in risk/reward decision making.

8.3.3 Statistical Analysis

The statistical analysis procedures performed in this Chapter were the same as the ones performed in **Chapter 6**. Analysis of righting reflex and win-stay/lose-shift data was performed using GraphPad Prism software (GraphPad Software, San Diego CA). Analysis of choice behavior as well as response and magazine latencies were performed using SPSS

software (IBM, SPSS Inc.). Male and female righting reflex data were analyzed separately using two-way repeated measures ANOVAs with surgery day (day 1, day 2, and day 3) as the within-subjects factor and injury condition (sham, smTBI, and rmTBI) as the between-subjects factor. For post-surgery PDT behavior, choice behavior as well as response and magazine latency, data were averaged across 3 consecutive sessions, respectively, and analyzed using three-way mixed-design ANOVAs with trial block (100%, 50%, 25%, 12.5%, and 6.25%) as the within-subjects factor and injury condition (sham, smTBI, and rmTBI) and sex (male and female) as the between-subjects factors. The effect of trial block was always significant ($p < 0.05$) and will not be discussed further. Win-stay and lose-shift behavior were computed across 3 consecutive sessions and analyzed using two-way repeated measures ANOVAs with feedback (win-stay and lose-shift) and phase (pre-surgery and post-surgery) as the within-subjects factors. Dunnett's or Sidak's multiple comparisons tests, when appropriate, were used to compare individual differences when overall significance was found. For all results, statistical significance was determined by a p value < 0.05.

8.4 Experimental Results

8.4.1 Acute Response to Injury

Immediately following sham injury or mTBI, the latency to regain righting reflex was recorded. rmTBI in male rats resulted in longer righting reflex times compared to sham animals across all three surgical days (**Table 8**). Analysis of righting reflex data revealed significant main effects of both day [$F (1.924, 78.89) = 5.489$, $p = 0.0064$] and injury [$F (2, 41) = 57.35$, $p < 0.0001$] as well as a significant day x injury interaction [$F (4, 82) =$

19.24, $p < 0.0001$]. Dunnett's multiple comparisons analysis revealed that rmTBI rats demonstrated longer righting reflex times compared to sham rats on days 1 ($p < 0.0001$), 2 ($p < 0.0001$), and 3 ($p < 0.0001$) whereas smTBI rats displayed longer righting reflex times on day 3 ($p < 0.0001$).

mTBI in female rats also resulted in longer righting reflex times compared to sham animals across all three surgical days (**Table 8**). Analysis of righting reflex data failed to reveal a significant main effect of day [F (1.581, 56.92) = 2.313, p 0.1192], but did reveal a significant main effect and injury [F (2, 36) = 43.72, $p < 0.0001$] as well as a significant day x injury interaction [F (4, 72) = 15.81, $p < 0.0001$]. Dunnett's multiple comparisons analysis revealed rmTBI rats demonstrated longer righting reflex times compared to sham rats on days 1 ($p < 0.0001$), 2 ($p < 0.0001$), and 3 ($p = 0.0084$) whereas smTBI rats displayed longer righting reflex times on day 3 ($p = 0.0131$).

Table 8

Righting Reflex Times of Pretrained Animals (3.5mm Impact Depth)

Sex	Injury Condition	N	Righting Reflex (sec)		
			Surgery Day 1	Surgery Day 2	Surgery Day 3
Male	sham	12	149.5 ± 18.3	181.1 ± 16.4	156.5 ± 19.3
Female	sham	12	171.8 ± 17.2	247.6 ± 16.0	215.6 ± 26.2
Male	smTBI	16	176. 3 ± 15.5	165.4 ± 22.0	353.7 ± 14.9*
Female	smTBI	13	175.2 ± 13.4	199.3 ± 17.2	325.7 ± 26.4*
Male	rmTBI	16	372.4 ± 16.2*	367.4 ± 18.3*	317.3 ± 24.0*
Female	rmTBI	14	411.4 ± 22.8*	369.6 ± 18.2*	310.4 ± 10.7*

Note. Average righting reflex times (seconds) of male and female sham, single (smTBI), and repetitive (rmTBI) injury groups across all three surgery days. Across surgery days, rmTBI males and females exhibited longer righting reflex times compared to their respective sham groups. On surgery day 3, smTBI males and females demonstrated longer righting reflex times compared to their respective sham groups. Values represent mean ± SEM. * denotes $p < 0.05$ from shams analyzed with Dunnett's multiple comparisons tests.

8.4.2 Effects of mTBI on Choice Behavior

We first compared performances in the PDT between injury groups. In the first and second week post-final surgery (**Fig. 24**), analysis of choice data failed to reveal a significant main effect of injury [$F(2, 76) = 1.531$, $p = 0.223$; $F(2, 77) = 0.320$, $p = 0.3727$, respectively], and sex [$F(1, 76) = 0.034$, $p = 0.853$; $F(1, 77) = 0.954$, $p = 0.332$, respectively], but did reveal a significant block x sex interaction [$F(3.027, 230.078) = 3.235$, $p = 0.023$; $F(2.793, 215.026) = 5.218$, $p = 0.002$, respectively]. No other interactions of injury x sex, block x injury, or block x injury x sex ($p > 0.05$) were observed in weeks 1 and 2.

In weeks 3 and 4 post-final surgery, analysis of choice data failed to reveal a significant main effect of injury [$F(2, 77) = 0.237$, $p = 0.790$; $F(2, 77) = 0.373$, $p = 0.690$, respectively], but did reveal a significant main effect of sex [$F(1, 77) = 11.914$, $p < 0.001$; $F(1, 77) = 5.294$, $p = 0.024$, respectively]. A significant block x sex interaction was observed at week 3 [$F(2.721, 209.531) = 3.747$, $p = 0.015$], but not at week 4 [$F(2.747, 211.547) = 1.241$, $p = 0.296$]. No other interactions of injury x sex, block x injury, or block x injury x sex ($p > 0.05$) were observed in weeks 3 and 4. Taken together, these analyses failed to detect differences between sham, smTBI, and rmTBI groups following mTBI.

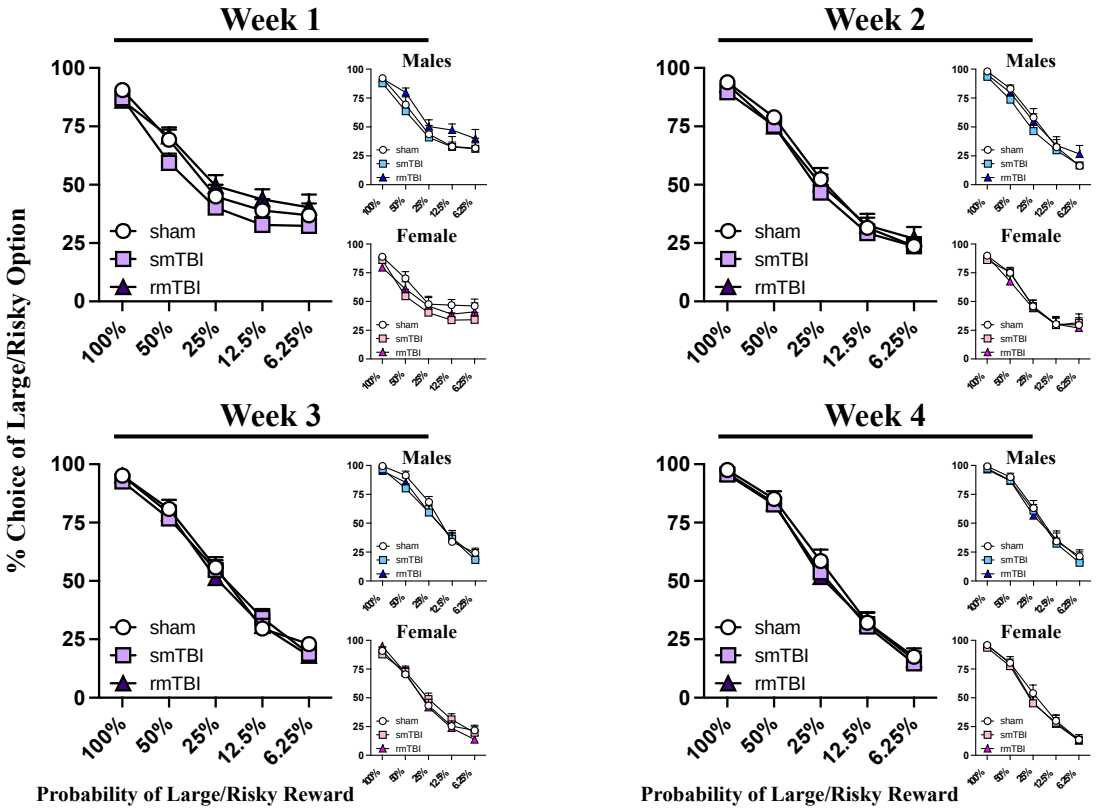


Figure 24. Post-surgery choice performance between-subjects (3.5mm impact depth). Choice behavior is illustrated across four weeks post-final surgery. Line graphs represent percent choice of the large/risky option across five trial blocks. No differences in choice behavior were found between sham, single (smTBI), or repetitive (rmTBI) injury groups in weeks 1-4 when males and females were combined for analysis (purple graph). Insets display choice behavior of males (blue) and females (pink) separately. No significant differences in choice behavior were found between sham, smTBI, or rmTBI groups for either males or females separately in weeks 1-4. Symbols represent mean ± SEM.

Although we did not observe differences in choice performance between surgery groups, we wanted to see if there were inter-subject difference within our injured groups following mTBI. As such, additional within-subjects analyses were conducted.

In week 1 post-injury, analyses of choice data revealed a significant main effect of phase in the smTBI and rmTBI groups [F (1.000, 28.00) = 4.759, p = 0.0377; F (1.000, 29.00) = 10.84, p = 0.0026, respectively] but not in the sham group [F (1.000, 23.00) = 2.421, p = 0.1334] (**Fig. 25**). A significant block x phase interaction was additionally observed within sham, smTBI, and rmTBI groups [F (3.393, 78.04) = 6.872, p = 0.0002; F (2.693, 74.07) = 10.02, p < 0.0001; F (2.976, 86.30) = 5.659, p = 0.014 respectively]. Dunnett's multiple comparisons analysis revealed that sham rats displayed a significant increase in risky choice in the 6.25% block (p = 0.0066) compared to their pre-surgery performances, whereas smTBI and rmTBI rats displayed significant increases in risky choice in both the 12.5% (p < 0.05 for both groups) and 6.25% (p < 0.05 for both groups) blocks. Taken together, these results show evidence of mTBI-induced increases in risky choice preference during the first week following injury.

To understand whether these effects were sex-driven, subsequent 2-way ANOVA analyses were conducted (**Fig. 25 insets, top row**). When males were evaluated during week 1 post-surgery, analyses of choice data revealed a significant main effect of phase in the rmTBI group [F (1.000, 15.00) = 6.579, p = 0.0216] but not in the sham or smTBI groups [F (1.000, 11.00) = 0.007497, p = 0.9326; F (1.000, 15.00) = 2.283, p = 0.1516, respectively]. However, a significant block x phase interaction was observed within sham and smTBI groups [F (2.867, 31.54) = 3.377, p = 0.0321; F (2.309, 34.63) = 4.881, p = 0.0105, respectively] whereas a trend towards a significant interaction was observed in the rmTBI group [F (2.269, 34.03) = 2.590, p = 0.0834]. Sidak's multiple comparisons analysis revealed that smTBI rats displayed a significant increase in risky choice in the 6.25% block (p = 0.0203) and a trend towards a significant increase in risky choice in the 12.5% block

($p = 0.0829$). The main effect of phase reflected the observation that rmTBI resulted in an overall increase in risky choice preference following injury, whereas the effects of smTBI only appeared within the low probability blocks of the task.

When females were evaluated during week 1 post-surgery, analyses of choice data revealed a significant main effect of phase in the sham and rmTBI groups [$F (1.000, 11.00) = 8.647$, $p = 0.0134$; $F (1.000, 13.00) = 4.698$, $p = 0.0493$, respectively] but not in the smTBI group [$F (1.000, 12.00) = 2.783$, $p = 0.1211$]. A significant block x phase interaction across sham, smTBI, and rmTBI groups [$F (2.947, 32.41) = 4.021$, $p = 0.0159$; $F (2.114, 24.31) = 5.166$, $p = 0.0124$; $F (2.349, 30.54) = 4.546$, $p = 0.0146$, respectively] was observed. Sidak's multiple comparisons analysis revealed that sham and rmTBI rats displayed a significant increase in risky choice in the 6.25% block ($p = 0.0197$; $p = 0.0291$, respectively), whereas smTBI rats only displayed a trend towards a significant increase in risky choice in the 6.25% block ($p = 0.0609$). smTBI rats additionally displayed a significant increase in risky choice in the 12.5% block ($p = 0.0492$). These data suggest the interim between training and testing significantly impacted female performance to the same or possibly greater extent than the injury, itself. However, similar to males, the effects of smTBI in females only appeared within the low probability blocks of the task.

In week 2 post-surgery, analyses of choice data revealed a significant main effect of phase in the smTBI and rmTBI group [$F (1.000, 28.00) = 7.864$, $p = 0.0091$; $F (1.000, 29.00) = 9.442$, $p = 0.0046$] but not in the sham group [$F (1.000, 23.00) = 1.906$, $p = 0.1807$]. No significant block x phase interactions were observed at weeks 2 for all groups [$F (3.253, 74.81) = 0.4121$, $p = 0.7605$; $F (3.251, 91.02) = 0.5737$, $p = 0.6473$; $F (3.261, 94.56) = 0.7982$, $p = 0.5070$, respectively.

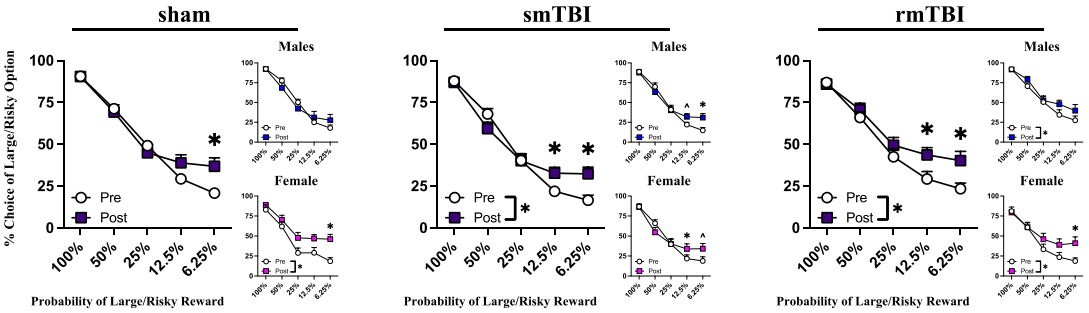
To, again, understand whether these effects were also sex-driven, additional 2-way ANOVA analyses were conducted (**Fig. 25 insets, second row**). During week 2 post-surgery, analysis of choice data in males failed to reveal a significant main effect of phase in the sham and rmTBI groups [F (1.000, 11.00) = 1.702, p = 0.2187; F (1.000, 15.00) = 1.492, p = 0.2408, respectively], but revealed a slight trend towards a significant main effect of phase in the smTBI group [F (1.000, 15.00) = 3.289, p = 0.0898]. Additionally, there were no interactions of block x phase observed across sham, smTBI, and rmTBI groups [F (2.662, 29.29) = 0.5202, p = 0.6507; F (3.122, 46.84) = 0.2493, p = 0.8686; F (3.116, 46.74) = 1.871, p = 0.1456, respectively], reflecting that the effects of mTBI on choice behavior in males resolve after the first week of post-injury testing.

When females were evaluated during week 2 post-surgery, analysis of choice data in failed to reveal a significant main effect of phase in the sham group [F (1.000, 11.00) = 0.3505, p = 0.5658], but did reveal a trend towards a significant main effect of phase in the smTBI group [F (1.000, 12.00) = 4.397, p = 0.0579] and a significant main effect in the rmTBI group [F (1.000, 13.00) = 17.71, p = 0.0010]. Additionally, there were no interactions of block x phase observed across sham, smTBI, and rmTBI groups [F (2.990, 32.89) = 1.431, p = 0.2515; F (2.612, 31.34) = 1.007, p = 0.3942; F (2.729, 35.47) = 0.1203, p = 0.9359, respectively]. These data indicate that females are still experiencing elevated risk preferences by Week 2.

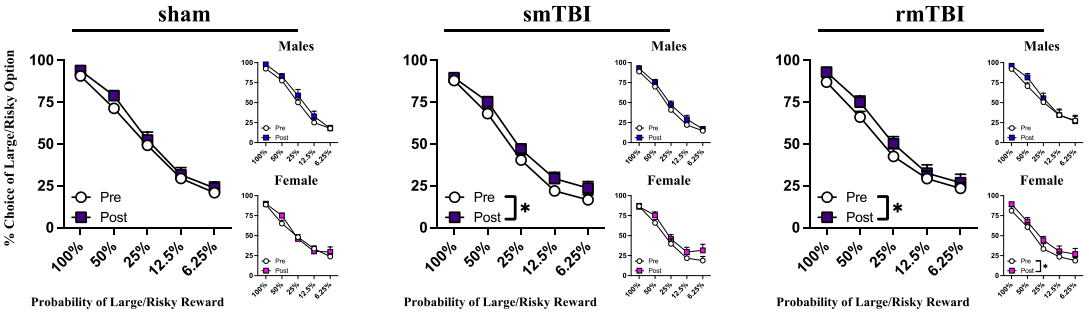
In weeks 3 and 4 post-surgery, the effects of mTBI appeared to resolve back to, and eventually exceed, pre-surgery baseline performances. When choice data was evaluated weeks 3 and 4 post injury (**Fig. 25**), our analyses failed to reveal a significant main effect of phase in sham animals [F (1.000, 21.00) = 0.9539, p = 0.3399; F (1.000, 23.00) = 3.585,

p = 0.0710, respectively]. No significant block x phase interactions were observed at week 3 [F (2.691, 61.90) = 1.321, p = 0.2761] but a significant block x phase interaction was observed at week 4 [F (2.906, 66.85) = 4.217, p = 0.0092]. For smTBI animals, a significant main effect of phase was observed in week 3 [F (1.000, 28.00) = 13.50, p = 0.001], but no significant block x phase interaction was observed [F (3.197, 89.50) = 2.236, p = 0.0855]. In rmTBI animals, a trend towards a significant main effect of phase was observed in rmTBI animals in week 3 [F (1.000, 29.00) = 4.158, p = 0.0506]; however, there was a significant block x phase interaction [F (3.223, 93.46) = 9.008, p < 0.0001]. By week 4, both smTBI and rmTBI groups demonstrated significant main effects of phase [F (1.000, 28.00) = 12.05, p = 0.0017; F (1.000, 29.00) = 4.730, p = 0.0379, respectively] and block x phase interactions [F (3.065, 85.81) = 4.699; p = 0.0041; F (3.370, 97.73) = 8.986, p < 0.0001, respectively]. Taken together, these data indicate that mTBI transiently increases risky choice during probabilistic discounting; however, these effects appear to resolve after extended re-training.

