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The Effect of Injury Severity on Behavioral Tasks Used for the Assessment of Cognitive Functioning Following Traumatic Brain Injury

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THE EFFECT OF INJURY SEVERITY ON BEHAVIORAL TASKS USED FOR THE ASSESSMENT OF COGNITIVE FUNCTIONING FOLLOWING TRAUMATIC BRAIN INJURY

by

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Cognitive impairment is the most frequent cause of disability in humans following traumatic brain injury (TBI), yet the behavioral tasks used to assess cognitive behavior in rodent models of brain injury are underrepresented in the field. Additionally, few of these tasks have been used to assess behavior across degrees of injury severity. The goal of the present study was to compare four behavioral tasks commonly used in the field in frontally-injured rats with both mild and moderate-to-severe brain injuries. At the start of the study, rats were assigned to two of the following behavioral tasks: Dig scent discrimination (Dig) task, novel object recognition (NOR) task, Morris water maze (MWM), and passive avoidance (PA) task. Four days prior to injury, Dig rats were trained to dig in unscented sand and MWM rats were trained to locate a hidden platform positioned in the northeast quadrant of the MWM. Following training, bilateral controlled cortical impact injuries were induced (mild bilateral frontal TBI, moderate-to-severe bilateral frontal TBI, or non-injured, sham). Following a seven day recovery period, rats were tested on the two assigned behavioral tasks. Following testing, linear mixed effects modeling was performed assessing performance differences on the four tasks as a function of injury (injured vs. non-injured), injury severity (mild TBI vs. moderate-to-severe TBI), and task interaction. The results indicated that, while all four behavioral tasks were effective at assessing injury, some of the tasks were more effective at differentiating between injury severities than

others. Specifically, the Dig task and MWM were effective at differentiating between rats with mild TBIs and rats with moderate-to-severe TBIs. Interactions between tasks also occurred such that Dig rats also assigned to the NOR task had significantly higher learning curves on the scent discriminations. The results from the current study indicate that all four behavioral tasks have the potential to assess cognitive impairment after TBI. However, these results are only a beginning. More work is needed before we can fully understand the efficacy of each of these tasks as behavioral assessment measures for cognitive functioning after TBI.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Traumatic brain injury (TBI) has been identified as a major health problem facing the United States today. The Centers for Disease Control and Prevention (CDC) estimates that, every year, 1.7 million people experience a TBI in the United States. Of these 1.7 million, approximately 1.4 million (82%) are treated and released from the hospital emergency room, 275,000 (16%) require hospitalization, and 52,000 (2%) die from complications resulting from the TBI (CDC, 2011). Sadly, these statistics do not account for the injuries that go unreported including individuals who do not seek medical care and those who are treated in military facilities either in the United States or abroad. Estimates for these injuries range from an additional 200,000 to nearly 1 million (Langlois, Rutland-Brown, & Wald, 2006). In the United States today it is estimated that approximately 5.3 million Americans, approximately 2% of the population, are living with the need for assistance due to a TBI (Langlois et al., 2006; Thurman, Alverson, Dunn, Guerrero, & Sniezek, 1999).

The leading cause of TBI in the United States is from falls. According to the CDC (2011), falls account for 35.2% of cases per year. Falls are the leading cause of TBI among adults aged 65 years and older and account for approximately half of the TBIs among children aged 0 to 14 years. After falls, 17.3% of annual TBI cases are caused by motor vehicle accidents. Compared to any other age group, teenagers aged 16 to 19 years are at the greatest risk of being involved in a motor vehicle accident. To date motor vehicle accidents remain the leading cause of death for U.S. teens, accounting for more than one in three deaths. After motor vehicle accidents, 16.5% of annual TBI cases are caused by struck by/against events. Struck by/against events involve colliding with a moving or stationary object and are the second leading cause of

TBI among children aged 0 to 14 years, accounting for approximately 25% of the TBIs in this age group. After struck by/against events, 10% of annual TBI cases are caused by assault. Of the remaining 21% of annual TBI cases, the causes are unknown (CDC, 2011).

When combining TBI cases from emergency department visits, hospitalizations, and deaths, children aged 0 to 4 years and teens aged 15 to 19 years were more likely to sustain a TBI than persons in any other age group (Langlois et al., 2006). However, when assessing hospitalizations only, adults aged 75 years or older have the highest incidence of TBI. Independent of age group, the incidence of TBI among males is higher than it is among females, accounting for 59% of TBI cases that occur each year (Summers, Ivins, & Schwab, 2009). The most common etiologies for males include falls, motor vehicle accidents, and being struck by or against an object.

The statistics presented thus far clearly demonstrate the importance of TBI as a public health problem; however, with the newly published addition of injuries sustained by military personnel in the Iraq (Operation Iraqi Freedom) and Afghanistan (Operation Enduring Freedom) theaters of operation the numbers become staggering. While better equipped to protect against penetrating injuries caused by bullets and bombs, soldiers are extremely vulnerable to blast injuries caused by improvised explosive devices, rocket-propelled grenades, and land mines (Galarneau, Woodruff, Dye, Mohrle, & Wade, 2008; McCrea, Jaffee, Guskiewicz, & Doncevic, 2009). These injuries occur as a direct result of blast wave-induced changes in atmospheric pressure, from projectiles (i.e., objects put in motion by the blast) hitting people, and from people being put into motion by the blast (Taber, Warden, & Hurley, 2006). Experimental research has shown that the brain is vulnerable to each type of blast injury (Taber et al., 2006). Blast injuries among military personnel have become so prevalent that they are considered by some to be the

"signature wound" of the wars in Iraq and Afghanistan (Galarneau et al., 2008; McCrea et al., 2009).To put the prevalence of TBI as a result of blast injury in perspective, ten years prior to the start of Operation Enduring Freedom approximately 20% of military personnel serving in the Persian Gulf War had primary or concurrent head injuries (Carey, 1996; Leadham, Newland, & Blood, 1993). The estimates from the most recent war in the Middle East, while not known for certain, are estimated to be as high as 60% (Galarneau et al., 2008). According to Fischer (2010), the incidence of TBI among military personnel from 2000-2010 who survived their injuries is estimated to be around 180,000 with 140,000 mild TBI cases, 30,000 moderate TBI cases, and 2,000 severe. The remaining 8,000 cases were not classified. The return of these soldiers increases the already immense burden of TBI.