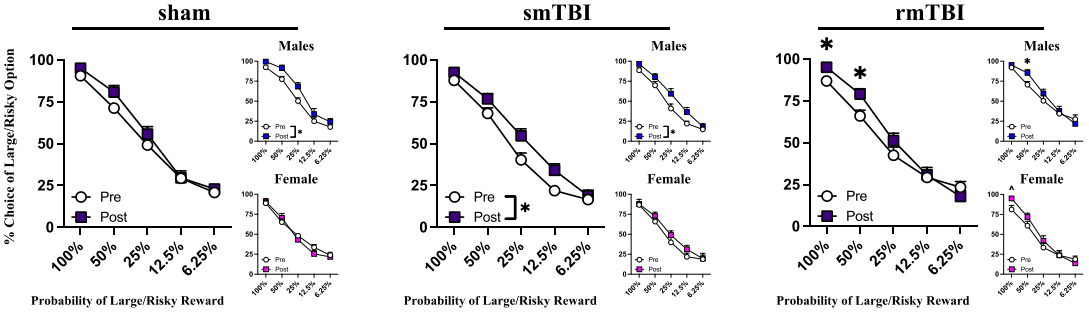
Choice Behavior: Week 1 Post-Surgery



Choice Behavior: Week 2 Post-Surgery



Choice Behavior: Week 3 Post-Surgery



Choice Behavior: Week 4 Post-Surgery

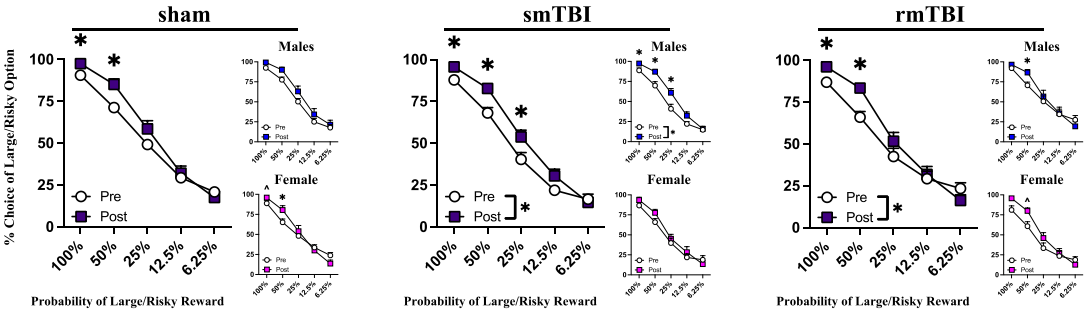


Figure 25. Post-surgery choice performance within-subjects (3.5mm impact depth). Pre- versus post-surgery choice behavior is illustrated across four weeks post-final surgery. Insets display choice behavior of males (blue) and females (pink) separately. Line graphs represent percent choice of the large/risky option across five trial blocks. When males and females were combined for analysis (purple graph) in week 1, single (smTBI) and repetitive (rmTBI) injury groups demonstrated a main effect of injury reflecting increased risky choice preference compared to pre-surgery performances. These effects appear to be greater in rmTBI with separate male and female performances also demonstrating increased risky choice preferences (top right insets). In week 2, rmTBI females still displayed increased risky choice, whereas the effects in males resolved. In weeks 3 and 4, all groups were performing similarly to, or in some cases, exceeding their pre-surgery performances. Symbols represent mean ± SEM. * denotes $p < 0.05$.

8.4.3 Effects of mTBI on Win-Stay/Lose-Shift Behavior

In a between-subjects analysis of win-stay/lose-shift performance, we observed no meaningful differences in win-stay/lose-shift behavior. Across weeks 1-4 of testing, analysis of win-stay/lose-shift data revealed a significant main effect of feedback ($p < 0.05$), reflecting a difference in the proportion of win-stay and lose-shift tendencies made. However, these analyses did not yield a main effect of injury [$F(2, 77) = 2.579$, $p = 0.0824$; $F(2, 77) = 0.6360$, $p = 0.5321$; $F(2, 77) = 1.118$, $p = 0.3321$; $F(2, 77) = 1.391$, $p = 0.2549$, respectively] or sex [$F(1, 77) = 0.5456$, $p = 0.4623$; $F(1, 77) = 0.006330$, $p = 0.9368$; $F(2, 77) = 1.118$, $p = 0.5072$; $F(1, 77) = 0.4401$, $p = 0.5090$, respectively] in weeks 1-4. There were no interactions of feedback x injury, injury x sex, feedback x sex, and feedback x injury x sex ($p > 0.05$) in weeks 1 and 2. However, in week 3 post-final injury, significant interactions of injury x sex [$F(2, 77) = 4.095$, $p = 0.0204$] and feedback x sex [$F(1, 77) = 12.92$, $p = 0.0006$] were observed whereas only a significant feedback x sex interaction [$F(1, 77) = 6.640$, $p = 0.0119$] was observed in week 4. No other interactions of feedback x injury or injury x sex ($p > 0.05$) were observed in weeks 3 and 4.

To assess whether the within-subjects alterations observed in discounting behavior, specifically those demonstrated in Week 1 and 2, were the result of alterations in feedback sensitivity to reward and non-rewarded risky choices, we additionally conducted a within-subjects on win-stay/lose-shift behavior. Analysis of win-stay/lose-shift data revealed a significant main effect of phase in the sham and rmTBI groups [F (1.000, 23.00) = 0.0252, p = 0.0377; F (1.000, 29.00) = 9.008, p = 0.0055, respectively] but not in the smTBI group [F (1.000, 28.00) = 0.08192, p = 0.7768] (**Fig. 26**) in week 1. These analyses failed to reveal a significant feedback x phase interaction in sham and smTBI groups [F (1.000, 23.00) = 3.800, p = 0.0636; F (1.000, 28.00) = 0.3265, p = 0.5723, respectively] but did reveal a significant feedback x phase interaction in the rmTBI group [F (1.000, 29.00) = 7.640, p = 0.0098]. Sidak's multiple comparisons analysis revealed that rmTBI rats displayed a significant decrease in lose-shift behavior (p = 0.0042), indicating that the observed increases in risky choice in week 1 appear to be driven by a reduced sensitivity to non-rewarded outcomes following a risky decision.

To understand whether these effects were sex-driven, subsequent 2-way ANOVA analyses were conducted (**Fig. 26 insets, top row**). When males were evaluated during week 1 post-surgery, analyses of win-stay/lose-shift data failed to reveal a significant main effect of phase in the sham, smTBI, and rmTBI groups [F (1.000, 11.00) = 1.376, p = 0.2655; F (1.000, 15.00) = 0.1502, p = 0.7038; F (1.000, 15.00) = 2.934, p = 0.1073, respectively]. There was no significant feedback x phase interaction observed in the sham and smTBI groups [F (1.000, 11.00) = 0.03730, p = 0.8504; F (1.000, 15.00) = 0.01612, p = 0.9007, respectively]; however, a significant interaction was observed in the rmTBI group [F (1.000, 15.00) = 7.861, p = 0.0134]. Sidak's multiple comparisons analysis

revealed that rmTBI rats displayed a significant decrease in lose-shift behavior ($p = 0.0288$), indicating that the observed increases in risky choice in week 1 in males appeared to be driven by a reduced sensitivity to non-rewarded outcomes following a risky decision.

When females were evaluated during week 1 post-surgery, analyses of win-stay/lose-shift data revealed a significant main effect of phase in the sham and rmTBI groups [$F (1.000, 11.00) = 4.982$, $p = 0.0473$; $F (1.000, 13.00) = 6.153$, $p = 0.0276$, respectively] but not in the smTBI group [$F (1.000, 12.00) = 0.0007306$, $p = 0.9789$]. A significant feedback x phase interaction was observed in the sham group [$F (1.000, 11.00) = 7.282$, $p = 0.0207$]; however, there was no significant interaction observed in the smTBI and rmTBI groups [$F (1.000, 12.00) = 2.682$, $p = 0.1274$; $F (1.000, 13.00) = 2.580$, $p = 0.1323$, respectively]. Sidak's multiple comparisons analysis revealed that sham rats displayed a significant decrease in lose-shift behavior ($p = 0.0221$), indicating that the observed increases in risky choice in week 1 appeared to be driven by a reduced sensitivity to non-rewarded outcomes following a risky decision. To determine whether the observed main effect of phase in rmTBI rats was specifically driven by either win-stay or lose-shift behavior, subsequent Wilcoxon matched-pairs signed rank test (win-stay) and paired t-test (lose-shift) analyses were conducted. There were no differences in win-stay performance following injury ($p = 0.9515$); however, it was revealed that rmTBI rats did display a significant decrease in lose-shift tendencies following injury ($p = 0.0414$), indicating that the observed increases in risky choice in week 1 in females were also driven by a reduced sensitivity to non-rewarded outcomes following a risky decision.

In week 2 post-surgery, analysis of win-stay/lose-shift behavior failed to reveal a significant main effect of phase in shams in week 2 post-injury [$F (1.000, 23.00) = 2.531$,

p = 0.1253] but did reveal a significant main effect of phase in the smTBI and rmTBI groups [F (1.000, 28.00) = 4.865, p = 0.0358; F (1.000, 29.00) = 5.428, p = 0.0270, respectively]. No significant block x phase interactions were observed at weeks 2 for all groups [F (3.253, 74.81) = 0.4121, p = 0.7605; F (3.251, 91.02) = 0.5737, p = 0.6473; F (3.261, 94.56) = 0.7982, p = 0.5070, respectively.

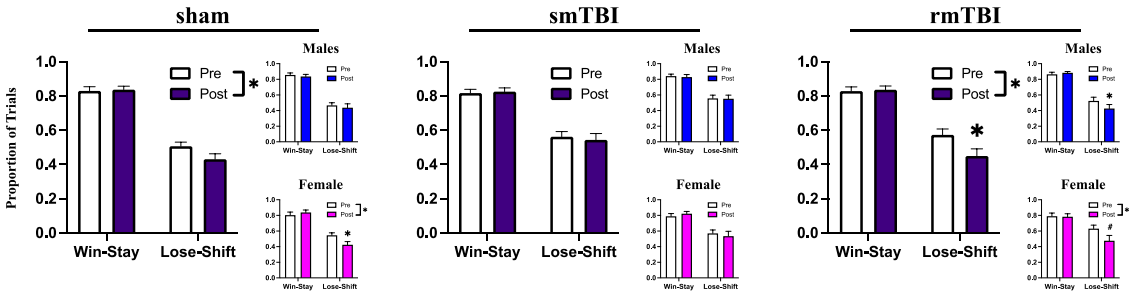
To, again, understand whether these effects were also sex-driven, additional 2-way ANOVA analyses were conducted for males and females separately (**Fig. 26 insets, second row**). When males were evaluated during week 2 post-surgery, analysis of win-stay/lose-shift data failed to reveal a significant main effect of phase in the sham and smTBI groups [F (1.000, 11.00) = 0.3328, p = 0.5756; F (1.000, 15.00) = 2.966, p = 0.1056, respectively], but did reveal a significant main effect of phase in the rmTBI group [F (1.000, 15.00) = 7.601, p = 0.0147]. There was no significant feedback x phase interaction observed in the sham and smTBI groups [F (1.000, 11.00) = 2.542, p = 0.1392; F (1.000, 15.00) = 0.8688, p = 0.3660, respectively]; however, a significant interaction was observed in the rmTBI group [F (1.000, 15.00) = 9.408, p = 0.0078]. Sidak's multiple comparisons analysis revealed that rmTBI rats displayed a significant decrease in lose-shift behavior (p = 0.0041). This would indicate that rmTBI's effects on feedback sensitivity persist despite these changes in lose-shift behavior no longer driving risk preference in week 2.

When females were evaluated during week 2 post-surgery, analysis of win-stay/lose-shift data failed to reveal a significant main effect of phase in the smTBI and rmTBI groups [F (1.000, 12.00) = 1.748, p = 0.2107; F (1.000, 13.00) = 1.318, p = 0.2716, respectively], but did reveal a trend towards a significant main effect of phase in the sham group [F (1.000, 11.00) = 3.872, p = 0.0748]. A trend towards a significant feedback x

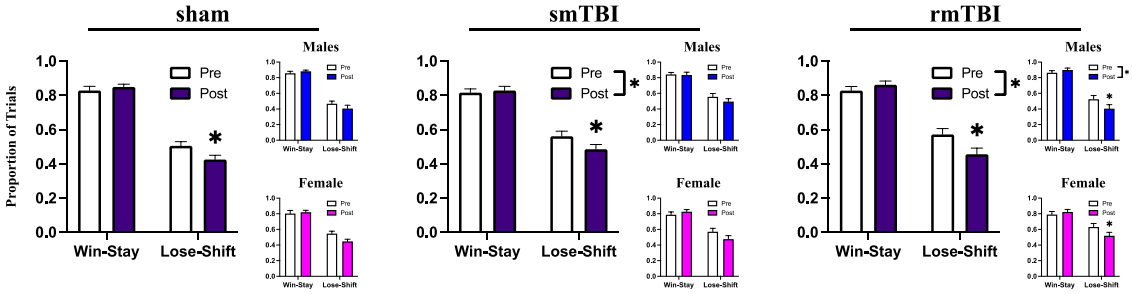
phase interaction was observed in the sham and smTBI groups [F (1.000, 11.00) = 4.505, p = 0.0573; F (1.000, 12.00) = 4.057, p = 0.0670, respectively] whereas a significant interaction was observed in the rmTBI group [F (1.000, 13.00) = 7.163, p = 0.0190]. Sidak's multiple comparisons analysis revealed that rmTBI rats displayed a significant decrease in lose-shift behavior (p = 0.0459), indicating that the observed increases in risky choice in week 2 were also driven by a reduced sensitivity to non-rewarded outcomes following a risky decision.

In weeks 3 and 4 (**Fig. 26**), analysis of win-stay/lose-shift behavior failed to reveal a significant main effect of phase in sham [F (1.000, 23.00) = 0.02322, p = 0.8802; F (1.000, 23.00) = 0.1195, p = 0.7327, respectively] and rmTBI groups [F (1.000, 29.00) = 2.345, p = 0.1365; F (1.000, 29.00) = 0.8395, p = 0.3671, respectively], but revealed a significant main effect of phase in smTBI animals [F (1.000, 28.00) = 5.626, p = 0.0248; F (1.000, 28.00) = 5.822, p = 0.0226, respectively]. A significant feedback x phase interaction was observed in all sham, smTBI, and rmTBI groups for both weeks 3 and 4 (all p values < 0.05). Given that sham groups were demonstrating similar trends in win-stay/lose-shift performances as to the mTBI groups, we, again, attributed these effects to animals improving in the PDT following extended re-training.

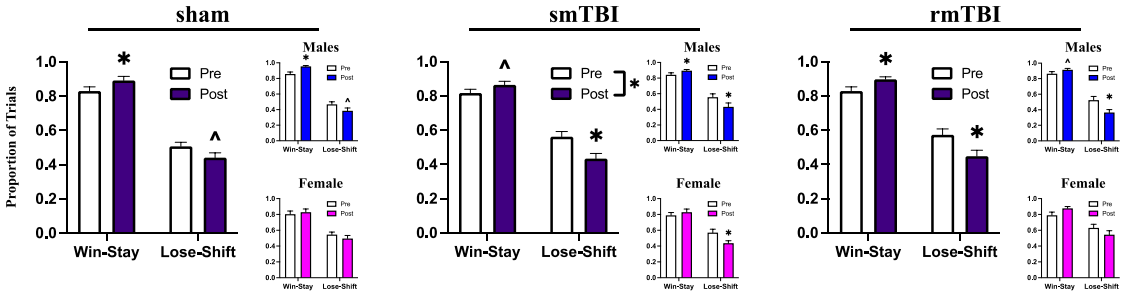
Win-Stay/Lose-Shift: Week 1 Post-Surgery



Win-Stay/Lose-Shift: Week 2 Post-Surgery



Win-Stay/Lose-Shift: Week 3 Post-Surgery



Win-Stay/Lose-Shift: Week 4 Post-Surgery

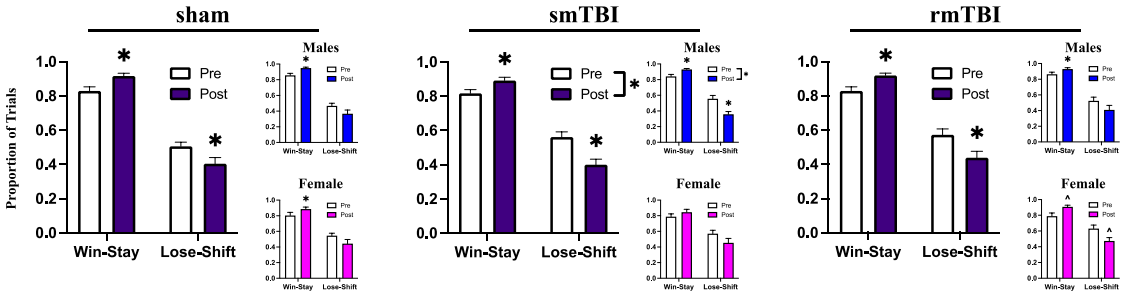


Figure 26. Post-surgery win-stay/lose-shift performance within-subjects (3.5mm impact depth). Pre- versus post-surgery win-stay/lose-shift performance is illustrated across four weeks post-final surgery. Insets display win-stay/lose-shift performances of males (blue) and females (pink) separately. In week 1, repetitive mild traumatic brain injury (rmTBI) significantly reduced lose-shift tendencies in comparison to pre-surgery performance. In week 2, rmTBI's effects on lose-shift behavior persisted in both males and females. In weeks 3 and 4, sham, single (smTBI), and rmTBI injury groups were performing similarly to, or in some cases, exceeding their pre-surgery performances. Bars represent mean ± SEM. * denotes $p < 0.05$ and ^ denotes $p < 0.1$.

8.4.4 Effects of mTBI on Response Latency

Separate analyses compared how TBI affected response latencies between injury groups. Across weeks 1-4 of testing (**Fig. 27**), analysis of response latency revealed a significant main effect of sex [$F(1, 76) = 16.018$, $p < 0.001$; $F(1, 77) = 11.547$, $p = 0.001$; $F(1, 77) = 10.660$, $p = 0.002$; $F(1, 77) = 9.668$, $p = 0.003$, respectively] as well as a block x sex interaction [$F(2.874, 218.458) = 2.957$, $p = 0.035$; $F(1.952, 150.289) = 7.205$, $p = 0.001$; $F(2.296, 176.803) = 7.035$, $p < 0.01$; $F(2.770, 213.269) = 3.659$, $p = 0.016$, respectively], once again reflecting that females were generally slower to make choices compared to males. However, these analyses did not yield a main effect of injury [$F(2, 76) = 2.413$, $p = 0.096$; $F(2, 77) = 0.608$, $p = 0.547$; $F(2, 77) = 1.307$, $p = 0.276$; $F(2, 77) = 0.508$, $p = 0.604$, respectively] or injury x sex interaction [$F(2, 76) = 0.923$, $p = 0.402$; $F(2, 77) = 0.596$, $p = 0.544$; $F(2, 77) = 2.619$, $p = 0.079$; $F(2, 77) = 0.004$, $p = 0.996$, respectively]. There were no other interactions of injury x sex or block x injury ($p > 0.05$) in weeks 1-4. A significant block x injury x sex interaction [$F(4.592, 176.803)$, $p = 0.043$] was observed only at week 3, but not in weeks 1, 2, and 4 post-final injury ($p > 0.05$).

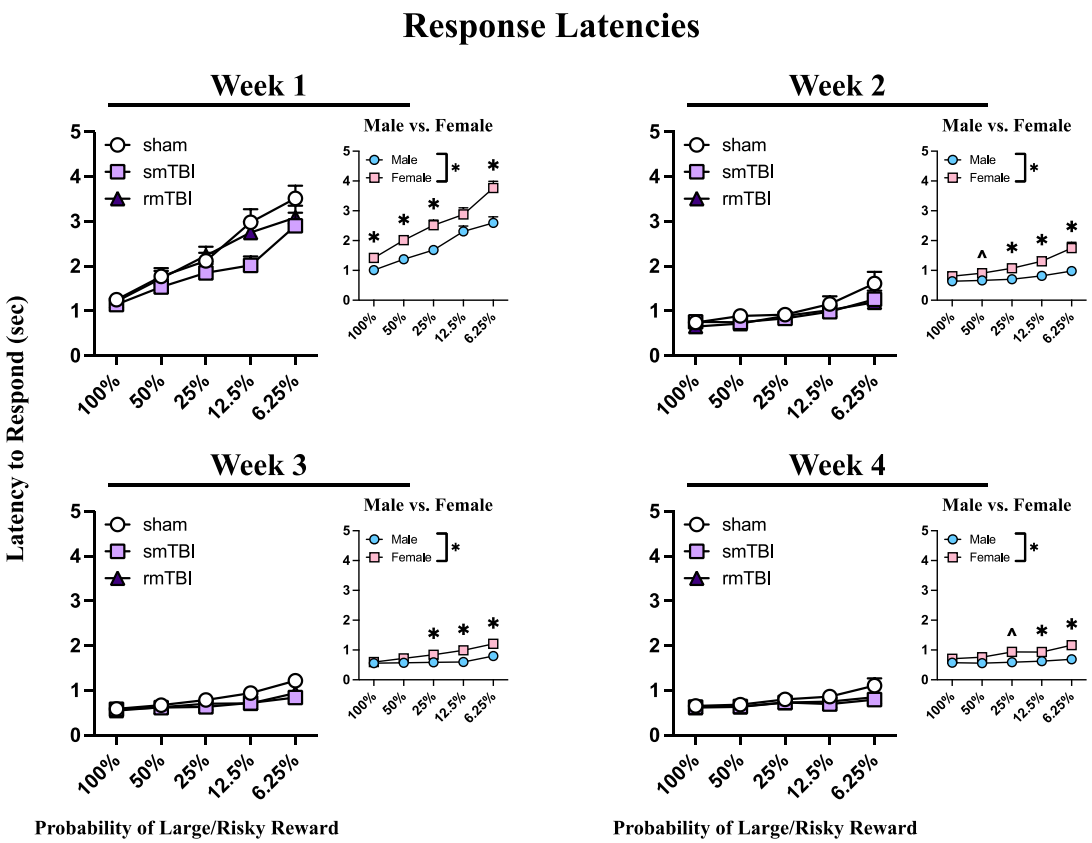


Figure 27. Post-surgery response latency performance between-subjects (3.5mm impact depth). Response latencies are illustrated across four weeks post-final surgery. Line graphs represent response latencies across five trial blocks. No differences in response latencies were found between sham, single (smTBI), or repetitive (rmTBI) injury groups in weeks 1-4 post-surgery. Insets display overall male vs. female response latencies across all trial blocks. Across all 4 weeks, females demonstrated slower response latencies compared to males. Symbols represent mean ± SEM. On legend, * denotes $p < 0.05$. * denotes $p < 0.05$ main effect of injury analyzed with three-way ANOVA.

Since we again found clear differences in response latencies between males and females, we subsequently assessed whether there were differences in latencies to make risky or certain choices. Subsequent three-way ANOVA analyses were therefore conducted to compare response latencies of trials that resulted in a risky choice versus a certain one (**Fig. 28**). Across weeks 1-4 of testing, analysis of response latency revealed a significant

main effect of sex [F (1, 77) = 17.05, p <0.0001; F (1, 77) = 13.62, p = 0.0004; F (1, 77) = 8.731, p = 0.0041; F (1, 77) = 11.94, p = 0.0009, respectively], reflecting, again, that females were slower to make choices compared to males. A significant main effect of choice type was observed during week 1 [F (1, 77) = 4.214, p = 0.0435] but not in weeks 2-4 [F (1, 77) = 0.6277, p = 0.4307; F (1, 77) = 1.020, p = 0.3157; F (1, 77) = 0.0007147, p = 0.9787, respectively]. Additionally, these analyses did not yield a main effect of injury [F (2, 77) = 0.8691, p = 0.4234; F (2, 77) = 0.4613, p = 0.6322; F (2, 77) = 1.460, p = 0.2387; F (2, 77) = 1.191, p = 0.3096, respectively] or injury x sex interaction [F (2, 77) = 0.5233, p = 0.5947; F (2, 77) = 0.4500, p = 0.6393; F (2, 77) = 3.032, p = 0.0540; F (2, 77) = 0.05820, p = 0.9435, respectively]. Lastly, significant main injury x choice interactions were only identified at weeks 2 and 4 post-final surgery [F (2, 77) = 4.422, p = 0.0152; F (2, 77) = 3.532, p = 0.0341, respectively] There were no other interactions of choice x sex or choice x injury x sex ($p > 0.05$) in weeks 1-4.

When the latencies of risky and certain choices were assessed separately, males showed no differences in response latencies of trials that ended in a risky or certain choice (all p values > 0.1) weeks 1-4 post-final injury (**Fig. 28A**). There were no significant differences between female injury groups in response latencies of trials that ended in a risky (all p values > 0.1) choice across weeks 1-4 (**Fig. 28B**). In weeks 1, 2, and 4, there were no differences between female injury groups in response latencies of trials that ended in certain choices (all p values > 0.1). However, in week 3, there was a significant decrease in latencies of trials that ended in certain choices (H (2) = 7.105, p =0.0287). Dunn's multiple comparisons analysis revealed that smTBI rats were quicker to make certain choices (p = 0.0208) compared to sham. Therefore, we can conclude that mTBI of

increased strength does not result in hesitation to make risky choices in males as observed with our milder 2.5mm depth model.

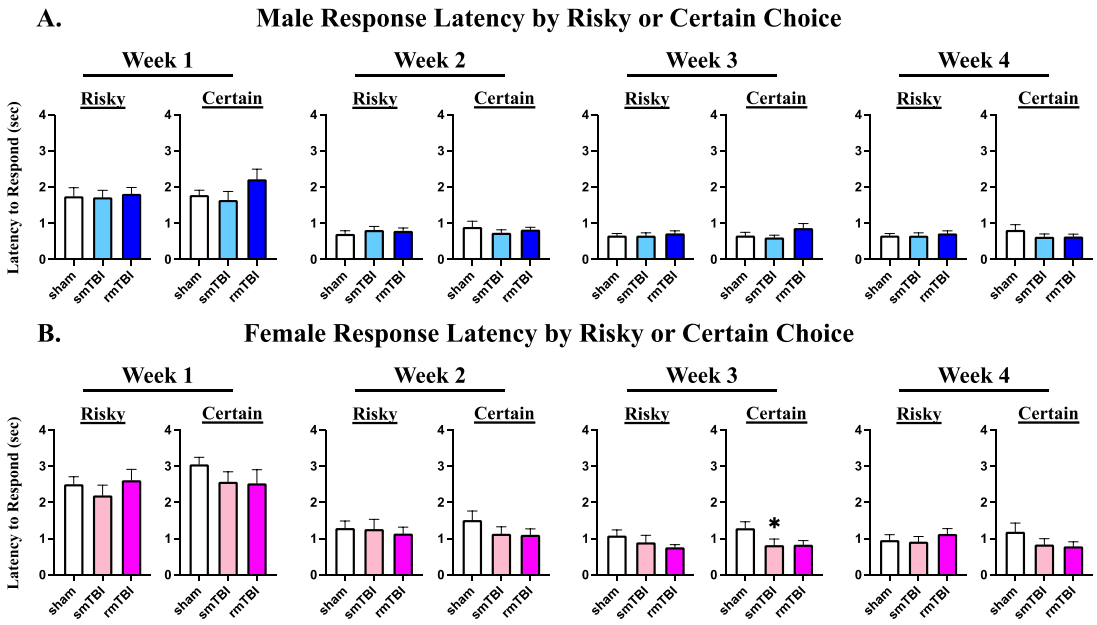


Figure 28. Response latency by risky or certain choice (3.5mm impact depth). Response latencies are illustrated by choice type across four weeks post-final surgery. Bar graphs represent the averaged total percentage of response latencies for trials that ended in either a risky or certain choice across all trial blocks. **A)** Males: No differences in response latencies were found between sham, single (smTBI), or repetitive (rmTBI) injury groups in weeks 1-4 post-final surgery. **B)** Females: No differences in response latencies were found between sham, smTBI, or rmTBI groups in weeks 1, 2, and 4. In week 3, smTBI reduced response latency in certain choice trials. Bars represent mean ± SEM. * denotes $p < 0.05$ from shams analyzed with Dunn's multiple comparisons tests.

8.4.5 Effects of mTBI on Magazine Latency

Lastly, we assessed the effects of mTBI on the latency to collect rewards. In week 1 post-final surgery (**Fig. 29**), analysis of magazine latency data revealed a significant main effect of injury [$F(2, 75) = 3.442$, $p = 0.037$], but failed to reveal a significant main effect

of sex [F (1, 75) = 0.851, p = 0.359]. Dunnett's multiple comparisons tests revealed a slight trend towards increased magazine latencies (p = 0.08) following rmTBI. In opposite fashion, during weeks 2-4, analysis of magazine latency data failed to reveal a significant main effect of injury [F (2, 77) = 1.056, p = 0.353; F (2, 77) = 0.285, p = 0.753; F (2, 77) = 0.142, p = 0.868, respectively], but did reveal significant main effects of sex in weeks 2 and 3 [F (1, 77) = 4.308, p = 0.041; F (1, 77) = 4.489, p = 0.037, respectively] and a trend towards a significant main effect of sex in week 4 [F (1, 77) = 3.065, p = 0.084], reflecting that females were generally slower to collect rewards compared to males regardless of injury condition (**Fig. 29, insets**). Across weeks 1-4 of testing, analysis of magazine latency data revealed significant block x injury x sex interaction [F (4.687, 175.765) = 2.323, p = 0.049; F (4.307, 165.801) = 2.448, p = 0.044; F (3.780, 145. 517) = 2.756, p = 0.033; F (3.772, 145.223) = 3.498, p = 0.011, respectively]. There were no other interactions of injury x sex, block x injury, and block x sex (p > 0.05) in weeks 1-4. Taken together, these results indicate that rmTBI animals are slower to claim rewards following a risk-related decision within the first week of testing; however, these increases were not associated with either males or females, specifically. Although there were inherent differences in magazine latencies between male and female subjects, these effects were only significantly apparent in weeks 2 and 3 post-injury. As such, additional analyses were not conducted.

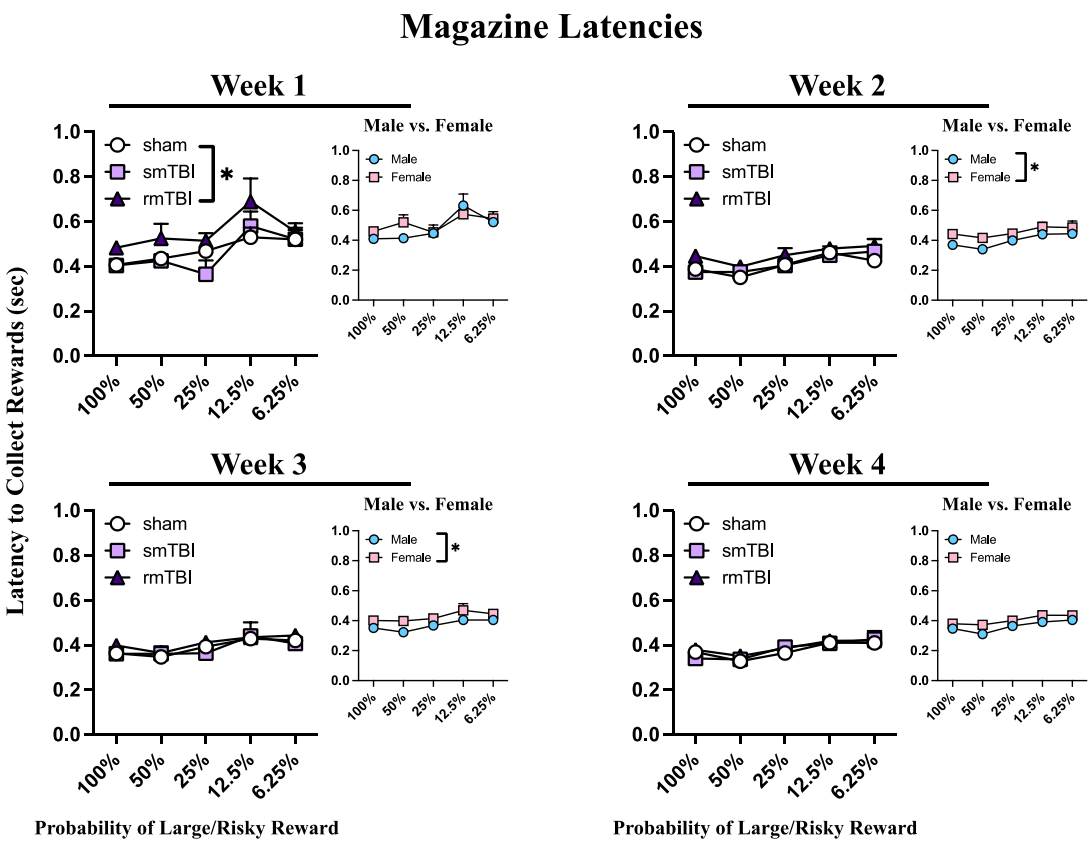


Figure 29. Post-surgery magazine latency between-subjects (3.5mm impact depth). Magazine latencies are illustrated across four weeks post-final surgery. Line graphs represent magazine latencies across five trial blocks. In week 1, a main effect of injury was observed when males and females were combined for analysis (purple graph). No differences in magazine latencies were found between sham, single (smTBI), or repetitive (rmTBI) injury groups in weeks 2-4 post-surgery. Insets display overall male vs. female magazine latencies across all trial blocks. Across weeks 2-4, females demonstrated slower magazine latencies compared to males. Symbols represent mean ± SEM. * denotes $p < 0.05$ main effect of injury or sex (insets) analyzed with three-way ANOVA.

8.5 Discussion

The focus of these experiments was to determine if a stronger form of rmTBI amplifies the effects observed in females and enables detection of increased risky choice behavior in males. Following mTBI of increased depth, we did not initially detect

differences in performance between injury groups. However, when we evaluated performances within each group, both single and repetitive injuries resulted in significant increases in risky choice preference in the PDT. When males and females were assessed separately, we observed increased risky choice preference in males following rmTBI during the first week of post-surgery testing. Interestingly, the smTBI-induced increases in risky choice preference were only observed in the low probability blocks, where seeking the larger reward was disadvantageous. The effects of rmTBI were accompanied by reduced lose-shift tendencies, indicative of a decreased sensitivity to non-rewarded risky choices. These alterations to feedback sensitivity were likely driving the increased risky choices observed during week 1; however, following extended testing, the effects of mTBI on choice behavior resolved by week 2, indicating these effects are transient. Interestingly, the effects of rmTBI on lose-shift behavior persisted into week 2 post-surgery despite these changes no longer driving risk preferences beyond week 1. These results would suggest that mTBI can produce differential time-dependent effects on different components of decision making in male rats.

When female performance was evaluated, all surgical groups, including shams, demonstrated increased risky choice in the first week post-final surgery. Similar to males, the effects of smTBI were only apparent in the low probability blocks. The effects of sham and rmTBI animals were accompanied by reduced lose-shift tendencies, suggesting that the increases in choice preference were largely driven by a reduced sensitivity to non-rewarded outcomes following a risky decision. By week 2, the effects in sham animals resolved to pre-surgery levels, whereas the effects of rmTBI on risky choice preference and lose-shift behavior persisted. These results would suggest that the interim between training

and testing may have significantly impacted female performance during week 1 to the same or possibly greater extent than the injury itself. However, through extended re-training, sham animals were able to re-establish optimal choice patterns, whereas repetitive injured animals could not. Given that males did not demonstrate this behavior suggests that females may require additional exposure to the PDT prior to this interim. Nevertheless, by weeks 3 and 4 post-surgery, the effects of rmTBI had resolved and all surgery groups were performing at or exceeding their respective pre-surgery performances, indicating that the duration of rmTBI's effects on risky choice preference in females is approximately 2 weeks.

This study additionally confirmed that male rats are generally quicker to make choices compared to females regardless of injury condition. We further observed that males were generally quicker to collect rewards following a risk-related decision. Again, these effects were not injury-dependent, indicating that males are generally quicker to respond and collect rewards in this particular assay. Interestingly, we did not observe slower deliberation times to make risky choices in males as we did with our milder mTBI model. One potential explanation could be that we are now experiencing a ceiling effect within our male animals that we previously observed in females in the 2.5mm depth model. Nevertheless, the results of these experiments expand our understanding of the effects of single versus repetitive TBI on risk/reward decision making. Consistent with our findings from **Chapter 6**, smTBI increased risky choice. However, these increases occurred only when the probability of obtaining larger rewards was relatively low. This pattern of risky choice was observed in both sexes, indicating that smTBI produces similar choice profiles in male and female rats and confirm our suspicion that our previous injury parameters were

sub-threshold for revealing effects in males. RmTBI extended the duration of these effects in females, whereas the effects of rmTBI in males remained transient. These results confirm that females are more susceptible to alterations in risk preferences following mTBI. RmTBI animals also exhibited reduced sensitivity to non-rewarded outcomes, suggesting that multiple TBIs can disrupt processes that facilitate appropriate adjustments in choice biases following non-rewarded actions. In cases of single TBI, brain injured patients exhibit reduced sensitivity to the contingencies (Schlund, 2002a, 2002b; Schlund & Pace, 2000) and outcomes (Bechara et al., 1994; Bechara et al., 1998; Bechara et al., 2000; Larson et al., 2007) associated with risk-related decisions. Many of these cases involved moderate to severe TBI, suggesting that rmTBI produces alterations to feedback sensitivity characteristic of a moderate-severe injury as opposed to mTBI. As such, these results confirm the overall assertion that mTBI alters risk/reward decision making behavior and that these effects are being magnified following repeated injuries.