The burden of TBI is immense and often underrepresented. Economically, a loss in productivity and medical treatment for the individual is costly. As of 2000, the direct medical costs and indirect costs of TBI totaled an estimated $60 billion in the U.S. with the per person costs totaling an estimating $45 thousand (CDC, 2011; Corrigan, Selassie, & Langlois, 2010). Individually, physical and/or psychological impairment and an increased risk for neurological disorders as the individual ages can have a lifelong financial and emotional impact on the individual. While impairment following injury depends upon multiple factors (e.g., injury location and severity, age, and general health of the individual), even a mild TBI can cause long-term deficits in cognition (i.e., complex behavioral phenomena such as memory, language, attention, problem solving, and emotion that cannot be observed) that affect the individual's ability to perform daily activities including returning back to work (Thurman et al., 1999). As the individual ages, their risk for various health conditions and neurological disorders increases. Post-injury, these individuals are 11 times more likely to develop epilepsy and have a 1.5 times

increased risk for depression compared to non-injured controls (Langlois et al., 2006). In addition, these individuals have shown a 2.3 and 4.5 times increased risk of Alzheimer's disease associated with moderate and severe head injury, respectively (Langlois et al., 2006). However, despite these widespread socio-economic effects there is currently no approved, standardized treatment for victims of head trauma. While numerous attempts have been made to develop a treatment for TBI, of the 21 clinical trials run since 1985, none have succeeded (Maas, Marmarou, Murray, Teasdale, & Steyerberg, 2007). The current protocol, as recommended by the National Institute of Neurological Disorders and Stroke (NINDS), is to try to stabilize the patient through a reduction in bleeding and an increase in oxygen supply to the brain all while controlling blood pressure (NINDS, 2011). As evidenced by the 52,000 individuals who die from TBI every year and the 5.3 million Americans who are living with the need for assistance due to a TBI, it is imperative that this simplistic protocol be improved upon. In order to develop a standardized treatment for victims of TBI, the deficits and pathology seen after injury must be reproduced in animal models of testing. Working toward this goal, the present study will attempt to further the field's understanding of the deficits that occur in frontally-mediated cognitive functioning after both mild and moderate-to-severe TBI. This will be accomplished by conducting a comparison of the behavioral tasks used in the field for the assessment of cognitive functioning in the rat. However, prior to outlining this study, more information regarding the injury process, the types of animal models used to study TBI, the cognitive deficits seen in humans after injury, and the evaluation of cognitive deficits in rodents after TBI must occur.

Pathophysiology of Traumatic Brain Injury

The injury process following TBI can be characterized by two major phases: primary damage and secondary injury. Head injury typically results from either a direct impact to the

head or from an indirect impact resulting from rapid acceleration being applied to the head and neck when the torso is stopped or accelerated rapidly. Primary damage results from the direct mechanical deformation in the form of linear acceleration, rotational head movement, or both of the brain tissue (Greve & Zink, 2009). It is believed that linear acceleration produces focal brain injuries leading to damage of the gray matter closest to the surface of the brain causing cortical contusions and hemorrhage (Greve & Zink, 2009). Rotational movements, on the other hand, are believed to produce both focal and diffuse brain injuries leading to shearing and diffuse axonal injury of the deep cerebral white matter axons (Greve & Zink, 2009). The primary phase of injury is untreatable except through prevention. Therapeutics can only focus on treating the secondary injury (McIntosh, 1993; Werner & Engelhard, 2007).

Secondary injury results from the biomolecular and physiological changes that follow the insult. Compared to the primary damage, it is a much more complex process that involves a multitude of actions depending on the type of injury, severity, and relative chemical balance of the brain. Some of the biomolecular responses to TBI include: edema, hemorrhage, inflammation, ischemia, excitotoxicity, neuronal cell death, and free radical production.

Edema.

Edema is an increase in net brain water content leading to an increase in tissue volume (Unterberg, Stover, Kress, & Kiening, 2004). The following types of edema may occur after injury: vasogenic edema, cytotoxic edema, and osmotic edema. Vasogenic edema is characterized by a breakdown of the tight endothelial junctions which make up the blood-brain barrier (BBB) resulting in increases in extracellular fluid and plasma proteins (Roof, Duvdevani, & Stein, 1993; Unterberg et al., 2004). Cytotoxic edema occurs independently of BBB compromise and is characterized by sustained intracellular fluid accumulation involving both

astrocytes and neurons. The mechanisms that account for this swelling consist of increases in sodium (Na^+) and potassium (K^+) permeability of the cell membrane, energy depletion followed by a failure of the sodium-potassium pumps (Na^+/K^+ pumps), and sustained uptake of osmotically active solutes (Unterberg et al., 2004). Osmotic edema is characterized by an increase in osmotic pressure, resulting in a large influx of water into the injury area (Unterberg et al., 2004).

While it was originally believed that brain edema following TBI was mostly vasogenic in nature, recent evidence from numerous experimental studies is pointing toward cytotoxic edema. While transient and moderate opening of the BBB has been shown to occur, implicating vasogenic edema, it appears to occur later in the second injury cascade (Unterberg et al., 2004). Cytotoxic edema can be traced directly to influxes in Calcium (Ca^{2+}) and Na^+. As the contents of the cell become saturated with Na^+, the osmotic pressure of the cell increases and begins to draw in more water. With the cell's energy source, adenosine triphosphate (ATP), depleted, the Na^+/K^+ pumps fail resulting in increased swelling as Na^+ continues to enter the cell (Unterberg et al., 2004). This increased swelling is detrimental to the neurons. Cytotoxic edema has also been shown to be detrimental to glial cells, particularly astrocytes. A major process associated with astrocytes after injury is the increased amount of the protein water channel, aquaporin-4. Aquaporin-4 upregulation has been shown to exist exclusively on activated, swollen astrocytes and increase progressively with increased edema (Kiening et al., 2002).

Hemorrhage.