8.6 Conclusion

Overall, we have validated the existence of mTBI-induced changes to probabilistic discounting behavior using two versions of our mTBI model. In **Chapter 6**, the effects of mTBI from our milder model resulted in increased risky choice behavior. Those changes in risk preference were mainly driven by single injury with the effects of rmTBI being just shy of significance. Following mTBI of increased depth, both single and repetitive injuries resulted in significant increases in risky choice preference in the PDT, demonstrating that this modified version of our injury model succeeded in amplifying the effects of mTBI on risky choice.

Chapter 9

Effects of Repetitive Mild Traumatic Brain Injury on Catecholamine Regulatory Proteins Levels within the Prefrontal Sub-Regions

Disclaimer: Portions of this chapter were adapted from: Knapp CP, Papadopoulos E, Loweth JA, et al. Perturbations in risk/reward decision making and frontal cortical catecholamine regulation induced by mild traumatic brain injury. *Behav Brain Res*. 467:115002. doi:10.1016/j.bbr.2024.115002

9.1 Introduction

In **Chapter 6**, we observed increases in risky choice preference in the probabilistic discounting task (PDT) 48 hours following mild traumatic brain injury (mTBI) with our milder (2.5mm impact depth) model. However, the neural mechanisms responsible for these behavioral changes remain unknown. The medial prefrontal cortex (mPFC), anterior cingulate cortex (ACC), and orbitofrontal cortex (OFC) receive dense innervation from catecholaminergic fibers that modulate each region's respective role in the risk/reward decision making process. As described in **Chapter 1**, previous studies have reported aberrant catecholamine activity within the PFC following TBI (Kobori et al., 2006; Massucci et al., 2004), suggesting that imbalanced dopamine (DA) and norepinephrine (NE) levels may underlie TBI-induced increases in risky behavior. Catecholamine synthetic, packaging, reuptake, and degradation proteins maintain optimal catecholaminergic activity within the PFC; however, no studies have examined the levels

of these proteins following repetitive injury (rmTBI). Our goal in this Chapter was to investigate how these catecholamine regulatory proteins might be affected by rmTBI.

9.2 Rationale

Catecholamine regulatory proteins are essential for maintaining optimal catecholaminergic signaling within the brain. These proteins include: 1) synthetic enzymes, tyrosine hydroxylase (TH) and dopamine-β-hydroxylase (DBH); 2) packaging enzyme, vesicular monoamine transporter-2 (VMAT2); 3) DA and NE transporters (DAT and NET, respectively); and 4) degradation enzymes, catechol-O-methyltransferase (COMT) and monoamine oxidase (MAO). TH is the rate-limiting enzyme for catecholamine synthesis and is present in both dopaminergic and noradrenergic neurons whereas DBH converts DA to NE and is located only in noradrenergic neurons. Biosynthesis of DA and NE can either increase or decrease depending on alterations to TH and DBH activity. Increased levels of TH, have been observed throughout the brain following experimental TBI (Huger & Patrick, 1979; Kobori et al., 2006; Wagner, Sokoloski, et al., 2005; Yan et al., 2001; Yan et al., 2007), suggesting a potential surge in catecholamine production. In regards to DBH, no changes in DBH expression have been observed within the PFC (Yan et al., 2001) or in other forebrain regions (Schmidt & Grady, 1995) following experimental TBI. However, a separate study did observe injury-induced loss of DBH-immunoreactive fibers and terminals within specific areas of the hippocampus 15 days following moderate fluid percussion injury (Zhu et al., 2000), suggesting potentially reduced NE production.

VMAT2 is an enzyme that is located in the membrane of monoaminergic vesicles and is responsible for packaging DA and NE into these vesicles for storage and subsequent

release. Following experimental TBI, sex-specific inhibition of VMAT2 within the striatum has been observed in mice, resulting in impaired DA storage (Xu et al., 2016). However, VMAT2 expression in the PFC following mTBI has yet to be explored.

Reuptake transporter proteins, DAT and NET, are located in the membranes of dopaminergic and noradrenergic neurons, respectively, and are responsible for removing extracellular DA and NE after their release to maintain efficient catecholaminergic signaling. These catecholamines can then be recycled back into vesicles by the actions of VMAT2. NET is the primary transporter for DA and NE in the PFC (Carboni et al., 1990; Morón et al., 2002; Sesack et al., 1998); however, no studies have examined changes in NET expression after TBI despite brain-wide, and sometimes sex-dependent, decreases in DAT being reported (Wagner, Chen, et al., 2005; Wagner, Sokoloski, et al., 2005; Wilson et al., 2005; Yan et al., 2002).

In addition to transporter activity, DA and NE are degraded through the actions of COMT and MAO. COMT works to degrade extracellular DA and NE, whereas MAO degrades cytosolic DA and NE. There are two major isoforms of COMT: soluble (S-COMT) and membrane-bound (MB-COMT). S-COMT is the main form of COMT in peripheral tissues, including the liver, whereas MB-COMT is the main form of COMT in the brain, and is specifically located in the cell body, axons and dendrites of neurons (Chen et al., 2011; Segura-Aguilar et al., 2014). The C-terminal catalytic domain of MB-COMT is oriented towards the extracellular space, allowing COMT to interact with extracellular DA and NE and inactivate them (Chen et al., 2011). Inhibition or damage to this catalytic site would interfere with COMT's ability to maintain proper catecholamine levels. MAO is located on the outer membranes of mitochondria in neurons and glia cells, and is also

classified into two types: MAO-A and MAO-B. MAO-A is primarily expressed in catecholaminergic neurons (Westlund et al., 1988) whereas MAO-B is mainly expressed in serotonergic neurons and astrocytes (Levitt et al., 1982; Westlund et al., 1985; Westlund et al., 1988). The role of MAO-A is to maintain low concentrations of DA and NE in the cytosol; however, given that MAO generates hydrogen peroxide, which is a precursor of hydroxyl radicals, overactive MAO activity can lead to oxidative stress (Segura-Aguilar et al., 2014). Conversely, reduced MAO activity can lead to an overaccumulation of cytosolic DA and NE, resulting in cytotoxicity of catecholamine neurons.

While a subset of these catecholamine regulatory proteins have been assessed after TBI, a comprehensive evaluation of all these proteins within a single study has not yet been performed. Furthermore, how repetitive injury affects these proteins and whether changes in protein expression are similar across all prefrontal areas remains to be explored. Given the dissociable contributions of the mPFC, ACC, and OFC to risk/reward decision making, differential disruptions to catecholamine activity within each sub-region would result in distinct changes to the processing and execution of probabilistic-based decisions. Therefore, to further investigate our behavioral observations and potential mechanisms of catecholamine imbalance within the PFC, Western blotting experiments were performed to measure levels of these catecholamine synthetic, packaging, reuptake, and degradation proteins in PFC sub-regions 48 hours following rmTBI. *We hypothesized that rmTBI alters levels of these catecholamine regulatory proteins within the mPFC, ACC, and OFC*.

9.3 Methods

9.3.1 Animals

Twenty-four male and twenty-four female Long-Evans rats were used in this study. Animals were obtained at 5-6 weeks old/100-125g from Charles River Laboratories and underwent the housing, acclimation, and food regulation conditions described in **Chapter 3**.

9.3.2 Surgical and Western Blotting Procedures

At 9-10 weeks of age, rats were randomly assigned to one of three surgical groups: sham (uninjured), single injury (smTBI), or repetitive injury (rmTBI). Animals then underwent the surgical procedures described in **Chapter 3**, using the 2.5mm depth model. Forty-eight hours after their final surgery, anesthetized animals were decapitated, the mPFC, ACC, and OFC were dissected, and tissue underwent the Western blotting procedures described in **Chapter 3**. Briefly, membranes were probed with either rabbit anti-TH (1:1000; MilliporeSigma, Temecula, CA), rabbit anti-VMAT2 (1:1000; Abcam, Waltham, MA), rabbit anti-NET (1:1000; Abcam, Waltham, MA), rabbit anti-COMT (1:1000; ThermoFisher Scientific, Waltham, MA), or rabbit anti-MAO-A (1:1000; ThermoFisher Scientific, Waltham, MA) followed by goat anti-rabbit secondary antibody conjugated with peroxidase (1:10,000; Rockland Immunochemicals, Inc., Limerick, PA). β-actin (1:2000; MilliporeSigma, Temecula, CA) was used as the loading control. Chemiluminescence was detected using Clarity Western ECL substrate (Bio-Rad, Hercules, CA), imaged using Azure c400 Biosystems imaging system (Azure Biosystems,

Dublin, CA), and analyzed using AzureSpot Analysis Software (Azure Biosystems, Dublin, CA).

DBH, DAT, and MAO-B were not evaluated for the following reasons: DBH and DAT – poor/unanalyzable signal; MAO-B – mainly expressed in serotonergic cells and astrocytes, thus not applicable to the current questions asked in this study. These proteins will not be discussed further in this Chapter.

9.3.3 Statistical Analysis

Analysis of righting reflex and Western blotting data were performed using GraphPad Prism software (GraphPad Software, San Diego CA). Male and female righting reflex times were measured separately using two-way repeated measures ANOVAs with surgery day (day 1, day 2, and day 3) as the within-subjects factor and injury condition (sham, smTBI, and rmTBI) as the between-subjects factor. For Western blotting data, individual one-way ANOVAs were used to analyze group differences (sham vs. smTBI vs. rmTBI) in protein levels for each PFC sub-region. This analysis was performed for males and females. Data was excluded only if there were bubbles/imperfections in the band that interfered with signal detection. Therefore, the "n" for all Western blotting data might vary from the total number of animals in each injury group but can be determined by the degrees of freedom provided in the Results section. These exclusion criteria are consistent with previous studies (Ferrario et al., 2011; Loweth et al., 2014; Loweth et al., 2010). Dunnett's multiple comparisons tests, when appropriate, were used to compare individual differences when overall significance was found. For all results, statistical significance was determined by a p value < 0.05.

9.4 Experimental Results

9.4.1 Acute Response to Injury

Immediately following sham injury or mTBI, the latency to regain righting reflex was recorded. For males (**Table 9**), analysis of righting reflex data revealed significant main effects of day [$F (1.464, 30.74) = 9.004$, $p = 0.0021$] and injury [$F (2, 21) = 7.623$, $p = 0.0032$] as well as a significant day x injury interaction [$F (4, 42) = 3.365$, $p = 0.0178$]. Dunnett's multiple comparisons analysis revealed rmTBI rats demonstrated a trend towards longer righting reflex times compared to sham rats on days 1 ($p = 0.0638$), which became significant by day 3 ($p = 0.313$). smTBI rats displayed a trend towards longer righting reflex times on day 2 ($p = 0.0885$), which became significant by day 3 ($p < 0.0001$).

mTBI in females also resulted in longer righting reflex times compared to sham animals across all three surgical days (**Table 9**). Analysis of righting reflex data revealed significant main effects of both day [$F (1.656, 34.77) = 9.168$, $p = 0.0012$] and injury [$F (2, 21) = 4.027$, $p = 0.0331$] as well as a significant day x injury interaction [$F (4, 42) = 7.131$, $p = 0.0002$]. Dunnett's multiple comparisons analysis revealed smTBI rats demonstrated longer righting reflex times compared to sham rats on day 1 ($p = 0.0125$) and a trend towards longer righting reflex times on day 3 ($p = 0.0846$).

Table 9

Righting Reflex Times of Protein Analysis Animals (2.5mm Impact Depth)

Sex	Injury Condition	N	Righting Reflex (sec)		
			Surgery Day 1	Surgery Day 2	Surgery Day 3
Male	sham	8	356.5 ± 74.4	204.6 ± 34.3	219.6 ± 17.2
Female	sham	8	493.8 ± 70.8	319.4 ± 50.3	204.3 ± 43.8
Male	smTBI	8	376.8 ± 38.3	352.4 ± 58.1^	440.0 ± 24.2*
Female	smTBI	8	220.6 ± 43.6*	201.6 ± 26.0	316.3 ± 24.8^
Male	rmTBI	8	562.5 ± 44.2^	317.6 ± 45.2	344.0 ± 39.8*
Female	rmTBI	8	454.0 ± 16.1	365.8 ± 43.5	254.3 ± 46.7

Note. Average righting reflex times (seconds) of male and female sham, single (smTBI), and repetitive (rmTBI) injury groups across all three surgery days. On surgery days 1 and 3, rmTBI males exhibited longer righting reflex times compared to sham animals whereas smTBI males exhibited longer righting reflex times on surgery days 2 and 3. smTBI females demonstrated longer righting reflex times compared to sham animals on surgery days 1 and 3. Values represent mean ± SEM. * denotes $p < 0.05$ and ^ denotes $p < 0.1$ from same-sex shams analyzed with Dunnett's multiple comparisons tests.

9.4.2 Effects of mTBI on TH

In this set of experiments, we examined the effects of TBI on expression of markers associated with catecholamine transmission. Analysis of TH protein levels (**Fig. 30**) within the mPFC and ACC collected from tissue 48 hours after final surgery revealed no significant changes in TH levels in males [$F(2, 18) = 0.170$, $p = 0.8454$; $F(2, 17) = 0.440$, $p = 0.6512$, respectively] or females [$F(2, 21) = 0.415$, $p = 0.6658$; $F(2, 17) = 0.799$, $p = 0.4661$, respectively] following injury. In the OFC, there were no significant changes in TH [$F(2, 20) = 0.945$, $p = 0.4054$] levels in males. However, in the OFC of females, we did observe a significant effect of injury on TH levels [$F(2, 20) = 3.593$, $p = 0.0464$].

Dunnett's multiple comparisons analysis determined that smTBI rats displayed a significant increase in TH levels ($p = 0.0296$) compared to sham rats. Although rmTBI did not cause a statistically significant increase in female OFC TH levels, it is notable that these treatments did alter these levels in the same direction as smTBI, causing an 89.5% increase relative to shams.

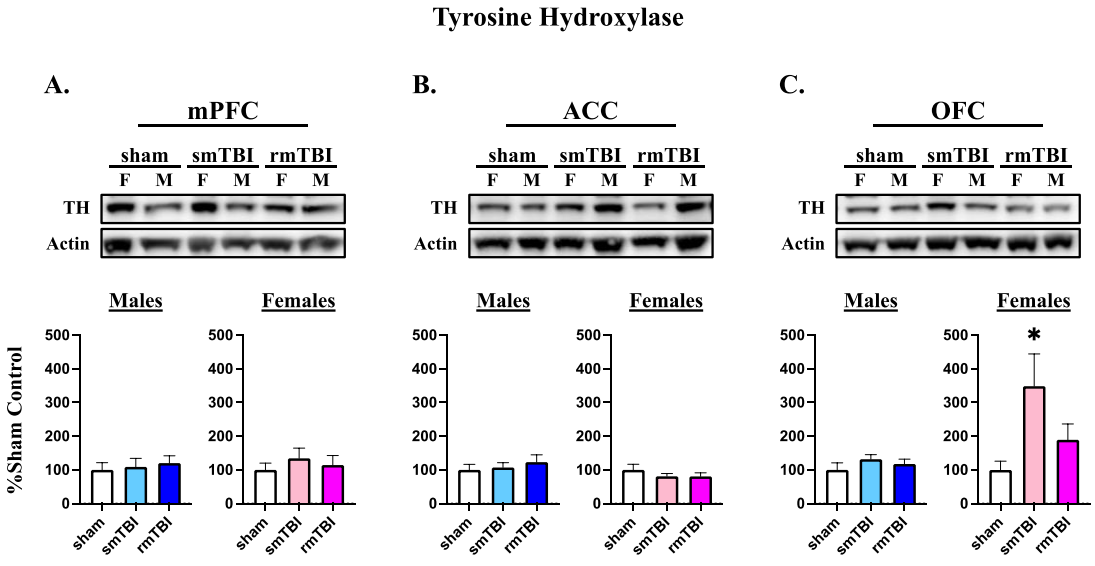


Figure 30. Post-surgery tyrosine hydroxylase levels in subregions of the PFC (2.5mm impact depth). Male and female levels of tyrosine hydroxylase (TH) in the A) medial prefrontal cortex, B) anterior cingulate cortex, and C) orbitofrontal cortex. Graphs represent percent total protein levels at 48 hours post-final surgery. No differences in TH levels were found between sham, single (smTBI), or repetitive (rmTBI) injury groups in the mPFC or ACC of either sex. In the OFC, smTBI significantly increased TH levels in females only. Bars represent mean ± SEM. * denotes $p < 0.05$ from sham analyzed with Dunnett's Multiple Comparisons Tests.

9.4.3 Effects of mTBI on VMAT

Analysis of VMAT protein levels (**Fig. 31**) within the mPFC, ACC, and OFC revealed no significant changes in VMAT levels in males [$F (2, 17) = 3.100$, $p = 0.0711$; F

(2, 16) = 1.215, p = 0.3226; F (2, 14) = 0.4932, p = 0.6209, respectively] or females [F (2, 18) = 1.312, p = 0.2937; F (2, 17) = 0.4513, p =0.6442; F (2, 17) = 1.193, p = 0.3275, respectively] following mTBI.

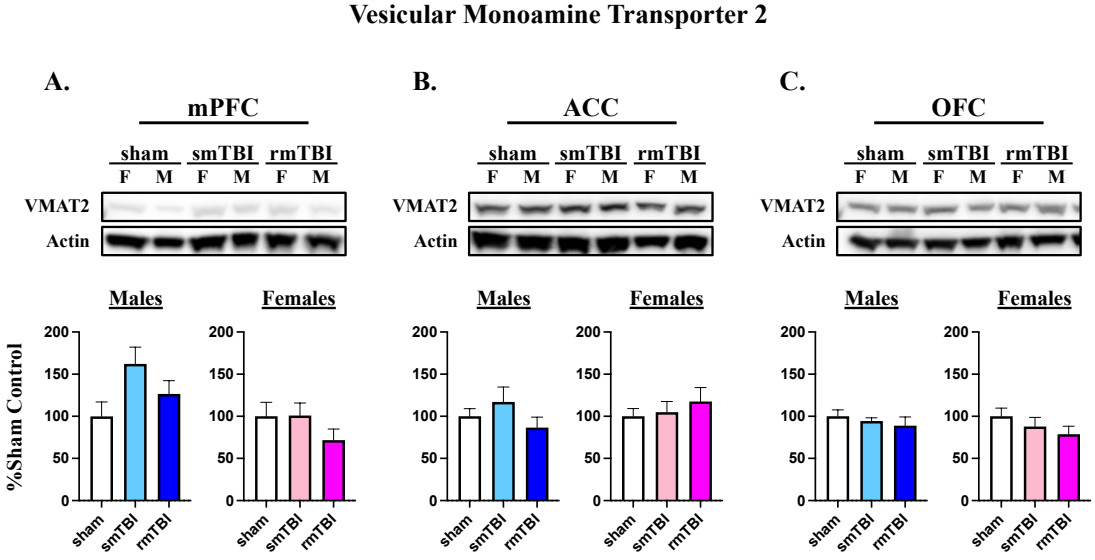


Figure 31. Post-surgery vesicular monoamine transporter 2 levels in subregions of the PFC (2.5mm impact depth). Male and female levels of vesicular monoamine transporter 2 (VMAT2) in the A) medial prefrontal cortex, B) anterior cingulate cortex, and C) orbitofrontal cortex. Graphs represent percent total protein levels at 48 hours post-final surgery. No differences in VMAT2 levels were found between sham, single (smTBI), or repetitive (rmTBI) injury groups in the mPFC, ACC, or OFC of

9.4.4 Effects of mTBI on NET

Analysis of NET protein levels (**Fig. 32**) within the mPFC and ACC revealed TBI caused no significant changes in NET levels in males [F (2, 19) = 1.166, p = 0.3328; F (2,19) = 0.368, p = 0.6971, respectively] or females [F (2,19) = 0.558, p = 0.5814; F (2,21) = 0.545, p = 0.5879, respectively]. On the other hand, in the OFC, there was a significant

effect of injury on NET levels that was apparent in both males [F (2, 12) = 3.953, p = 0.0480] and females [F (2, 17) = 3.854, p = 0.0417]. Dunnett's multiple comparisons determined that rmTBI induced a significant decrease in NET ($p < 0.05$) levels compared to sham rats.

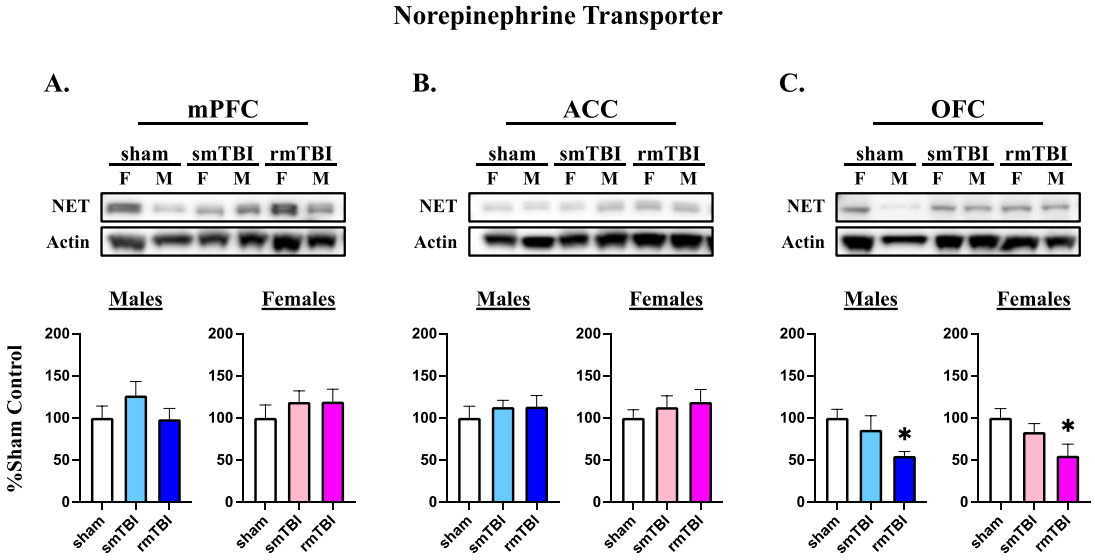


Figure 32. Post-surgery norepinephrine transporter levels in subregions of the PFC (2.5mm impact depth). Male and female levels of norepinephrine transporter (NET) in the A) medial prefrontal cortex, B) anterior cingulate cortex, and C) orbitofrontal cortex. Graphs represent percent total protein levels at 48 hours post-final surgery. No differences in NET levels were found between sham, single (smTBI), or repetitive (rmTBI) injury groups in the mPFC or ACC of either sex. In the OFC, rmTBI significantly reduced NET levels in both males and females. Bars represent mean ± SEM. * denotes $p < 0.05$ from sham analyzed with Dunnett's Multiple Comparisons Tests.

9.4.5 Effects of mTBI on COMT

Analysis of COMT protein levels (**Fig. 33**) within the mPFC, ACC, and OFC revealed no significant changes in COMT levels in males [F (2, 12) = 2.116, p = 0.1633; F (2, 12) = 2.179, p = 0.1559; F (2, 21) = 0.3381, p = 0.7169, respectively] or females [F (2,

16) = 1.015, p = 0.3847; F (2, 21) = 0.5985, p = 0.5587; F (2, 13) = 1.523, p = 0.2546, respectively] following mTBI.

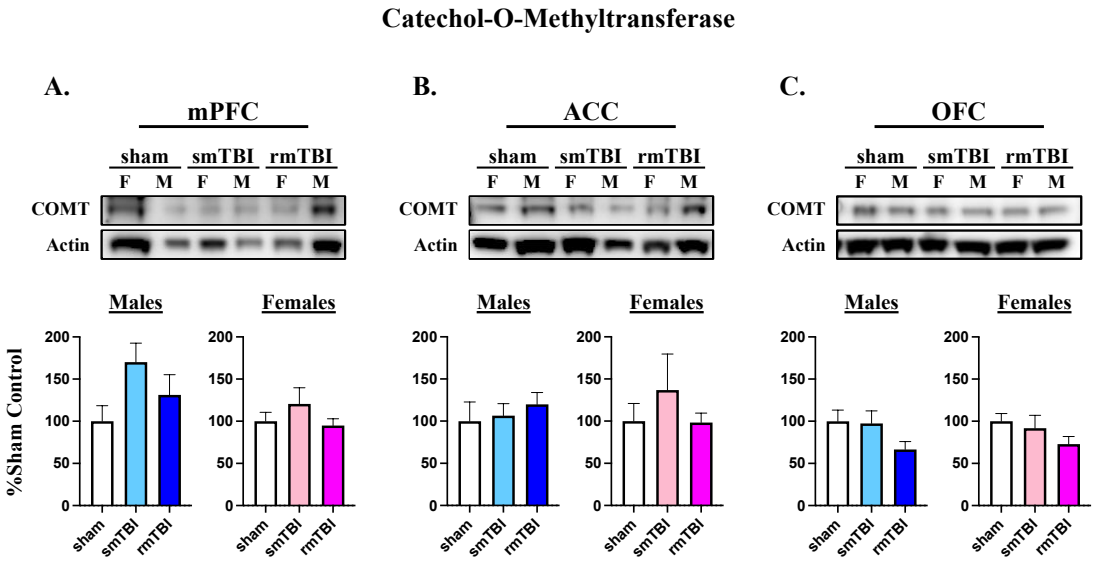


Figure 33. Post-surgery catechol-O-methyltransferase levels in subregions of the PFC (2.5mm impact depth). Male and female levels of catechol-O-methyltransferase (COMT) in the A) medial prefrontal cortex, B) anterior cingulate cortex, and C) orbitofrontal cortex. Graphs represent percent total protein levels at 48 hours post-final surgery. No differences in COMT levels were found between sham, single (smTBI), or repetitive (rmTBI) injury groups in the mPFC, ACC, or OFC of either

9.4.6 Effects of mTBI on MAO-A

Analysis of MAO-A protein levels (**Fig. 34**) within the mPFC, ACC, and OFC revealed no significant changes in MAO-A levels in males [F (2, 14) = 0.1990, p = 0.8219; F (2, 17) = 0.1056, p = 0.9004; F (2, 16) = 0.3018, p = 0.7436, respectively] or females [F (2, 14) = 1.396, p = 0.2801; F (2, 13) = 1.843, p = 0.1974; F (2, 16) = 1.712, p = 0.2120, respectively] following mTBI.

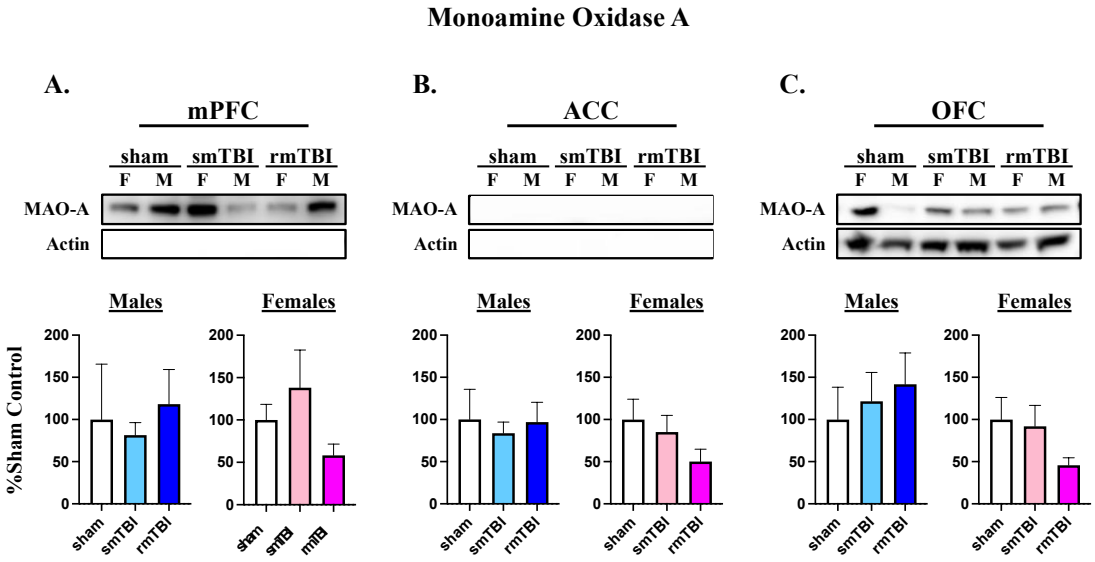


Figure 34. Post-surgery monoamine oxidase-A levels in subregions of the PFC (2.5mm impact depth). Male and female levels of monoamine oxidase-A (MAO-A) in the A) medial prefrontal cortex, B) anterior cingulate cortex, and C) orbitofrontal cortex. Graphs represent percent total protein levels at 48 hours post-final surgery. No differences in MAO-A levels were found between sham, single (smTBI), or repetitive (rmTBI) injury groups in the mPFC, ACC, or OFC of either sex. Bars represent mean ± SEM.

9.5 Discussion

The present study investigated the effects of rmTBI on catecholamine synthetic, packaging, reuptake transporter, and degradation proteins within prefrontal regions that mediate risk/reward decision making processes. In the OFC, smTBI produced dramatic increases in TH within female, but not male, rats. While not statistically significant, rmTBI produced a roughly 90% increase in TH expression in females only. rmTBI also significantly reduced levels of NET in the OFC of both males and females. These results suggest that the OFC may be more susceptible to catecholamine dysregulation following repetitive mild head injuries.

Previous studies have demonstrated increased TH and decreased DAT within the PFC and striatum following experimental TBI (Wagner, Sokoloski, et al., 2005; Yan et al., 2001; Yan et al., 2002). During impact, mechanical forces result in neuronal hyperexcitability and the opening of calcium channels, which enhances the activity of TH (Lan et al., 2019; Louin et al., 2004). This activation of TH accelerates catecholamine synthesis, leading to the accumulation of large amounts of DA and NE within the tissue. This "catecholamine storm" is typically short-lived, often occurring immediately after injury and lasting for up to twenty-four hours in some brain regions (Huger & Patrick, 1979; Kawa et al., 2015; Massucci et al., 2004; McIntosh et al., 1994). In areas such as the striatum, this dramatic increase is followed by a hypo-dopaminergic state, which has been observed as long as two weeks following experimental TBI (Wagner, Sokoloski, et al., 2005). In comparison, TBI induces a different pattern of alterations in post-injury catecholamine levels within the PFC. Increased TH activity and catecholamine levels have been observed in the mPFC of male rats up to two weeks following TBI (Kobori et al., 2006). At four weeks, the same study noted that TH activity within the mPFC was reduced, suggesting that the PFC might not follow the traditional pattern of a brief catecholamine storm followed by a lasting depression of catecholamine synthesis. In response to this sustained increase in DA and NE synthesis, downregulation of transporter expression may serve as a compensatory mechanism to prevent overaccumulation of intracellular catecholamines. Increased cytosolic DA, in particular, can result in oxidative stress, neurotoxicity, and eventual death of catecholaminergic cells (Graham et al., 1978; Masoud et al., 2015; Mosharov et al., 2009).

Interestingly, rmTBI's effects on TH were less robust than those of smTBI. Catecholaminergic neurons exposed to repetitive injury may not have sufficient stores of DA and NE by the third impact to mount another storm of the same magnitude produced by the first injury. Nevertheless, this increased expression of TH at 48 hours post-injury suggests that the PFC continues to experience an elevated catecholaminergic state following rmTBI, leading to a decrease in NET as a compensatory response to increased intracellular levels of DA and NE. This may also explain why there is a lack of change in NET levels following single injury. smTBI rats experienced only 48 hours of catecholaminergic disruption before regulatory protein levels were assessed, whereas rmTBI rats experienced eight days of irregular catecholamine activity from the first injury to 48 hours post the final impact. Therefore, reduced NET may be both a time-dependent response to increased TH as well as a rmTBI-driven response to maintain catecholamine homeostasis following prolonged exposure to catecholamine instability. The lack of changes in male TH expression at 48 hours post mTBI could suggest that males experience a shorter duration of elevated TH that subsides prior to our selected timepoint. Following rmTBI, it is possible that while levels of TH are no longer elevated 48 hours post-injury, the TH-induced increases of DA and NE may still linger resulting in the observed decreases in NET expression in male rats.

The decrease in OFC NET expression induced by TBI would be expected to result in enhanced NE levels within this region. With this in mind, it is interesting to note that enhancing NE transmission with systemic treatment of the α2 antagonist yohimbine increased risky choice in a manner comparable to the effects reported in **Chapter 6**, using a version of the PDT similar to that used by our laboratory (Montes et al., 2015). Although

the specific neural locus where pharmacological enhancements in NE activity may promote risky choice remains to be clarified, the similarity of effects observed this previous study, and the current results allude to the possibility that abnormal increases in OFC NE transmission may contribute to alterations in risk/reward decision making induced by TBI.

Contrary to our expectations, we did not observe significant changes in VMAT2, COMT, or MAO-A levels in the OFC; however, visual inspection of these data suggest that rmTBI may be altering MAO-A expression. Levels of MAO-A were noticeably reduced in female rats, suggestive of decreased degradation of cytosolic DA and NE. These reductions appear to be present across all three prefrontal regions despite lack of statistical significance. In humans, a recent study found that females have significantly higher MAO-A gene expression levels than males in many brain regions, including the PFC (Sanfilippo et al., 2021). An in-depth protein study would be required to confirm if MAO protein expression correlates with RNA expression mentioned in that study; however, it is possible that females may be more suspectable to injury-induced decreases in MAO-A levels following TBI. As such, this sex-specific decrease of MAO-A serves as a novel variable to further elucidate differential rmTBI-induced mechanisms of increased risk preference.

The lack of significant changes in protein expression levels in the mPFC and ACC were initially surprising, given that TBI-induced alterations in catecholaminergic activity have been reported in the mPFC (Kobori et al., 2006). While visual inspection of the data suggests that there may be potential alterations of MAO-A in these regions, we cannot make any definitive conclusions at the present moment. One explanation could be that the injury parameters used in these experiments (2.5mm depth and 5.5 m/s velocity) were sub-threshold for altering catecholamine regulation in the mPFC and ACC regions. The

previously mentioned study of increased TH activity and levels of catecholamines in the mPFC was performed using an open skull CCI model (Kobori et al., 2006). Open-skull models of TBI often induce a more severe injury than a closed skull which can lead to increased detection of TBI-induced effects; however, these models are less suitable for repetitive TBI studies. Increasing our injury parameters to induce a stronger form of rmTBI may allow us to detect changes in levels of catecholamine regulatory proteins within these sub-regions. Nevertheless, this injury model has proved effective in revealing the susceptibility of the OFC to catecholamine dysregulation following TBI.

9.6 Conclusion

By evaluating specific subregions of the PFC, we determined that the OFC is more susceptible to catecholamine instability after rmTBI, a finding indicating that not all areas of the PFC contribute equally to the observed TBI-induced catecholamine imbalances. This observation has not been previously reported and reveals new avenues for exploration into the neurochemical changes within the OFC following repeated head trauma. These studies also identified individual catecholamine associated proteins that were more affected by rmTBI, thus revealing novel targets for future therapeutic strategies.

Chapter 10

The Impact of Repetitive Mild Traumatic Brain Injury on Noradrenergic Innervation in the Orbitofrontal Cortex

10.1 Introduction

The experiments conducted in **Chapter 9** revealed reduced levels of norepinephrine transporter (NET) within the orbitofrontal cortex (OFC) following repetitive mild traumatic brain injury (rmTBI). While we speculated that these decreases were in response to prolonged exposure to high amounts of catecholamines caused by TBI-induced increases in tyrosine hydroxylase (TH), we cannot rule out the possibility that these changes may also be due to loss of noradrenergic fibers within the OFC. Reduced noradrenergic innervation within the prefrontal cortex (PFC) would impact higher order executive functions that guide risk/reward decision-making. These alterations might be responsible for the changes in choice behavior observed in the probabilistic discounting task (PDT). Thus, the goal of this Chapter was to elucidate the injury-induced mechanisms behind our observed decreases in NET to determine whether these changes are compensatory or the result of reduced fiber presence.

10.2 Rationale

In cases of mTBI, axonal injuries are most commonly caused by intra-axonal changes that occur within the first 12 hours after impact (Christman et al., 1994; Grady et al., 1993; Povlishock et al., 1983; Povlishock & Christman, 1995). Following injury, misalignment of the cytoskeletal network results in impaired anterograde axoplasmic

transport, leading to axonal swelling and eventual disconnection. As a result, downstream synaptic fields are lost and damaged axons are eventually engulfed by reactive glia. Surviving fibers may undergo adaptive plasticity, leading to eventual synaptic recovery (Erb & Povlishock, 1991); however, this process often takes several months. Following the results of **Chapter 9**, it remained unclear whether our observed changes in noradrenergic innervation were caused by a reduction in NET protein within existing fibers or if the fibers themselves were damaged or lost. Moreover, it is unclear how long these potential changes in fiber density last. Given that we only observed transient increases in risky choice within our milder (2.5mm depth) rmTBI model, it is possible that these potential changes to NE innervation may also be short-lived. Thus, our objective was to conduct a fiber analysis study to investigate the potential changes in NE fiber density within the OFC after rmTBI and to determine how long these changes last. *We hypothesized that rmTBI reduces NE fiber density in the OFC, but that these effects are transient similar to the behavioral changes observed in the PDT.*

10.3 Methods

10.3.1 Animals and Surgery

Fifteen male and sixteen female Long-Evans rats were used in this study. Animals were obtained at 5-6 weeks old/100-125g from Envigo Laboratories and underwent the housing, acclimation, and food regulation conditions described in **Chapter 3**. Rats were randomly assigned to one of two surgical groups at 9-10 weeks of age: sham or rmTBI (n = 3-4 per group), and underwent the surgical procedures described in **Chapter 3**.