A hemorrhage is a leaking of blood from the blood vessels inside the brain. After head trauma, hemorrhaging can be the result of the initial insult (i.e., tearing of blood vessels at the moment of head impact) or the product of secondary injuries (Finnie & Blumbergs, 2002; Greve

& Zink, 2009). The following types of hemorrhaging may occur after head trauma: petechial hemorrhage, subdural hemorrhage, subarachnoid hemorrhage, intraventricular hemorrhage, and intraparenchymal hemorrhage. A petechial hemorrhage is a minor hemorrhage that can occur after injury. A single petechial hemorrhage can occur or numerous petechial hemorrhages scattered throughout the brain (i.e., diffuse vascular injury). In the case of the latter, survival after injury is rare (Finnie & Blumbergs, 2002). A subdural hemorrhage occurs when blood gathers within the outermost meningeal layer, between the dura and arachnoid mater. A subdural hemorrhage is commonly produced in inertial acceleration models where bridging veins are ruptured by rapid angular acceleration forces (Finnie & Blumbergs, 2002). This type of hemorrhage is more extensive compared to the petechial hemorrhage and may extend over an entire hemisphere. A subarachnoid hemorrhage occurs when blood gathers within the subarachnoid space, between the arachnoid and pia mater. A subarachnoid hemorrhage is the most common form of vascular injury after head trauma (Finnie & Blumbergs, 2002). This type of hemorrhage is usually minor, but may evolve into a significant lesion. An intraventricular hemorrhage occurs when blood gathers within the brain's ventricular system and has been found to occur in 35% of moderate-to-severe TBIs (Barkley, Morales, Hayman, & Diaz-Marchan, 2007). This type of hemorrhage requires a great deal of force to cause; thus, this hemorrhage usually does not occur without extensive associated damage (Dawodu, 2007). An intraparenchymal hemorrhage occurs when blood gathers in the brain's parenchyma (i.e., neurons and glial cells). In percussion models an intraparenchymal hemorrhage is often principally distributed throughout the brainstem (Finnie & Blumbergs, 2002).

Hemorrhaging may affect the brain in a variety of ways. It can increase intracranial pressure (ICP), aid in the formation of free radicals, and/or increase the brain's inflammatory response. In

regards to ICP, as the brain is housed in a rigid cavity it has a very limited ability to compensate for increases in intracranial blood volume and pressure. Hemorrhaging, in combination with edema, is a major contributor to the increase in ICP (Finnie & Blumbergs, 2002). In regards to free radical formation, blood is a rich source of iron which has been shown to catalyze free-radical formation. In addition, blood is a major mode of transportation for other excitatory amino acids (see **Free radical production.**). In regards to the brain's inflammatory response, blood within the interstitial space has been shown to be proinflammatory (see **Inflammation.**) (Greve & Zink, 2009).

Inflammation.

TBI triggers an inflammatory response that is initiated by the release of pro-inflammatory cytokines. As the various processes of edema and hemorrhage cause cell death following injury, the brain and body generate an immunological response to the tissue damage that can continue for months to years (Holmin & Mathiesen, 1999). This occurs due to an increase in the permeability of the BBB that allows for the influx of blood-borne proteins into the brain. These proteins are part of the body's immune system and help to defend the body against disease. While this immune response should clear away dead cells at the site of injury, the influx of new cells only appear to increase cell death due to swelling and inflammation (Holmin & Mathiesen, 1999; Lenzinger, Morganti-Kossman, Laurer, & McIntosh, 2001). Inflammation right after injury appears to be good, continued inflammation is bad. In humans, this immune response has been associated with an increased risk for disorders such as Alzheimer's and Parkinson's as well as certain types of dementia.

Ischemia.

Ischemia is characterized by a lack of oxygen and glucose after injury. As a result of the primary injury, blood flow is interrupted to the damaged areas resulting in a lack of sugars and lipids used for the synthesis of ATP. As ATP is depleted, the cells switch over to anaerobic mechanisms of energy which are unable to maintain cellular energy states, a large portion of which is used to maintain the Na^+/K^+ pumps (Werner & Engelhard, 2007). As the ion-pumps fail to remove Na^+ trickling into the cell, the intracellular voltage increases. As the threshold of excitation is met, the voltage-gated Na^+ channels open resulting in a large influx of Na^+ and an efflux of K^+ (McIntosh, 1994). The depolarization of the neuron causes it to fire, releasing large amounts of neurotransmitter, specifically the excitatory amino acid neurotransmitter glutamate (McIntosh, 1994). As the failed Na^+/K^+ pumps are unable to regain neural homeostasis, the neuron is no longer able to function and cell death occurs (see **Neuronal cell death.**). Energy depletion has also been shown to lead to the depolarization of astroglia, a type of glial cell, increasing the amount of excitatory transmitter in the synapse (Dirnagl & Priller, 2005). As energy-dependent reuptake is unable to occur in the compromised presynaptic neuron or astroglia, the overabundance of excitatory transmitter in the synapse leads to the excitation of other postsynaptic neurons who succumb to the same fate (see **Excitotoxity.**).

Excitotoxicity.

Excitotoxity is the pathological process by which nerve cells are damaged and killed by excessive stimulation of excitatory chemicals (e.g., glutamate). Excitotoxity occurs as a result of ischemic stroke and TBI. After primary injury, localized ischemia results in a lack of available cellular energy. With the depletion of ATP, neuronal cells are unable to maintain basic cellular functions and, as a result, the Na^+/K^+ pumps fail to remove Na^+ trickling into the cell leading to

an increase in the intracellular voltage. As the threshold of excitation is met the neuron fires releasing large amounts of excitatory neurotransmitter into the synapse stimulating other neurons (McIntosh, 1994). As these neurons fire, more glutamate is released into the synapse resulting in an increase in concentration of extracellular glutamate. In the uninjured brain, extracellular glutamate is regulated (via clearing) by the astrocytic glutamate transporters excitatory amino acid transporter-1 (EAAT1) and EAAT2 (Mongin & Kimelberg, 2004). However, after injury, astrocytes coexpressing EAAT1or EAAT2 have been shown to decrease due to astrocytic degeneration (Van Landeghem, Weiss, Oehmichen, & Von Deimling, 2006). The repetitive release of glutamate stimulates local NMDA and AMPA receptors. As these receptors are activated, Ca^{2+} enters the cell and begins to accumulate. This accumulation, combined with increases in intracellular Na^{+}, leads to neuronal swelling (Obrenovitch & Urenjak, 1997). In addition to neuronal swelling, the excessive accumulation of Ca^{2+} has also been shown to have toxic effects within the cell. Calcium has been shown to not only increase free radical production, but, due to its nature as an endogenous secondary messenger, to activate several detrimental chemicals including phospholipases and proteases (Choi, 1988; Werner & Engelhard, 2007). Phospholipases break down the phospholipid bilayer that makes up the cell wall. Proteases (calpains) degrade cytoskeletal proteins (Kampfl et al., 1997). Excitotoxicity, in combination with other secondary processes, leads to necrotic cell death.