10.3.2 Immunohistochemistry

At 48 hours and 4 weeks post-final surgery, rats underwent the immunohistochemistry procedures described in **Chapter 3**. Briefly, tissue containing the OFC was probed with mouse anti-DBH (1:2000, MilliporeSigma, Temecula, CA) primary antibody followed by Alexafluor 488 donkey anti-mouse (1:250, Invitrogen, Waltham, MA) secondary antibody. Images of the OFC [3-4 tissue sections per animal; coordinates: 4.20mm to 3.24mm from bregma (Paxinos & Watson, 2007)] were taken at 20X using a Keyence BZ-X710 microscope and processed with Image J software. Briefly, the Hessian feature was used to compute the eigenvalues of each image element (pixel/vowel), which were subsequently used to quantify the density of DBH^+ fibers. The threshold feature was used to identify DBH^+ fibers within each image, which were then measured using the analyze feature. The percent area of DBH^+ fibers in each tissue section was then averaged together for each rat.

10.3.3 Statistical Analysis

All data analysis was performed using GraphPad Prism software (GraphPad Software, San Diego CA). Male and female righting reflex times were measured separately using two-way repeated measures ANOVAs with surgery day (day 1, day 2, and day 3) as the within-subjects factor and injury condition (sham and rmTBI) as the between-subjects factor. Fiber density was analyzed using a three-way ANOVA with injury condition (sham and rmTBI), time (week 1 and week 4), and sex (male and female) as the between-subjects factors. Males and females were then combined in an additional analysis using an ordinary two-way ANOVA with injury condition (sham and rmTBI) and time (week 1 and week 4)

as the between-subjects factors. Sidak's multiple comparisons tests, when appropriate, were used to compare individual differences when overall significance was found. For all results, statistical significance was determined by a p value < 0.05.

10.4 Experimental Results

10.4.1 Acute Response to Injury

rmTBI in male rats resulted in longer righting reflex times compared to those of sham animals (**Table 10**). Analysis of righting reflex data failed to reveal a main effect of day [$F (1.671, 21.73) = 1.137$, $p = 0.3299$], but did reveal a significant main effect of injury [$F (1, 13) = 12.26$, $p = 0.0039$] as well as a significant day x injury interaction [$F (2, 26) = 4.860$, $p = 0.0161$]. Sidak's multiple comparisons analysis revealed that rmTBI rats demonstrated longer righting reflex times compared to sham rats on day 1 ($p = 0.034$).

rmTBI in female rats also resulted in longer righting reflex times compared to those of sham animals (**Table 10**). Analysis of righting reflex data revealed significant main effects of both day [$F (1.878, 26.29) = 9.855$, $p = 0.0008$] and injury [$F (1, 14) = 14.99$, $p = 0.0017$] as well as a significant day x injury interaction [$F (2, 28) = 5.350$, $p = 0.0108$]. Sidak's multiple comparisons analysis revealed rmTBI rats demonstrated longer righting reflex times compared to sham rats on day 1 ($p = 0.0063$).

Table 10

Righting Reflex Times of Fiber Analysis Animals (2.5mm Impact Depth)

Sex	Injury Condition	N	Righting Reflex (sec)		
			Surgery Day 1	Surgery Day 2	Surgery Day 3
Male	sham	8	107.3 ± 12.5	160.3 ± 13.6	144.3 ± 16.8
Female	sham	8	150.1 ± 24.0	124.4 ± 25.2	117.4 ± 13.7
Male	rmTBI	7	328.6 ± 40.5*	252.3 ± 53.4	214.6 ± 49.7
Female	rmTBI	8	383.0 ± 51.1*	228.3 ± 44.4	162.3 ± 26.7

Note. Average righting reflex times (seconds) of sham and rmTBI injury groups across all three surgery days. On surgery day 1, rmTBI males and females demonstrated longer righting reflex times compared to male and female sham animals, respectively. No differences in righting reflex times were observed on surgery days 2 and 3. Values represent mean ± SEM. * denotes $p < 0.05$ from shams analyzed with Sidak's multiple comparisons tests.

10.4.2 Effects of rmTBI on DBH

At 48-hours post-injury (**Fig. 35** and **36**), analysis of DBH fiber density failed to reveal a main effect of injury [$F(1, 23) = 0.03288$, $p = 0.8577$], time [$F(1, 23) = 0.4679$, $p = 0.5008$), sex [$F(1, 23) = 0.7964$, $p = 0.3814$], or injury x sex interaction [$F(1, 23) = 2.204$, $p = 0.1512$]. Additionally, there were no other interactions of time x injury, time x sex, and time x injury x sex ($p > 0.05$).

When male and female data were combined (**Fig. 34** and **35**), analysis of DBH fiber density failed to reveal a main effect of injury [$F(1, 27) = 0.1003$, $p = 0.7539$], time [$F(1, 27) = 0.6343$, $p = 0.4327$), or injury x time interaction [$F(1, 27) = 0.08913$, $p = 0.7676$). Taken together, rmTBI does not alter NE fiber density within the OFC.

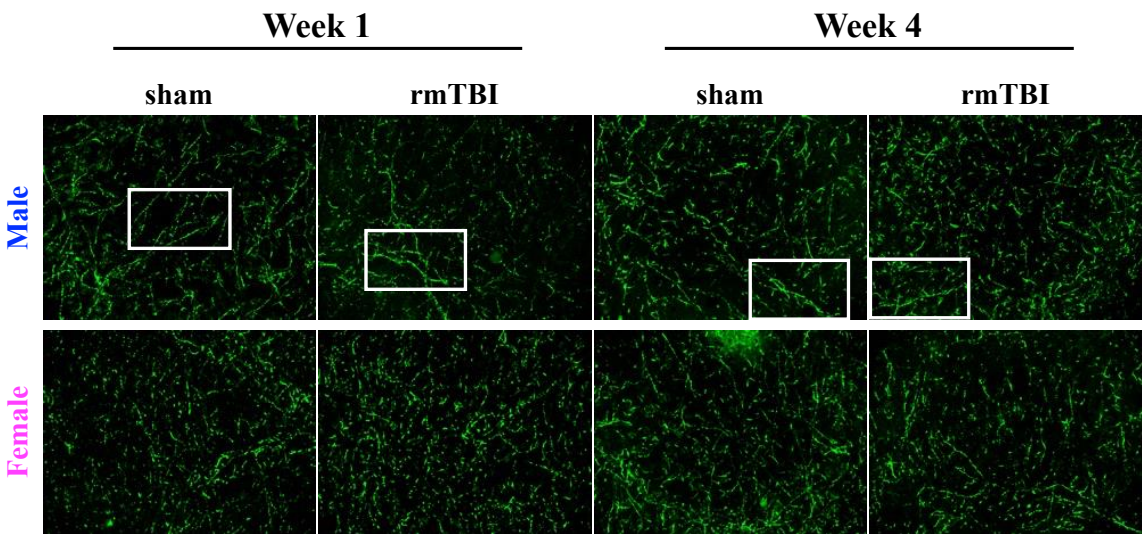


Figure 35. Immunofluorescent imaging of post-surgery dopamine β-hydroxylase fiber density. Immunohistochemical staining of dopamine β-hydroxylase (DBH; green) expressing fibers in the orbitofrontal cortex (OFC) of sham and repetitive mild traumatic brain injury (rmTBI) male and female rats at weeks 1 and 4 post-final injury. Images are captured at 20x magnification. Rectangles are zoomed in for subsequent figure. No differences in DBH fiber density were found between surgical groups, sex, or timepoints after injury.

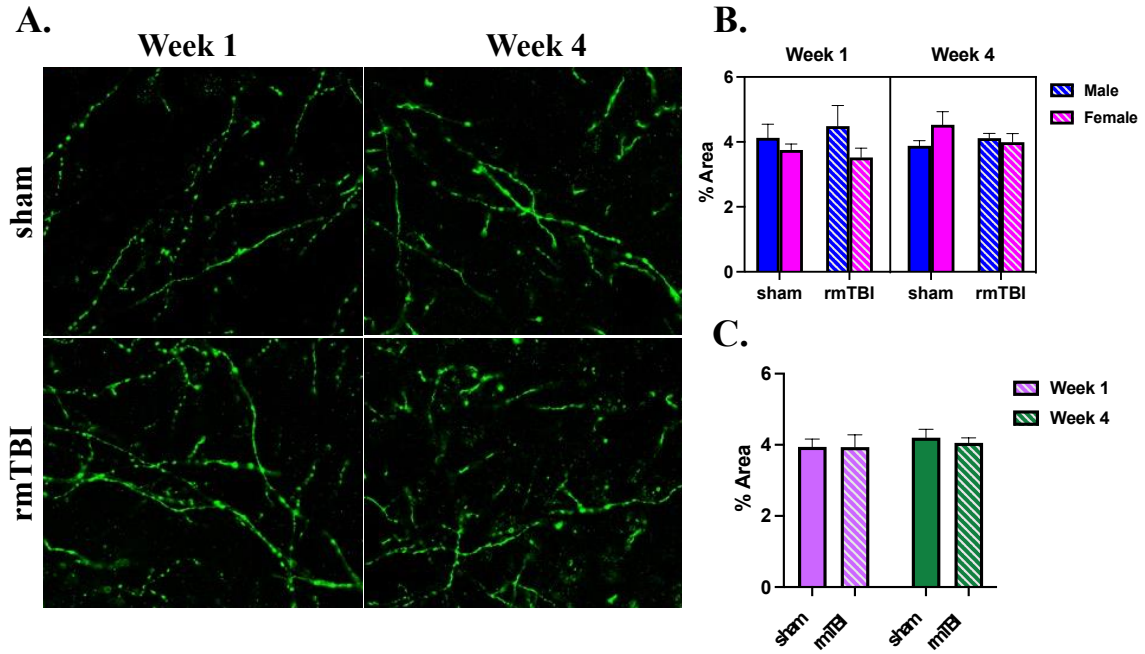


Figure 36. Quantification of post-surgery dopamine β-hydroxylase fiber density. A) Immunohistochemical staining of dopamine β-hydroxylase (DBH; green) expressing fibers in the orbitofrontal cortex (OFC) of sham and repetitive mild traumatic brain injury (rmTBI) rats at weeks 1 and 4 post-final injury. B) Bar graphs represent quantification of DBH expressing fibers within the OFC. No differences in DBH fiber density were found between surgical groups, sex, or timepoints after injury. C) Bar graphs represent a combined male and female analysis of DBH expressing fibers within the OFC. No differences in DBH fiber density were found between surgical groups at weeks 1 and 4 after injury. Bars represent mean ± SEM.

10.5 Discussion

Using immunohistochemistry, the present study evaluated whether the reductions in NET protein levels observed in **Chapter 9** were the result of damaged or absent fibers in the OFC following rmTBI. No differences in DBH-positive fiber density were observed between sham and rmTBI animals within or between weeks 1 and 4 post-final injury. Additionally, no sex differences were observed. Together, these results indicate that the fibers within the OFC are still present after rmTBI, suggesting that the previously demonstrated decreases in NET are most likely due to less NET protein available within the fibers rather than loss or damage to the DBH fibers, themselves.

These results are consistent with previous studies that have also found no changes in DBH expression in the PFC following experimental TBI (Schmidt & Grady, 1995; Yan et al., 2001). Considering the OFC's location in relation to the impact site of our rmTBI model, noradrenergic fibers in this region may be too distal to experience direct trauma from these injuries. As such, the results of this study add support to our proposed theory that reduced NET may be a compensatory response to maintain catecholamine homeostasis following prolonged exposure to catecholamine instability.

Future research is warranted to further investigate whether the compensatory actions of NET following rmTBI persist beyond 48 hours post-final injury. Moreover, the effects of rmTBI on dopamine transporter (DAT) levels have yet to be determined. In the experiments conducted in this dissertation, we were unable to detect DAT protein within any prefrontal sub-region. Expression of DAT is relatively low in the PFC (Sesack et al., 1998) and likely even lower within individual sub-regions. A more sensitive method for detecting low-abundance proteins like an Enzyme-Linked Immunosorbent Assay (ELISA) may be an alternative approach for future studies. Nevertheless, the findings obtained in this study, along with those described in **Chapter 9**, provide a better understanding of how the brain invokes compensatory actions via the NE transporter to recover and maintain optimal catecholaminergic transmission following rmTBI.

10.6 Conclusion

Overall, there was no evidence of reduced DBH fiber presence in the OFC following rmTBI at either the 48 hour or 4 week timepoints. This information suggests that the observed decreases in OFC NET are the result of there being less NET protein available within existing fibers. Future research is required to determine the duration of these changes in NET expression and whether the DAT also experiences similar reductions following rmTBI.

Chapter 11

Evaluating the Effects of Repetitive Mild Traumatic Brain Injury of Increased Depth on Catecholamine Regulatory Proteins within the Prefrontal Sub-Regions

11.1 Introduction

As discussed in **Chapters 9** and **10** of this dissertation, there is strong evidence of mild traumatic brain injury (mTBI)-induced disruptions to catecholamine transmitter regulation within specific prefrontal (PFC) subregions 48 hours after injury, specifically in the orbitofrontal cortex (OFC). These findings begin to suggest that the OFC may be a primary focal point of TBI-induced catecholamine imbalances within the PFC. Levels of tyrosine hydroxylase (TH) and norepinephrine transporter (NET) were significantly altered in the OFC following mTBI, i.e. smTBI dramatically increased TH levels in females only, while rmTBI decreased NET levels in both sexes. However, we additionally observed that levels of monoamine oxidase-A (MAO-A), an enzyme responsible for degrading cytosolic dopamine (DA) and norepinephrine (NE) in catecholaminergic neurons, were also noticeably reduced within the medial (mPFC), anterior cingulate (ACC), and orbitofrontal regions of the PFC. These observations, along with those observed in the probabilistic discounting task (PDT) following mTBI at a 2.5mm depth, warranted suspicion that our current injury parameters may have been sub-threshold for revealing significant alterations in protein levels in these regions, particularly the mPFC and ACC. In **Chapter 7**, we modified the parameters of our injury model of rmTBI to allow improved detection of these potential effects and solidify those produced by our milder 2.5mm depth model. As shown in **Chapter 8**, our 3.5mm depth injury model succeeded in revealing effects of repetitive

mTBI (rmTBI) on risky choice that were previously shy of significance in our milder model. The goal of this Chapter was to see whether this 3.5mm depth injury model would also amplify the effects of mTBI on levels of catecholamine regulatory proteins observed in **Chapter 9**.

11.2 Rationale

In **Chapter 9**, we observed changes in catecholamine associated regulatory protein levels that suggest increased catecholamine synthesis along with decreased clearance and degradation. These findings suggest greater availability of catecholamines within the PFC following mTBI. We suspected that these changes are responsible for driving the increased risky choice behavior observed in the PDT. Here, we aim to see whether these effects are magnified following a stronger mTBI. If they are, then this would strengthen our idea that there is a correlation between increased DA and NE presence in the PFC and increased risk/reward decision making behavior. We also aim to determine whether these changes remain relatively OFC-specific. If they are, this would reinforce our hypothesis that the OFC may be the primary contributor of TBI-induced catecholamine imbalances observed within the PFC. If we begin detecting significant changes in protein levels within the mPFC and/or ACC, this may indicate that the OFC is more susceptible to milder injuries but that greater impact force is needed in order to disrupt catecholamine balance within the mPFC and ACC.

As observed in **Chapter 9**, the effects of smTBI on TH levels were female-specific while the effects of rmTBI on NET levels were observed in both sexes. If we are able to detect additional changes in catecholamine associated protein levels within males

following a 3.5mm depth injury, this would confirm our suspicion that our previous injury parameters may have been sub-threshold for producing effects in males. However, if we do not observe any changes in protein levels following this deeper depth injury, this may indicate that TBIs of mild severity are not sufficient to further disrupt catecholamine activity within males. If catecholamine associated protein levels within the PFC of males change, but are not correlated with behavior assessed with the PDT, this could suggest that male risk preference may not be as reliant on the PFC and that other brain regions might have a more prominent role in male risk/reward decision making. As such, the focus of **Chapter 11** was to evaluate the effects of mTBIs of increased strength (3.5mm depth) on levels of catecholamine regulatory proteins in the mPFC, ACC, and OFC. *We hypothesized that rmTBI of increased depth produces greater changes in catecholamine regulatory protein levels in the OFC of females and produces more detectable changes in males. We further hypothesized that these stronger impacts (3.5mm depth) would enable detection of potential changes in the mPFC and ACC regions*.

11.3 Methods

11.3.1 Animals

Thirty-five male and thirty-six female Long-Evans rats were used in this study. Animals were obtained at 5-6 weeks old/100-125g from Envigo Laboratories and underwent the housing, acclimation, and food regulation conditions described in **Chapter 3**.

11.3.2 Surgical and Western Blotting Procedures

At 9-10 weeks of age, rats were randomly assigned to one of three surgical groups: sham (uninjured), single injury (smTBI), or repetitive injury (rmTBI). Animals then underwent the surgical procedures described in **Chapter 3**, using the 3.5mm depth model. Forty-eight hours after their final surgery, anesthetized animals were decapitated, the mPFC, ACC, and OFC were dissected, and tissue underwent the Western blotting procedures described in **Chapters 3 and 9**. As described in **Chapter 9**, membranes were probed with either rabbit anti-TH (1:1000; MilliporeSigma, Temecula, CA), rabbit anti-VMAT2 (1:1000; Abcam, Waltham, MA), rabbit anti-NET (1:1000; Abcam, Waltham, MA), rabbit anti-COMT (1:1000; ThermoFisher Scientific, Waltham, MA), or rabbit anti-MAO-A (1:1000; ThermoFisher Scientific, Waltham, MA), followed by goat anti-rabbit secondary antibody conjugated with peroxidase (1:10,000; Rockland Immunochemicals, Inc., Limerick, PA). β-actin (1:2000; MilliporeSigma, Temecula, CA) was used as the loading control. Chemiluminescence was detected using Clarity Western ECL substrate (Bio-Rad, Hercules, CA), imaged using Azure c400 Biosystems imaging system (Azure Biosystems, Dublin, CA), and analyzed using AzureSpot Analysis Software (Azure Biosystems, Dublin, CA). DBH, DAT, and MAO-B were not evaluated and will not be discussed further in this Chapter.

11.3.3 Statistical Analysis

The statistical analysis procedures performed in this Chapter were identical to those performed in **Chapter 9**. Analysis of righting reflex and Western blotting data were performed using GraphPad Prism software (GraphPad Software, San Diego CA). Male and

female righting reflex times were measured separately using two-way repeated measures ANOVAs with surgery day (day 1, day 2, and day 3) as the within-subjects factor and injury condition (sham, smTBI, and rmTBI) as the between-subjects factor. For Western blotting data, individual one-way ANOVAs were used to analyze group differences (sham vs. smTBI vs. rmTBI) in protein levels for each PFC sub-region. This analysis was performed for males and females. For data sets that were not normally distributed (i.e., male and female OFC – TH, and male and female ACC – VMAT2), data were subjected to Kruskal-Wallis tests. Data was excluded only if there were bubbles/imperfections in the band that interfered with signal detection. Dunnett's or Dunn's multiple comparisons tests, when appropriate, were used to compare individual differences when overall significance was found. For all results, statistical significance was determined by a p value < 0.05.

11.4 Experimental Results

11.4.1 Acute Response to Injury

Immediately following sham injury or mTBI, the latency to regain righting reflex was recorded. rmTBI in male rats resulted in longer righting reflex times compared to sham animals across all three surgical days (**Table 11**). Analysis of righting reflex data revealed significant main effects of both day [$F(1.768, 56.59) = 9.563$, $p = 0.0004$] and injury [$F(2, 32) = 56.99$, $p < 0.0001$] as well as a significant day x injury interaction [$F(4, 64) = 12.49$, $p < 0.0001$]. Dunnett's multiple comparisons analysis revealed that rmTBI rats demonstrated longer righting reflex times compared to sham rats on days 1 ($p < 0.0001$), 2 ($p < 0.0001$), and 3 ($p < 0.0001$) whereas smTBI rats displayed longer righting reflex times on day 3 ($p < 0.0001$).

mTBI in female rats also resulted in longer righting reflex times compared to sham animals across all three surgical days. Analysis of righting reflex data failed to reveal a significant main effect of day [$F (1.946, 64.23) = 0.5817$, $p = 0.5574$], but did reveal a significant main effect and injury [$F (2, 33) = 48.83$, $p < 0.0001$] as well as a significant day x injury interaction [$F (4, 66) = 19.48$, $p < 0.0001$]. Dunnett's multiple comparisons analysis revealed rmTBI rats demonstrated longer righting reflex times compared to sham rats on days 1 ($p < 0.0001$), 2 ($p < 0.0001$), and 3 ($p = 0.0259$) whereas smTBI rats displayed longer righting reflex times on day 3 ($p < 0.0001$).

Table 11

Righting Reflex Times of Protein Analysis Animals (3.5mm Impact Depth)

Sex	Injury Condition	N	Righting Reflex (sec)		
			Surgery Day 1	Surgery Day 2	Surgery Day 3
Male	sham	12	144.1 ± 13.1	148.4 ± 19.2	182.1 ± 19.9
Female	sham	12	195.4 ± 15.7	190.5 ± 10.7	202.8 ± 19.8
Male	smTBI	10	170.0 ± 21.9	168.2 ± 29.6	352.5 ± 07.6*
Female	smTBI	13	174.6 ± 15.1	188.3 ± 23.5	347.1 ± 16.7*
Male	rmTBI	13	361.1 ± 25.3*	354.8 ± 17.4*	311.8 ± 16.2*
Female	rmTBI	11	414.7 ± 24.6*	398.7 ± 31.1*	275.5 ± 18.6*

Note. Average righting reflex times (seconds) of male and female sham, single (smTBI), and repetitive (rmTBI) injury groups across all three surgery days. Across surgery days, rmTBI males and females exhibited longer righting reflex times compared to their respective sham groups. On surgery day 3, smTBI males and females demonstrated longer righting reflex times compared to their respective sham groups. Values represent mean ± SEM. * denotes $p < 0.05$ from shams analyzed with Dunnett's multiple comparisons tests.

11.4.2 Effects of mTBI on TH

In this set of experiments, we examined the effects of TBI on expression of markers associated with catecholamine transmission. Analysis of TH protein levels (**Fig. 37**) within the mPFC and ACC collected from tissue 48 hours after final surgery revealed no significant changes in TH levels in males [F (2, 21) = 0.4847, p = 0.6226; F (2, 21) = 0.5621, p = 0.5784, respectively] or females [F (2, 21) = 1.344, p = 0.2824; F (2, 21) = 1.333, p = 0.2852, respectively] following injury. In the OFC, there were no significant changes in TH [H (2) = 0.02968, p = 0.9877] levels in males. However, in the OFC of females, we did observe a significant effect of injury on TH levels [H (2) = 10.72, p = 0.0015]. Dunn's multiple comparisons analysis determined that rmTBI rats displayed a significant increase in TH levels (p = 0.0437) compared to sham rats.

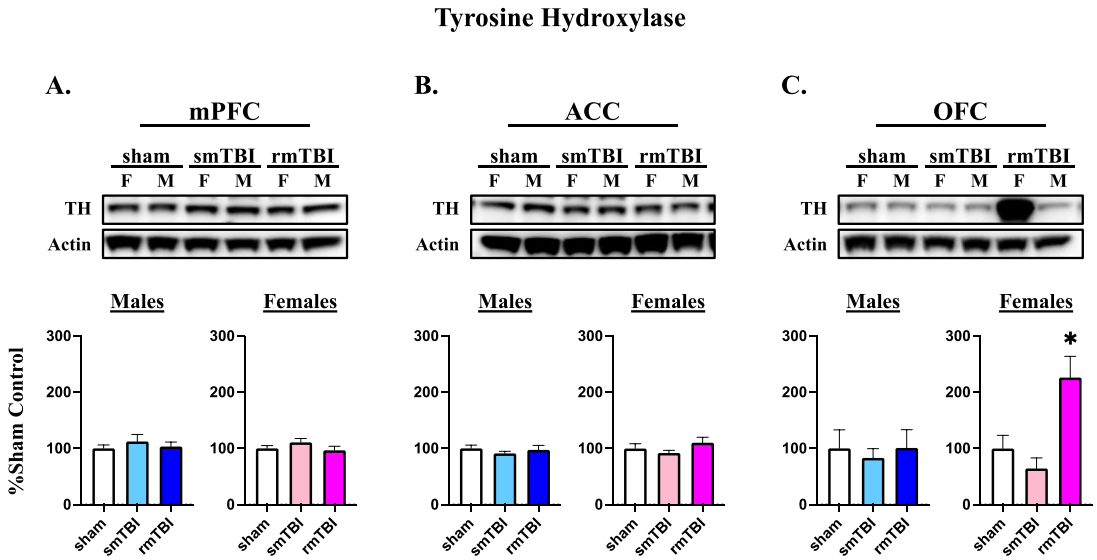


Figure 37. Post-surgery tyrosine hydroxylase levels in subregions of the PFC (3.5mm impact depth). Male and female levels of tyrosine hydroxylase (TH) in the A) medial prefrontal cortex (mPFC), B) anterior cingulate cortex (ACC), and C) orbitofrontal cortex (OFC). Graphs represent percent total protein levels at 48 hours post-final surgery. No differences in TH levels were found between sham, single (smTBI), or repetitive (rmTBI) injury groups in the mPFC or ACC of either sex. In the OFC, rmTBI significantly increased TH levels in females only. Bars represent mean ± SEM. * denotes $p < 0.05$ from sham analyzed with Dunn's Multiple Comparisons Tests.

11.4.3 Effects of mTBI on VMAT2

Analysis of VMAT protein levels (**Fig. 38**) within the mPFC, ACC, and OFC revealed no significant changes in VMAT levels in males [F (2, 20) = 3.237, p = 0.0605; H (2) = 1.887, p = 0.4021; F (2, 18) = 0.2600, p = 0.7739, respectively] or females [F (2, 21) = 0.6478, p = 0.5333; H (2) = 0.7435, p = 0.7039; F (2, 18) = 0.3123, p = 0.7356, respectively] following mTBI.

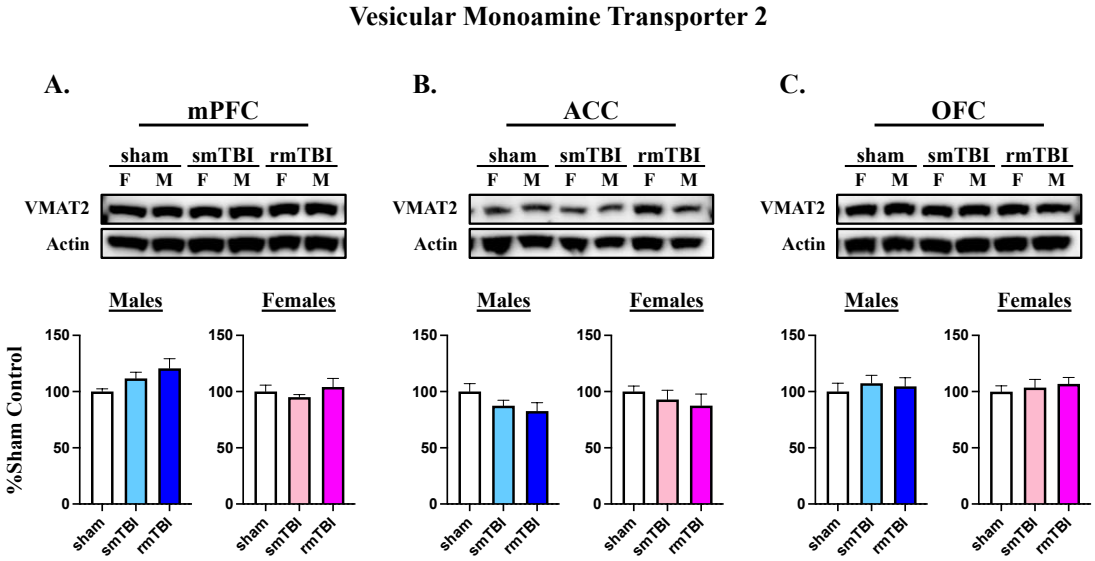


Figure 38. Post-surgery vesicular monoamine transporter 2 levels in subregions of the PFC (3.5mm impact depth). Male and female levels of vesicular monoamine transporter 2 (VMAT2) in the A) medial prefrontal cortex (mPFC), B) anterior cingulate cortex (ACC), and C) orbitofrontal cortex (OFC). Graphs represent percent total protein levels at 48 hours post-final surgery. No differences in VMAT2 levels were found between sham, single (smTBI), or repetitive (rmTBI) injury groups in the mPFC, ACC, or OFC of either sex. Bars represent mean ± SEM.

11.4.4 Effects of mTBI on NET

Analysis of NET protein levels (**Fig. 39**) within the mPFC, ACC, and OFC revealed no significant changes in NET levels in males [F (2, 21) = 0.03609, p = 0.9646; F (2, 21) = 0.3570, p = 0.7040; F (2, 21) = 0.1060, p = 0.8999, respectively] or females [F (2, 21) = 0.1145, p = 0.8924; F (2, 21) = 0.5880, p = 0.5643; F (2, 21) = 0.005247, p = 0.9948, respectively] following mTBI.

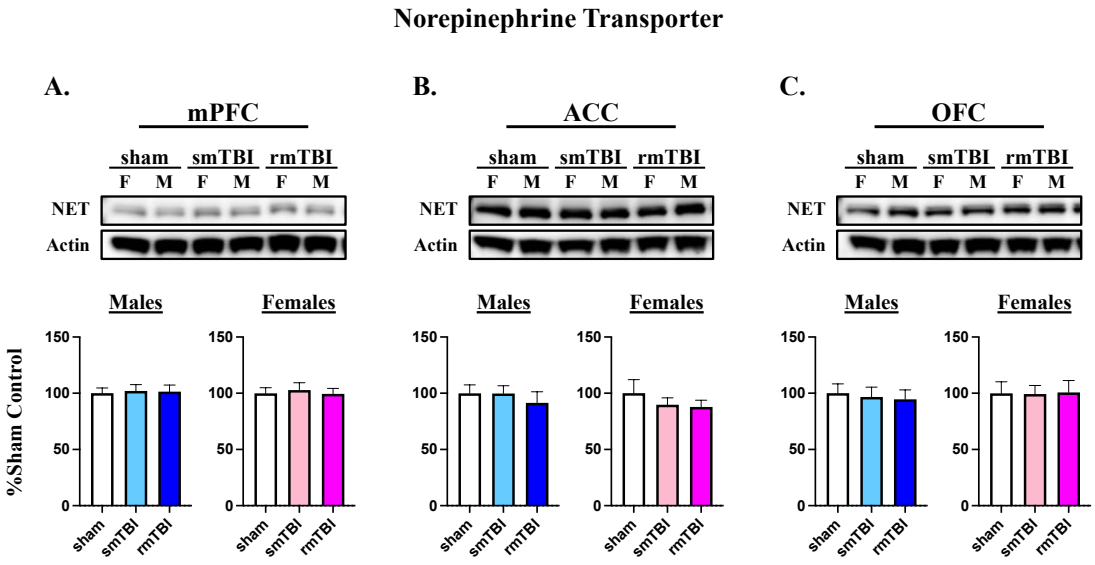


Figure 39. Post-surgery norepinephrine transporter levels in subregions of the PFC (3.5mm impact depth). Male and female levels of norepinephrine transporter (NET) in the A) medial prefrontal cortex (mPFC), B) anterior cingulate cortex (ACC), and C) orbitofrontal cortex (OFC). Graphs represent percent total protein levels at 48 hours post-final surgery. No differences in NET levels were found between sham, single (smTBI), or repetitive (rmTBI) injury groups in the mPFC, ACC, or OFC of either sex. Bars represent mean ± SEM.

11.4.5 Effects of mTBI on COMT

Analysis of COMT protein levels (**Fig. 40**) within the mPFC, ACC, and OFC revealed no significant changes in COMT levels in males [F (2, 21) = 0.6223, p = 0.5463; F (2, 19) = 0.04486, p = 0.9562; F (2, 21) = 0.05731, p = 0.9445, respectively] or females [F (2, 21) = 0.7497, p = 0.4848; F (2, 18) = 0.05779, p = 0.9440; F (2, 21) = 0.7836, p = 0.4696, respectively] following mTBI.

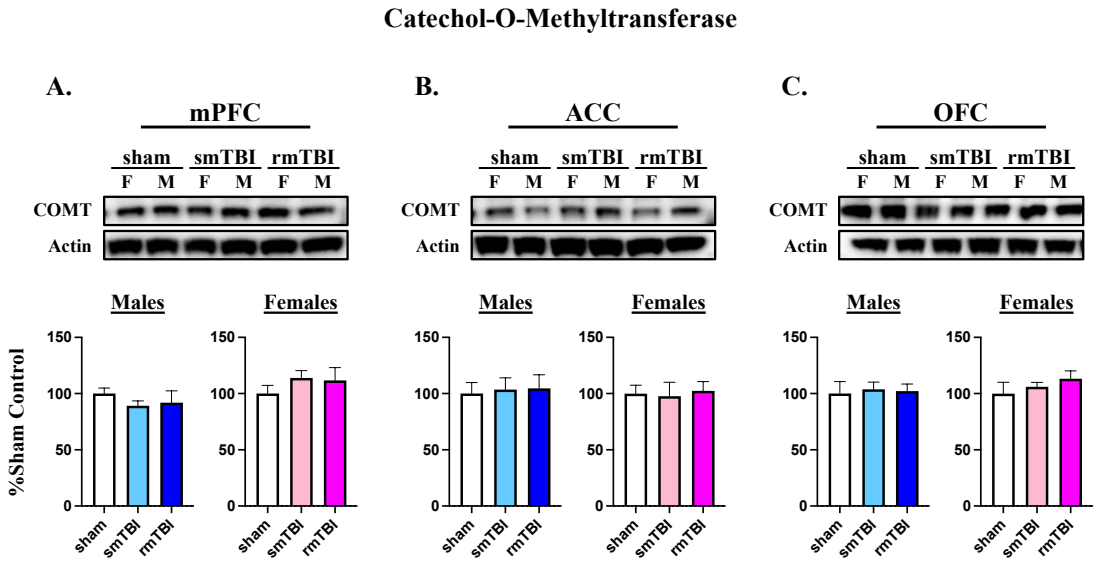


Figure 40. Post-surgery catechol-O-methyltransferase levels in subregions of the PFC (3.5mm impact depth). Male and female levels of catechol-O-methyltransferase (COMT) in the A) medial prefrontal cortex (mPFC), B) anterior cingulate cortex (ACC), and C) orbitofrontal cortex (OFC). Graphs represent percent total protein levels at 48 hours post-final surgery. No differences in COMT levels were found between sham, single (smTBI), or repetitive (rmTBI) injury groups in the mPFC, ACC, or OFC of either sex. Bars represent mean ± SEM.

11.4.6 Effects of mTBI on MAO-A

Analysis of MAO-A protein levels (**Fig. 41**) within the mPFC, ACC, and OFC revealed no significant changes in MAO-A levels in males [F (2, 19) = 0.08758, p = 0.9165; F (2, 21) = 0.1163, p = 0.8907; F (2, 19) = 0.1932, p = 0.8259, respectively] or females [F (2, 19) = 0.4103, p = 0.6692; F (2, 20) = 0.4250, p = 0.6595; F (2, 19) = 0.2497, p = 0.7815, respectively] following mTBI.

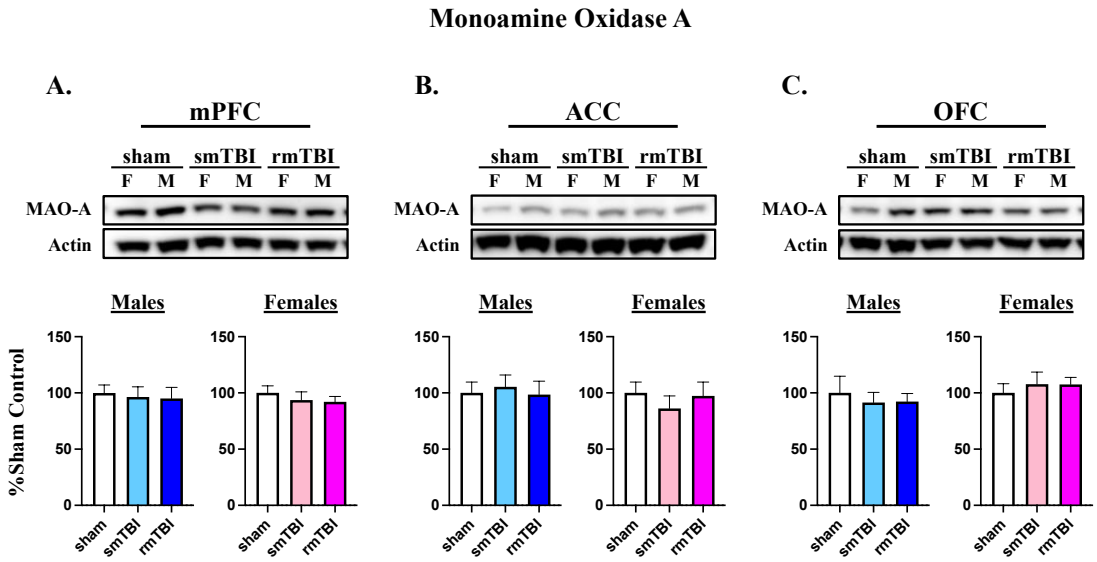


Figure 41. Post-surgery monoamine oxidase-A levels in subregions of the PFC (3.5mm impact depth). Male and female levels of monoamine oxidase-A (MAO-A) in the A) medial prefrontal cortex (mPFC), B) anterior cingulate cortex (ACC), and C) orbitofrontal cortex (OFC). Graphs represent percent total protein levels at 48 hours post-final surgery. No differences in MAO-A levels were found between sham, single (smTBI), or repetitive (rmTBI) injury groups in the mPFC, ACC, or OFC of either sex. Bars represent mean ± SEM.