Neuronal cell death.

The two types of neuronal cell death after TBI: apoptosis and necrosis. Apoptosis, termed programmed cell death, occurs when the cell undergoes a self-destruction protocol wherein it dismantles itself to be reabsorbed by other cells in the central nervous system (CNS) (Huttenlocher, 2002). This type of cell death typically occurs during development and is

characterized by mitochondrial degeneration and endoplasmic reticulum vacuolization after the rupture of the nuclear membrane (Stoica & Faden, 2010). Necrosis occurs when the cell dies without dismantling its components and requires the assistance of microglia to scavenge it (Stoica & Faden, 2010). Necrotic cell death is characterized by rupture of the mitochondria and endoplasmic reticulum, followed by the formation of small chromatin clumps at the center of the nucleus, and the rupture of the cell membrane (Stoica & Faden, 2010).

After injury the cells in the brain are likely to undergo apoptosis, necrosis, or both. High levels of intracellular ATP favors the initiation of apoptosis, a complex, energy-requiring process, while low intracellular levels of ATP are associated with necrosis. As necrosis has been shown to initialize an inflammatory response along with cell death, apoptosis is the slightly better alternative (Stoica & Faden, 2010). However, when ATP depletion occurs in the cell, it no longer has the resources to undertake apoptosis and instead succumbs to necrotic cell death (Raghupathi, Graham, & McIntosh, 2000).

Free radical production.

Free radicals (i.e., superoxide, nitric oxide) are ions with unpaired electrons making them highly chemically reactive. Excessive amounts of free radicals has been shown to lead to cell injury and death and may result in certain cancers and stroke as well as major disorders such as Parkinson's and Alzheimer's (Valko et al., 2007).

In regards to brain injury, the increased production of free radicals after TBI is one of the major detrimental events that occur in the secondary injury process. While this upregulation is the result of multiple processes, Ca^{2+} appears to play the largest role. Calcium-dependent free radical production occurs as a result of the following mechanisms: It leads to the utilization of arachidonic acid and it aids in the breakdown of the electron transport chain (Lewén, Matz, &

Chan, 2000). In regards to arachidonic acid utilization, increased levels of Ca^{2+} signaling increase the production of phosolipase C (PLC). The upregulation of PLC causes an increase in the release of arachidonic acid. The utilization of arachidonic acid results in the generation of superoxide (O-), a powerful free radical (Kontos & Wei, 1986). In regards to the breakdown of the electron transport chain, Ca^{2+} influx activates cellular enzymes which reduce the capacity of the electron transport chain to reduce oxidation (Lewén et al., 2000). As a result, more free radicals are produced. In a normal functioning cell, the free radicals would be scavenged. However, due to the ATP depletion and the breakdown of the electron transport chain, there is no energy to scavenge the increased free radicals. The marked increase in free radicals after injury leads to further damage to the cells. Free radicals are inherently unstable and attempt to acquire electrons from other sources. As the free radicals work their way into the extracellular space, they begin the process of lipid peroxidation wherein electrons are stolen from the cell membrane. This results in the compromise of the cellular membrane and causes further stress to the cell (Halliwell & Chirico, 1993). This free radical-induced cellular stress can be instrumental in initiating apoptosis in the cell (Raghupathi, 2004).

Animal Models of Traumatic Brain Injury

Treatment development for TBI requires the use of an animal injury model that exhibits both the pathophysiology and behavioral responses similar to those described clinically (Lighthall, 1988). While no single model has the ability to replicate the entire spectrum seen in human clinical TBI, several different models have been developed to attempt to imitate the various outcomes seen after head injury (Finnie & Blumbergs, 2002; Lighthall, Dixon, & Anderson, 1989; Morales et al., 2005; O'Connor, Smyth, & Gilchrist, 2011). Of these models, the two most commonly employed to model human focal injuries are the fluid percussion injury

(FPI) and the controlled cortical impact (CCI) models of TBI (Chen, Pickard, & Harris, 2003; Morales et al., 2005; O'Connor et al., 2011).

Originally developed by Lindgren and Rinder (1965), the FPI model makes use of a fluid pressure pulse delivered to the exposed, intact dura of the animal (Lighthall et al., 1989). The FPI model results in an injury severity that can range from mild to severe and has the ability to replicate both focal and diffuse injury characteristics (i.e., gray matter damage, subdural hematoma, subarachnoid hemorrhage, diffuse axonal injury) (Morales et al., 2005; Thompson et al., 2005). Unlike CCI, the anatomical consequences of FPI differ based on the injury location. A midline FPI leads to smaller contusions and herniation of the brainstem, adding to increased morbidity. A lateral FPI leads to widespread cortical damage without compression of the brainstem, closer to what is seen in clinical TBI (Lighthall et al., 1989; Thompson et al., 2005). The FPI model is used to mimic concussive events that occur as a result of rapid acceleration being applied to the head and neck when the torso is stopped or accelerated rapidly.

Originally developed by Lighthall (1988), the CCI model features a pneumatically driven piston that impacts the exposed, intact dura of the animal. Through the control of impact velocity, impact compression, and impactor size, the CCI model results in a precise injury that can range from mild to severe (Lighthall, 1988). The advantage of the CCI model is that it produces an anatomically and behaviorally accurate injury that replicates those seen in human clinical TBI; the injury is both controllable and replicable. This precision makes the CCI model an ideal candidate for examining the injury process as well as testing various pharmacological agents (Chen et al., 2003). The CCI model is used to mimic impact events that occur as a result of an automobile accident, fall, or struck by/against event.

Cognitive Functioning Deficits after Traumatic Brain Injury

Cognitive impairment is the most frequent cause of disability following TBI with deficits occurring in attention, learning, motivation, personality, and memory (Al-Adawi, Powell, & Greenwood, 1998; Draper & Ponsford, 2008; Fujimoto et al., 2004; Hamm et al., 1992; Hicks et al., 1993; Mathias, Beall, & Bigler, 2004; Serino et al., 2007; Thurman et al., 1999). These impairments occur as a result of injury to the head with an impact site over the frontal and/or temporal cortical regions and/or damage to any of the frontal-subcortical circuits. Dependent upon location site and injury severity, these impairments have the ability to affect the capacity of the individual to make normal everyday decisions leading to hardships in day-to-day living.

Location site.