11.5 Discussion

The present study investigated the effects of rmTBI of increased depth on levels of catecholamine regulatory proteins within the mPFC, ACC, and OFC. No changes in protein levels were observed in the mPFC or ACC. In the OFC, rmTBI produced robust increases in TH levels within female, but not male, rats. Using a milder version of our rmTBI model, we observed a roughly 90% increase in TH levels in the OFC of female rats following rmTBI (see **Chapter 9** for review). Using this stronger mTBI model, we managed to both amplify and confirm the effects of rmTBI on TH in females. Thus, in females, rmTBI enhances region-specific levels of the enzyme responsible for synthesizing catecholamines that modulate risk/reward decision making processes.

The lack of detectable changes in TH levels within males following this mTBI model suggests that males may be more resilient to changes in catecholamine regulation within the PFC. However, given the behavioral changes observed in the PDT, it is possible that other brain regions might have a more prominent role in male risk/reward decision making. Recently, the dorsal medial striatum (DMS) has been shown to guide choice biases in the PDT by monitoring changes in the frequency of non-rewarded choices (Schumacher et al., 2021). Through inactivation of the DMS, increased risky choice preference and reduced lose-shift behavior have been observed in the PDT (Schumacher et al., 2021),

similar to what was seen in our repetitive injured animals. These increases in risky choice are also similar to those induced by inactivation of different prefrontal regions, including the mPFC (St Onge & Floresco, 2010) and medial OFC (mOFC) (Stopper et al., 2014), which are known for mediating choice preference in the PDT. Following experimental TBI, the striatum has also been reported to experience aberrant catecholaminergic activity. Following TBI, increased levels of TH (Wagner, Sokoloski, et al., 2005; Yan et al., 2007) along with a brief "dopaminergic storm" (Massucci et al., 2004; McIntosh et al., 1994; Wagner, Sokoloski, et al., 2005) have been observed. Given that both TH and DA increases have also been reported in the PFC following TBI (Kobori et al., 2006; Massucci et al., 2004), it is likely the rmTBI may be altering catecholamine regulatory actions responsible for modulating risk/reward decision making processes in this region. Therefore, changes in catecholaminergic activity within the striatum, particularly the DMS, may be contributing to the behavioral changes observed in the PDT.

As discussed previously, high concentrations of cytosolic catecholamines, particularly DA, can lead to neurotoxicity and death of catecholaminergic neurons (Graham et al., 1978; Masoud et al., 2015; Mosharov et al., 2009). In **Chapters 9 and 10**, we reported that sustained increases in DA and NE synthesis caused by elevated TH presence may result in downregulation of NET expression as a compensatory response to prevent overaccumulation of intracellular catecholamines. In contrast, no changes in NET protein levels were observed in the OFC following rmTBI from our stronger injury model. While initially surprising, it is possible that the strength of these repetitive insults might be impairing the PFC's ability to respond to dramatic shifts in catecholamine levels. Further investigations are needed to confirm these speculations; however, this lack of

compensatory response to sustained elevations in DA and NE may explain the additional effects of rmTBI on feedback sensitivity (see **Chapter 8** for review) that were not observed previously in our milder model. As such, this information indicates that milder forms of mTBI are within the realm of promoting compensatory actions, whereas more severe mTBIs might impair these responses. With respect to the OFC, we have now demonstrated through two studies that the OFC experiences robust neurochemical disruption following rmTBI. These results reinforce our contention that the OFC is more susceptible to damage following mTBI and that this region may be the primary contributor to catecholamine imbalances in the PFC. Future research with this pre-clinical model provides an outlet for exploring potential treatment strategies to alleviate rmTBI-induced disruptions in cognitive processes that facilitate risk/reward decision making.

11.6 Conclusion

By exploring the effects of mTBI of increased injury depth on catecholamine regulatory proteins levels within subregions of the PFC, we only observed large increases in TH levels in the OFC of rmTBI females. While these results differ from those **obtained in Chapter 9**, it is clear that the OFC is more susceptible to catecholamine instability after rmTBI. Females are also more suspectable to alterations in protein expression following repetitive injuries in this region. As such, these collective findings directly contribute to our understanding of how rmTBI disrupts catecholamine activity within males vs females and sets a foundation for sex-specific therapeutic strategies.

Chapter 12

Concluding Remarks

12.1 Summary of Findings

The primary goal of this dissertation work was to assess the effects of repetitive mild traumatic brain injury (rmTBI) on risk/reward decision making behavior using a well-established probabilistic discounting task (PDT). We further aimed to evaluate the processes that contribute to TBI-induced imbalances in the prefrontal cortex (PFC) by targeting catecholamine transmitter regulatory proteins that maintain optimal catecholaminergic transmission. Finally, we wanted to determine whether these outcomes can be differentiated by sex. *The central hypothesis tested was that rmTBI produces greater increases in risk-taking behavior compared to single and uninjured animals, and that these effects are more severe and longer-lasting in females compared to males. We further hypothesized that these effects are driven by TBI-induced alterations of catecholamine regulatory protein levels*. To address this overarching hypothesis, a series of experiments were conducted, with the results being comprehensively discussed in each chapter. A summation of these results and their significance are discussed here.

12.1.1 Repetitive Mild Traumatic Brain Injury Increases Risky Choice in the Probabilistic Discounting Task

In the experiments conducted in **Chapter 6**, we found that mTBI transiently increases risky choice preference in the PDT and that these effects were seemingly driven by our female injury groups (**Fig. 15, Chapter 6)**. Interestingly, the effects of rmTBI were

not as robust as those of a single impact; however, repetitive injury did appear to disrupt adjustments in choice biases in response to changes in the value of large/risky rewards. Furthermore, we revealed that males were more likely to experience delayed disruptions in cost/benefit evaluations, resulting in longer deliberation periods when making risky choices (**Fig. 18, Chapter 6)**. When we repeated our behavioral experiments in **Chapter 8** using a stronger model of mTBI, we were able validate the existence of mTBI-induced increases in risky choice and reveal the previously subthreshold effects of repetitive injury. We revealed that rmTBI increases risky choice preference in both males and females, and that these increases in risk preferences were caused by reduced sensitivity to non-rewarded risky decision (**Fig. 25** and **26, Chapter 8**). Overall, these experiments were successful in effectively evaluating and differentiating the effects of mTBI on risk/reward decision making in male and female rodents using the PDT. We conclude that repetitive mild TBIs lead to increases in risk taking behavior.

12.1.2 Repetitive Mild Traumatic Brain Injury Alters Catecholamine Regulatory Actions in the Orbitofrontal Cortex

By evaluating specific subregions of the PFC, we conclude that the orbitofrontal cortex (OFC) is more susceptible to catecholamine instability after rmTBI. The medial (mPFC), anterior cingulate (ACC), and orbitofrontal regions of the PFC work collaboratively to mediate risk/reward decision making processes. The results of the experiments conducted in **Chapters 9** and **11** revealed that levels tyrosine hydroxylase (TH), an enzyme responsible for synthesizing dopamine (DA) and norepinephrine (NE), was substantially elevated in the OFC of female rats (**Fig. 30** and **37, Chapter 9** and

Chapter 11, respectively). In response to dramatic shifts in catecholamine presence response brought on by elevated TH levels, we observed reduced levels of norepinephrine transporter (NET) in both males and females within our milder mTBI model (**Fig 32, Chapter 9**), suggestive of a compensatory response to maintain efficient catecholamine signaling in the PFC. We confirmed this hypothesis in **Chapter 10**, where we found no evidence of short- or long-term reductions in DBH fiber presence within the OFC following rmTBI (**Fig. 35** and **36, Chapter 10**). Taken together, females may be more susceptible to alterations in protein expression following repetitive injuries, but both sexes can initiate compensatory actions in response to these catecholamine imbalances to maintain normal catecholamine activity. Our findings directly contribute to our understanding of how rmTBI disrupts catecholamine activity within males versus females and sets a foundation for sex-specific therapeutic strategies.

12.1.3 Developing of a Model for Assessing the Effects of Repetitive Mild Traumatic Brain Injury on Risk/Reward Decision Making and Catecholamine Regulation within the Prefrontal Cortex

Clinical studies have failed to adequately assess the effects of mTBI on risk/reward decision making. An essential step towards developing a model of rmTBI that would enable the characterization of rmTBI's effects on risk/reward decision making was to validate the PDT in our laboratory (see **Chapter 4** for review). Following the completion of these validation experiments, we designed a strategy for evaluating the impact of rmTBI on risk/reward decision making using the PDT. However, we found no differences in risky choice following mTBI when animals sustained injuries prior to PDT testing (see **Chapter**

5 for review). While these findings were initially surprising, we concluded that we may have missed the window for detecting possible injury-induced changes in risky choice behavior given the amount of time animals required to learn and establish stable profile of performance in the PDT combined with potentially transient effects of our mTBI model. Thus, we modified our experimental design to allow animals to first train and establish stable choice patterns in the PDT before receiving sham or mTBI surgeries. Although we managed to detect mTBI-induced changes in risky choice using this improved experimental strategy as well as alterations in levels of catecholamine regulatory proteins within the PFC, the effects of our injury model were subtle, reflecting that the current injury parameters of 2.5mm depth and 5.5 m/s velocity may have been sub-threshold for producing the full range of effects associated with mTBIs in the PDT. Therefore, in **Chapter 7**, we conducted experiments to design a stronger version of our rmTBI model that would enable detection of these previously sub-threshold effects. Using both models, we found various time-dependent and sex-specific changes in risk/reward decision making and catecholamine regulation, which ultimately underscores the importance of evaluating both males and females in pre-clinical TBI studies. This work additionally demonstrates the effectiveness of combining this repetitive injury model with operant-based behavioral paradigms and procedures to analyze levels of regulatory proteins within select brain regions that mediate specific behaviors of interest. Future research with this model will allow for further investigation into the underlying mechanisms responsible for these behavioral changes as well as for testing potential treatment strategies to alleviate rmTBI-induced cognitive deficits.

12.2 Clinical Significance

These pre-clinical findings indicate that males and females experiencing repetitive concussions are likely to exhibit increased tendencies to make riskier choices after injury. In females, these changes in risk preferences may be correlated to injury-induced neurochemical changes in the PFC. In head injured males, longer deliberation times could, by themselves, be debilitating when faced with cost/benefit decisions. Thus, patients should be mindful when partaking in risky behaviors such as gambling and substance use (see the **Risk/Reward Decision Making** section of **Chapter 1**).

This work establishes an avenue for exploring potential treatment strategies for alleviating rmTBI-induced deficits in risk/reward decision making processes. Ongoing experiments in the Navarra laboratory are focused on exploring the therapeutic efficacy of psychostimulants such as methylphenidate (MPH) for resolving increased risky choice behavior in the PDT. As described in **Chapter 1** of this dissertation, few clinical studies have explored the beneficial effects of MPH on TBI-induced cognitive deficits and none have explored its potential for alleviating deficits in executive functioning following repeated mild head trauma.

These findings further underscore the need for evaluating changes in executive function in patients with concussive injuries. The current work demonstrates that alterations to probabilistic discounting occur within the first week after injury. Thus, the implementation of cognitive assessments in initial concussion assessment protocols may be advantageous to detect and treat immediate cognitive deficits that arise following TBI incidents. As described in **Chapter 1**, a battery of neuropsychological tests is often used to assess changes in cognitive performance in athletes; however, these tests are not always

administered immediately after TBI events. Future concussion evaluation protocols would benefit from incorporating common neuropsychological tests such as: Controlled Oral Word Association Test – assesses verbal fluency; Hopkins Verbal Learning Test – assesses verbal learning and delayed memory; Trail Making Tests, Forms A and B – assesses visual scanning and executive functioning; Wechsler Digit Span Test – assesses attention and concentration; and Symbol Digit Modalities Test – assesses information processing speed (Guskiewicz et al., 2004; Merritt et al., 2017). These tests are administered through a paper-and-pencil method; however, computerized neurophysiological tests such as the Immediate Postconcussion Assessment and Cognitive Testing (ImPACT) (Covassin et al., 2009), which evaluates multiple cognitive domains, offer an alternative automated approach (Collie et al., 2001; Guskiewicz et al., 2004). By conducting these cognitive test batteries immediately after TBI events, health care professionals can screen for potential deficits in cognitive performance that may require therapeutic interventions and/or additional assessments of more complex cognitive processes (e.g., decision making).

12.3 Future Directions

12.3.1 Investigation of Other Neurochemical Disruptions Occurring in the Orbitofrontal Cortex Following Repetitive Mild Traumatic Brain Injury

This work raises new questions regarding other probable neurochemical disruptions occurring in the OFC following rmTBI, including the actual levels of DA and NE. Ongoing experiments in the Navarra laboratory are using high-performance liquid chromatography (HPLC) to measure catecholamine levels within the PFC 48 hours post-surgery to confirm whether our observed changes in catecholamine regulatory proteins directly result in

alterations in DA and NE. By assessing multiple timepoints, we could determine the potential duration of these changes. Given the observed sex-specific variation in catecholamine regulatory levels following mTBI, it is possible that there might also be sex differences in overall DA and NE levels following injury.

In addition to the catecholamines themselves, alterations in receptor expression after mTBI would also serve as an avenue for exploration. The mOFC D1 and D2 receptors have been shown to have opposing roles in the PDT (Jenni et al., 2021). D1 aids in promoting profitable reward seeking tendencies. Pharmacologically inhibiting D1 receptor activity results in decreased risky choice whereas stimulating D1 receptor activity does not elicit any changes in risky choice behavior in the PDT (Jenni et al., 2021). The D2 receptor aids in mitigating strong choice biases, specifically in response to probabilistic wins and losses. Pharmacologically inhibiting D2 receptor activity results in increased risky choice, whereas stimulating D2 reduces risky choice preference (Jenni et al., 2021). It is possible that altered expression of these receptors, particularly D2, following rmTBI may be contributing to the increased risky choice observed in the PDT. Future immunoblotting studies could assess levels of D1 and D2 receptors as well as other dopaminergic and noradrenergic receptors that may be affected by mTBI within the OFC. The role of noradrenergic receptors in the OFC has yet to be investigated and no studies have explored their expression after TBI. This lack of information offers a novel route for exploration into how these receptors are affected in the OFC after single and repetitive injuries and how those changes may contribute to our observed behavioral outcomes.

12.3.2 Investigating the Effects of Repetitive Mild Traumatic Brain Injury on Gonadal Hormones That Modulate Risk/Reward Decision Making Processes

The sex-specific changes in risky choice behavior and levels of catecholamine regulatory proteins raise new questions regarding the mechanisms underlying these observed differences between males and females. In the PFC, gonadal hormones have been reported to be regulators of risk-based decision making (Orsini et al., 2022) with both ovarian hormones (Dazzi et al., 2007) and testosterone (Kritzer, 1997; Kritzer & Creutz, 2008) influencing DA neurotransmission. In females, DA levels have been shown to fluctuate across different stages of the estrous cycle (Dazzi et al., 2007). It is unclear whether rmTBI disrupts the estrus cycle, thereby disrupting normal fluctuations of estrogen and progesterone that influence cognitive processes involved in risk/reward decision making. Testosterone interacts with and activates androgen receptors (ARs) located within the ventral tegmental area (VTA) (Kritzer, 1997). These AR-expressing neurons in the VTA also express TH and project to the PFC, namely the mPFC, to regulate DA-dependent functions that may contribute to risky decision making (Kritzer & Creutz, 2008). However, it is unclear how rmTBI affects testosterone and AR levels in the brain and whether these effects are partially responsible for our observed behavioral and neurochemical changes.

Ongoing analyses in the Navarra laboratory are exploring whether risky choice preference in females following rmTBI differs in animals that are in the proestrus/estrus stages, where levels of estrogen and progesterone are higher, versus those that are in the metestrus/diestrus stages, where levels of hormones are low. In regard to future experiments, studies should aim to investigate whether rmTBI disrupts the normal fluctuation of gonadal hormones as well as the enzymes responsible for synthesizing

estrogen, progesterone, and testosterone. Additionally, receptor levels for estrogen [estrogen receptor α (ERα) and estrogen receptor β (ERβ] and progesterone [progesterone receptor A (PRA) and progesterone receptor B (PRB)], along with ARs within prefrontal regions should also be evaluated following rmTBI. Together, these assessments may provide insight into the mechanisms underlying the observed rmTBI-induced sex-specific changes in risky choice and levels of catecholamine regulatory proteins.

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Appendix

List of Abbreviations

α2 – Noradrenergic receptor alpha 2

ACC – Anterior cingulate cortex

ADHD – Attention deficit hyperactivity disorder

AMPH – Amphetamine

ANOVA – Analysis of variance

AR – Androgen receptor

BSA – Bovine serum albumin

CCI – Control cortical impact

CH-CCI – Closed head-Controlled cortical impact

cm – centimeter

COMT – Catechol-O-methyltransferase

CT – Computed tomography

D1 – Dopaminergic receptor D1

D2 – Dopaminergic receptor D2

D3 – Dopaminergic receptor D3

DA – Dopamine

DAT – Dopamine transporter

DBH – Dopamine β-hydroxylase

dlPFC – Dorsolateral prefrontal cortex

DMS – Dorsal medial striatum

DOD – Department of Defense

ELISA – Enzyme-Linked Immunosorbent Assay

ERα – Estrogen receptor α

Erβ – Estrogen receptor β

FPI – Fluid percussion injury

FR1 – Fixed-ratio one

g – grams

GCS – Glasgow Coma Scale

h – hours

H – Height

HPLC – high-performance liquid chromatography

IGT – Iowa gambling task

ImPACT – Immediate Postconcussion Assessment and Cognitive Testing

kg – kilogram

L – Length

LC – Locus coeruleus

lOFC – Lateral orbitofrontal cortex

MACE2 – Military Acute Concussion Evaluation 2

MAO-A – Monoamine oxidase A

MAO-B – Monoamine oxidase B

m – meter

mg – milligrams

mL – milliliter

mm – millimeter

ms – milliseconds

mOFC – Medial orbitofrontal cortex

mPFC – Medial prefrontal cortex

MPH – Methylphenidate

mTBI – Mild traumatic brain injury

NE – Norepinephrine

NET – Norepinephrine transporter

NBA – National Basketball Association

NFL – National Football League

NHL – National Hockey League

NS – nonsignificant

OFC – Orbitofrontal cortex

PBS – Phosphate buffer saline

PBS-t – Phosphate buffer saline tripton

PCS – post-concussion symptoms

PD – Probabilistic discounting

PDT – Probabilistic discounting task

PFC – Prefrontal cortex

PRA – Progesterone receptor A

PRB – Progesterone receptor B

rmTBI – Repetitive mild traumatic brain injury

s – second

SEM – Standard error of the mean

smTBI – Single mild traumatic brain injury

TBI – Traumatic brain injury

TH – Tyrosine hydroxylase

VMAT2 – Vesicular monoamine transporter 2

VTA – Ventral tegmental area

W – Width