Evidenced from lesion studies, it is widely acknowledged that the frontal lobe plays a major role in human behavior with damage to its anterior cortical regions and/or subcortical circuits producing distinctive symptoms (Cummings, 1993). In regards to the cortical regions, damage to the dorsolateral and/or orbitofrontal region of the prefrontal cortex has been shown to produce deficits in the various cognitive domains of planning, decision making, personality, and moderating correct social behavior (Cummings, 1993; Yang & Raine, 2009).

Damage to the dorsolateral prefrontal cortex (DLPFC) in both humans and animals has been shown to produce deficits in planning, working memory, attention, problem solving, verbal reasoning, inhibition, multi-tasking, initiation, and monitoring of actions (Cummings, 1993). For example, Milner (1963) found that patients with unilateral lesions of the DLPFC were incapable of set-shifting from one strategy to another on the Wisconsin Card Sorting Test. These individuals achieved fewer sorting categories and made larger perseveration errors compared to patients with lesions of the orbitofrontal cortex. In another study, Hornak and colleagues (2004)

found that patients with unilateral lesions of the DLPFC had difficulty in attending to feedback (i.e., the amount of money won or lost) during the reversal phase of a two-choice visual discrimination task (i.e., discriminating a simple pattern in order to earn or lose varying amounts of imaginary money). While these individuals were successful in determining the more "profitable" pattern during the initial phase of the task, they performed below chance during the reversal phase of the task. In another study, Levy and Goldman-Rakic (2000) found that lesions of the DLPFC in nonhuman primates disrupted eye movements to spatial cues on a series of spatial delayed-response tasks (i.e., remembering the spatial location of a peripheral cue while fixating on a central target). The spatial delayed-response task has been used as a primary instrument for assessing working memory capacity in nonhuman primates. In a final study, Broersen and Uylings (1999) found that lesions of the medial prefrontal cortex in rats decreased the percentage of correct responses on a three-choice serial reaction time task (i.e., responding to stimuli presented in one of three spatial locations for reinforcement).

Damage to the orbitofrontal cortex (OFC) in both humans and animals has been shown to produce impairments in decision making and impairments in emotional and social behavior (e.g., disinhibition, social inappropriateness) (Cummings, 1993). For example, Bechara and colleagues (1996) found that patients with bilateral lesions of the OFC performed significantly worse on a gambling task (i.e., consistently selected cards from one of the two "bad" decks with the largest immediate reward, but largest overall loss) compared to controls. Of utmost interest with this particular study, while individuals in the control group began to generate skin conductance responses prior to selecting a card from the "bad" decks patients with OFC lesions produced no conductance responses. In another study, Logue and colleagues (1968) found that survivors with OFC injuries as a result of ruptured anterior communicating artery aneurysms demonstrated

abrupt changes in personality. These changes included becoming more outspoken, more irritable, and less considerate or conscientiousness of the feelings of others. In another study, Deets and colleagues (1970) found that bilateral lesions of the OFC in nonhuman primates modified the social behavior of the animals. Compared to controls, the lesioned primates displayed more solitary behavior such that they spent less time near primates (unknown to the control/lesioned animals) used as social stimuli and reacted with passive submission toward the female social stimuli animals. The lesioned primates also displayed higher levels of distress (e.g., fear grimacing, cooing, screeching, compulsive body rocking, aggression directed toward its body) compared to the unoperated controls. In a final study, Rudebeck and colleagues (2006) found that lesions of the OFC in rats increased impulsive behavior on a simple T-maze cost benefit delay-based decision-making task (i.e., rats choosing the low-reward arm of the maze received one food pellet, rats choosing the high-reward arm of the maze received 10 food pellets after a five second delay). All rats were trained to criterion (choosing the high-reward arm on at least 80% of trials) prior to injury. While sham rats and rats with anterior cingulate cortex lesions continued to choose the high-reward arm post injury, rats with OFC lesions chose the low-reward arm (immediate, small reward) on the majority of trials.

While research on certain injury-induced cognitive deficits have thus far pointed to damage of one or more of the anterior cortical regions, dysfunction of any of the subcortical circuits that link regions of the frontal lobe to the subcortical structures have also been implicated. Each of these circuits form a loop that originates in the frontal lobe, projects to the striatal structures (caudate, putamen, ventral striatum), projects to specific thalamic nuclei, and terminates back in the frontal lobe closing the loop (Cummings, 1993). Similar to the anterior cortical regions, dysfunction of any of these circuits has been shown to produce a wide range of

cognitive impairments such as decreases in attention and motivation and personality changes (Cummings, 1993). For example, dysfunction of the subcortical circuit linking the anterior cingulate with the medial dorsal thalamus has been associated with decreases in motivation (Cummings, 1993). Dysfunction of the subcortical circuit linking the DLPFC with the head of the caudate has been associated with deficits in attention as well as abnormalities in motor programming (Cummings, 1993). Dysfunction of the subcortical circuit linking the OFC with the ventromedial caudate has been associated with changes in personality (Cummings, 1993).

Injury severity.

The extent to which impairments in cognitive functioning alter the way in which an individual leads his or her day-to-day life depends not only upon the injury location but, more importantly, upon the injury severity. The severity of an injury following TBI is most commonly assessed using the Glasgow Coma Scale (GCS). The GCS is a neurological scale that attempts to give a reliable, objective way of recording the conscious state of a person at the time of injury (Corrigan et al., 2010). Scores on the GCS range from 3 to 15, with higher numbers predicting better outcome following injury. Injury severity can also be assessed, although less frequently, by the duration of time the individual loses consciousness (LOC) and/or the duration of posttraumatic amnesia (PTA) experienced by the individual (Corrigan et al., 2010). Using these assessment measures the level of severity after injury can be categorized as being mild, moderate, or severe; however, it should be noted that the boundaries of these categories are somewhat ambiguous and can differ by the measurement scale used. According to the NINDS (2011), a mild injury is classified by GCS scores ranging from 13 to 15 with a LOC of less than 30 minutes and a PTA of less than 24 hours. A moderate injury is classified by GCS scores ranging from 9 to 12 with a LOC of 30 minutes to 24 hours and a PTA of approximately one

week (NINDS, 2011). A severe injury is classified by GCS scores ranging from 3 to 8 with a LOC of greater than 24 hours and a PTA of greater than one week (NINDS, 2011). When comparing the number of mild, moderate, and severe injuries that occur every year, mild injuries occur much more frequently compared to either moderate or severe injuries (Bohnen, Jolles, & Twijnstra, 1992).

With regard to the degree of impairment in cognitive functioning after TBI, research indicates that, most often, severity of injury (e.g., mild, moderate, severe) is predictive of outcome. For example, while decreases in processing speed and impairments in selective and sustained attention have been found to occur after mild TBI these impairments are subtle with recovery of function typically occurring 1 to 6 months post injury regardless of the age of the patient (Bohnen et al., 1992; Gentilini, Nichelli, & Schoenhuber, 1989; Gentilini et al., 1985; Iverson, 2005; Leininger, Gramling, Farrell, Kreutzer, & Peck, 1990; MacFlynn, Montgomery, Fenton, & Rutherford, 1984). Impairments in intelligence, memory, and verbal and non-verbal fluency have also been found to occur; however, these types of impairments are uncommon after mild TBI and typically only occur in select cases (Bohnen et al., 1992; Iverson, 2005; Leininger et al., 1990). In comparison, functional outcome after moderate and severe TBI is not as straightforward with impairments in memory, concentration, processing speed, personality, attention, mood, and various sensory or motor disturbances being reported (Draper & Ponsford, 2008; Hellawell, Taylor, & Pentland, 1999; Olver, Ponsford, & Curran, 1996). In contrast to mild TBI, these impairments have been shown to continue long after the initial injury. Results from neuropsychological testing found significant decreases in processing speed, memory, and executive functioning in individuals, 10 years post-TBI, compared to non-injured, demographically similar controls (Draper & Ponsford, 2008). The injury severity of these

individuals was found to correlate with test performance; the more severe the injury, the worse the performance. As these impairments can drastically affect the ability of the individual to remain independent, it is imperative more work be done to develop a standardized treatment for victims of TBI. This standardization is possible, but requires a more critical look at the assessment measures the TBI field relies upon to assess changes in animal behavior after injury.

Evaluating Behavior in Rodents after Traumatic Brain Injury

Traumatic brain injury results in a multitude of deficits following injury, the long-term consequences of which can cause impairments in sensation and perception, motor control, and cognition. In an attempt to assess the varied deficits following brain injury, many behavioral batteries of testing have been adopted in rodent models of TBI evaluating sensory, motor, and cognitive behavior (Fujimoto et al., 2004; Hamm et al., 1992; Schallert, Woodlee, & Fleming, 2002). While an extensive number of tasks have been developed for the assessment of sensory and motor functioning after injury, the tasks assessing cognitive behavior are lacking. Cognitive tasks typically consist of mazes that evaluate only one aspect of behavior, episodic memory (Fujimoto et al., 2004; Schallert et al., 2002). Few tasks assessing non-hippocampal aspects of cognitive behavior (e.g., application of rules, strategies, procedures) have been developed for use in rodent models of TBI. Of the tasks that exist, few are in current use (Fujimoto et al., 2004; Schallert et al., 2002). As deficits in cognitive functioning in humans are prevalent after TBI and cognition is not a process mediated solely by the hippocampus, it is important to examine multiple types of tasks that have the ability to assess non-hippocampal aspects of cognitive behavior.

Mazes.

Of the mazes available, the most widely used to investigate spatial learning and memory in rodents after experimental TBI is the Morris water maze (MWM) (Fujimoto et al., 2004; Schallert et al., 2002). The MWM consists of a circular tank filled with water. Hidden in the water is a small, usually submerged platform. The purpose of the task is for the rodent to locate the platform through the use of visual cues located outside of the tank. Training of the task prior to injury can occur, but not in all cases. Post injury, the rodent is placed into the maze at various locations and given a set amount of time to locate the platform. Rodents that do not locate the platform in the allotted time are guided there by the researcher and then removed from the maze. The latency or distance to locate the platform is used as an indicator of spatial learning and memory.

The MWM was first described by Morris (1981). In the study, rats were trained to escape from water under conditions where an escape platform was either visible or invisible, and occupied either a fixed or semi-random position. The results of the study showed that, through the use of distal cues, rats were able to locate an object (i.e., a platform) they could not see, hear, or smell by locating its position in a familiar space. The importance of the MWM was that it was the first maze to assess spatial learning through only the use of distal cues, supporting the theory that spatial learning differed from other forms of associative learning (Morris, 1984). Since Morris' description over 30 years ago, the MWM has been used to assess spatial learning and memory after a multitude of experimental manipulations.

The first study to use the MWM after TBI was conducted by Smith and colleagues (1991). In the study, the MWM was used as a measurement of post-TBI memory retention forty-two hours after moderate and severe lateral FPI in rats. The results of the study showed that

memory retention was significantly worse in the severely-injured group compared to the moderately-injured group and significantly worse in the moderately-injured group compared to the non-injured, control group as measured by latency to the platform. The results from this study indicated that the MWM had the ability to assess spatial learning and memory in rats after experimental TBI. The MWM has since become the number one behavioral task used for the assessment of cognitive behavior after TBI.

For the assessment of frontally-mediated behavior after injury, the working memory paradigm of the MWM is typically used. In the working memory paradigm, the location of the platform is moved at the start of each testing day requiring the rodent to relearn the location. Research utilizing the working memory paradigm of the MWM in frontally-injured rats has shown significant differences between injured and non-injured animals. According to the research, non-injured rats (i.e., sham rats) locate the platform significantly faster during each testing day compared to injured rats (i.e., vehicle rats) after both mild and moderate-to-severe CCI (Hoane, Akstulewicz, & Toppen, 2003; Hoane, Pierce, Holland, & Anderson, 2008; Hoane, Wolyniak, & Akstulewicz, 2005; Hoffman, Fulop, & Stein, 1994).

Avoidance tasks.

In avoidance tasks, non-injured rodents learn to avoid an environment in which an aversive stimulus was previously delivered (Fujimoto et al., 2004). Of the variations on the avoidance task available, one used for the assessment of non-spatial learning and memory after experimental TBI is the passive avoidance (PA) task (Fujimoto et al., 2004). The PA task involves an apparatus divided into two adjoining compartments, one illuminated and one darkened, divided by a guillotine door. The floor of the compartments consists of steel rods capable of delivering an electric footshock to the rodent. On the first day of testing, the rodent is

placed in the illuminated compartment of the apparatus and allowed to explore. When the rodent crosses over into the preferred darkened compartment, it receives an electric footshock. Non-injured rodents learn to associate properties of the darkened compartment with the foot shock and refrain from entering during subsequent testing (Adwanikar, Noble-Haeusslein, & Levin, 2011). The latency to enter into the darkened compartment post shock is used as an indicator of learning and memory (Fujimoto et al., 2004).

While the development of avoidance tasks is credited to Mowrer (1938) as a way to explain phobias in humans, it was first experimentally described in animals by Hudson (1939). In Hudson's study, rats were placed into a cage which contained an electrified food cup. Mounted on the cup was a striped pattern. Contact with the food cup resulted in a severe shock. Testing began one week post shock and continued as the rats aged. Learning established by the shock was measured by approaches to and withdrawals from the striped pattern. Early on, during testing, rats would push bedding material over the striped pattern and withdraw to the other end of the cage. As testing continued, and the rats aged, this behavior decreased. The results of the study showed that rats were able to demonstrate a conditioned fear reaction after one occurrence of an aversive stimulus. Apart from the substitution of a grid floor for the food cup by Maatsch (1959), the protocol for the PA task has remained relatively unchanged since Hudson's study over 70 years ago.

A major benefit of the PA task for the assessment of non-spatial learning and memory in frontally-injured rodents is that impairment in performance on the PA task has been shown to occur after lesions of the frontal cortex (Santucci, Kanof, & Haroutunian, 1989). These findings implicate the frontal cortex as an important area for the acquisition of the PA task. Lending further support to this implication, acquisition of the PA task does not appear dependent on

hippocampal processing as accessed via hippocampal lesion studies (Hamm, Lyeth, Jenkins, O'Dell, & Pike, 1993). Thus, behavioral differences that occur on the PA task between frontally-injured and non-injured rodents can be attributed to damage that occurs to an area of the frontal cortex critical for acquisition of the task. Research utilizing the PA task in frontally-injured rats has shown shorter latencies to enter into the darkened compartment for up to 14 days following both mild and moderate-to-severe lateral FPI compared to non-injured, controls (Hamm et al., 1993; Hogg, Moser, & Sanger, 1998; Yamaguchi et al., 1996).

Object exploration tasks.

Of the variations on the object exploration task, the novel object recognition (NOR) task is used as an assessment of recognition memory after experimental TBI (Ennaceur, Michalikova, Bradford, & Ahmed, 2005; Zhao, Loane, Murray II, Stoica, & Faden, 2012). The NOR task relies on the spontaneous inclination of non-injured rodents to explore novel over familiar objects in an environment and is often used to test a rodent's ability to recognize an object over a variable length of time (Ennaceur et al., 2005; Reger, Hovda, & Giza, 2009). In the NOR task, rodents are placed in an open field and exposed, for a limited period of time, to two identical (sample) objects. The rodents are then returned to their home cage. After a variable length of time has elapsed, the rodents are returned to the open field where they are exposed to two different objects, one identical to the familiar sample object and the other novel. The amount of time spent exploring the novel object compared to the familiar object is used as an indicator of recognition memory (Ennaceur & Delacour, 1988; Ennaceur et al., 2005; Ennaceur, Neave, & Aggleton, 1997).

The NOR task was first described by Ennaceur and Delacour (1988). Prior to the development of the NOR task, memory was frequently assessed in animals using delayed match

and nonmatch-to-sample procedures (Mishkin & Delacour, 1975). As it was difficult to tease apart the extent to which remembering or rule learning contributed to performance on the delayed match and nonmatch-to-sample task, Ennaceur and Delacour developed a one trial memory task based entirely on the spontaneous exploratory behavior of the rat (Ennaceur & Delacour, 1988). Over the course of five experiments, the protocol for the NOR task was refined to include two sample objects (from an original one) and intertrial intervals of one minute to 24 hours. The results of the study showed that normal rats preferentially explore new, compared to familiar, objects. The results of the study also showed that the recognition memory strength, defined as the ratio of time the new object was explored compared to the familiar object, could be influenced by the length of the intertrial interval (ITI) such that shorter ITIs resulted in increased exploration of the new object compared to the familiar.

The benefit of the NOR task is that it does not provoke high stress levels in rodents during testing (Davis, Shear, Chen, Lu, & Tortella, 2010). Another benefit of the NOR task is its versatility in experimental TBI research. While a large number of studies have used the NOR task to assess learning and memory after injury, its ability as an assessment measure of anxiety after post-traumatic stress is just now being discovered. Research utilizing the NOR task has shown significant differences between frontally-injured and non-injured, control rodents and between stressed and non-injured control rodents. According to the research, rats with a penetrating ballistic-like brain injury displayed significantly decreased object exploration at seven days post-injury compared to non-injured, control rats (Davis et al., 2010). In another study, mice with closed head injuries (induced via a weight-drop device) displayed varying degrees of impairment dependent upon injury severity such that mice with mild injuries performed at control level while mice with severe injuries showed no difference between the

novel and familiar object from day three to six post injury (Tsenter et al., 2008). Performance improved from day 14 to 28, but was always significantly worse from the mildly-injured group (Tsenter et al., 2008). In a final study, mice exposed to a stress procedure (i.e., a novel clean cage identical to the home cage) displayed significantly decreased object exploration five days after the final (of 16) stress exposures compared to controls (Scullion, Kendall, Sunter, Marsden, & Pardon, 2009).

Discrimination tasks.

The assessment of decision-making behavior in rodents has been extensively tested under many different paradigms (e.g., conditional discrimination, delayed matching-to-sample, differential reinforcement of low rate of responding, effort-based decision making) in the field of the experimental analysis of behavior (Burkett & Bunnell, 1966; Fantino, 1998; Herrnstein, 1970; Porter, Burk, & Mair, 2000; Walton, Bannerman, Alterescu, & Rushworth, 2003; Williams, 1994). Operant tasks have long utilized behavioral choice paradigms and have come to be known to some extent as the "gold standard" when it comes to evaluating decision-making behavior (Fantino, 1998). However, while these tasks are highly regarded for their ability to parse out subtle differences between animals and treatments, they are frequently both time and equipment intensive. Due to these barriers and the narrow therapeutic window following TBI, time intensive operant paradigms are rarely incorporated into the battery of TBI tests traditionally used. Of the few laboratories that are currently utilizing operant paradigms for the assessment of cognitive behavior after TBI, only one is using the paradigm in frontally-injured rats (Martens, Vonder Haar, Hutsell, & Hoane, 2012a; Martens, Vonder Haar, Hutsell, & Hoane, 2012b).

Regardless of the research field, simple discrimination paradigms have been and remain a popular method for the study of animal decision-making behavior. While the protocol for these tasks remains relatively consistent from laboratory to laboratory, the discriminative stimuli used vary depending on the dominant sensory modality of the animal being studied (Eichenbaum, Fagan, & Cohen, 1986). For the study of decision-making behavior in rodents, whose dominant sensory modality is olfaction, odor discrimination tasks are commonly employed (Slotnick & Katz, 1974). Of the variations on the odor discrimination task, the Dig task is used by the Hoane laboratory for the assessment of decision-making behavior in rodents after frontal TBI. The Dig task is a simple odor discrimination task that requires rats to dig in various scented sands for a reinforcer. Prior to injury, rats are trained to recover and consume a food reinforcer buried in unscented sand. Post injury, rats are tested on a series of simple scent discriminations: simple discrimination 1 (cocoa scented sand [baited] vs. basil scented sand), reversal 1 (basil baited), simple discrimination 2 (cumin scented sand [baited] vs. coffee scented sand), and reversal 2 (coffee baited) until a predetermined criterion level is achieved. Rats unable to reach criterion after a variable number of days are automatically moved to the next discrimination. The accuracy level on each of the scent discriminations is used as an indicator of both decision-making behavior and learning.

The first study to use odor discrimination in the rat was conducted by Jennings and Keefer (1969). In the study, water-deprived rats were presented with a pairing of positive (reinforced) and negative odorants. Correct discrimination of the positive odorant resulted in access to water. The results of the study showed that rats could learn a simple discrimination task using odor cues. The findings by Jennings and Keefer (1969) were supported a few years later by Slotnick and Katz (1974). In the Slotnick and Katz (1974) study, water-deprived rats were

trained on a go, no-go odor discrimination task utilizing 16 odorants. During the task, a positive (reinforced) or negative odorant was presented for 5 seconds. A correct response in the presence of the positive odorant resulted in access to water. Similar to the findings by Jennings and Keefer (1969), rats were able to discriminate between the positive and negative odorants.

The first study to use odor discrimination after TBI was conducted by Martens and colleagues (2012a, b). In the study, the Dig task was used as a measurement of decision-making behavior after severe CCI in rats. The results of the study indicated that the Dig task was successful in assessing deficits after frontal TBI such that frontally-injured rats had significantly lower accuracy levels on all four of the scent discriminations post injury compared to non-injured, sham rats (Martens et al., 2012a; Martens et al., 2012b). While data from the Dig task appears promising, there is concern that the poor performance by the frontal TBI rats was the result of the inability of the rats to perceive the odor of the scented sands (anosmia) due to damage to the olfactory bulb(s) or olfactory tract.

With regard to olfactory disruption after frontal TBI, prior research in the Hoane laboratory, utilizing the same injury coordinates, showed that frontally-injured rats responded strongly to predator scent introduced into the environment (Hoane, unpublished date). In addition, a body of research exist showing that lesions of the olfactory bulb and olfactory tract do not disrupt scent discrimination in rats (Lu & Slotnick, 1998; Slotnick, 1985; Slotnick, Bell, Panhuber, & Laing, 1997). In a study by Lu and Slotnick (1998), rats received a unilateral olfactory bulbectomy and removal of different parts (anterior, lateral, medial, or dorsal) of the contralateral bulb. Post-surgery the rats were tested on a preoperatively learned series of odor detection and discrimination tasks. The results of the study showed that only the rats with less than 21% remaining of the contralateral olfactory bulb showed deficits in the detection and

discrimination tasks. Rats with more than 21% remaining of the contralateral olfactory bulb performed as well or nearly as well as controls. These results demonstrated that relatively small remnants of one olfactory bulb can be sufficient for the detection of odor. In another study by Slotnick and Berman (1980), rats received lesions of the olfactory peduncle and part of the lateral olfactory tract and anterior commissure. Post-surgery the rats were tested on a preoperatively learned series of odor detection and discrimination tasks. The results of the study showed that lesions of the olfactory peduncle and lateral olfactory tract had little or no effect on odor detection and discrimination performance in the lesioned rats. While these animals had deficits in retention and relearning of the odor tasks, the lesions did not produce anosmia. In a final study by Slotnick (1985), rats received a posterior lateral olfactory tract and anterior amygdala lesion. Post-surgery the rats were tested on a preoperatively learned series of odor detection and discrimination tasks. The results showed that the lesioned rats had perfect or near-perfect retention of the detection task and there were no discernible differences between groups in learning new odor discriminations. With the compilation of results from Slotnick and colleagues, it seems unlikely that potential damage to the olfactory bulbs or olfactory tract after frontal TBI would result in an inability of the frontal TBI rats to detect odors on the Dig task. Thus, the poor performance on the Dig task by the frontally-injured rats appears to be related to a learning deficit as a function of damage to the frontal cortex and not anosmia.

Introduction Summary

Traumatic brain injury is a major health problem facing the United States. It has the ability to result in a multitude of deficits in sensory, motor, and cognitive functioning following injury that can drastically affect the ability of the individual to remain independent. The development of standardized treatments for victims of TBI requires the use of animal models.

While an extensive number of tasks have been developed for the assessment of sensory and motor functioning after injury, tasks assessing frontally-mediated behavior are lacking. Of the tasks that exist to assess frontally-mediated behavior, few are in current use. Of the few that are in use, none have been used to compare across differing degrees of injury severity. The goal of the present study will be to compare the following tasks: MWM, PA, NOR, and Dig in frontally-injured rats with two types of injury severity: mild and moderate-to-severe. The purpose of this comparison will determine the following: 1) the ability of each task to assess deficits after frontal injury, 2) the sensitivity of each task to assess deficits in mild and moderate-to-severe injuries, and 3) the influence of each task on a secondary task which consisted of the MWM, PA, NOR, and Dig task.

Specific Aims

1. Determine whether rats with frontal brain injuries demonstrate deficits on the MWM, PA, NOR, and Dig tasks.
2. Determine the sensitivity of the MWM, PA, NOR, and Dig tasks to assess deficits in mild and moderate-to-severe injuries.
3. Determine the extent of the influence of the MWM, PA, NOR, and Dig tasks on a secondary task. The secondary task consisted of the MWM, PA, NOR, and Dig tasks.

Research Hypotheses

1. Based upon the aforementioned literature, it is hypothesized that performance deficits on the four tasks will occur in frontally-injured rats compared to non-injured, sham rats. It is further hypothesized that the performance deficits will occur in the following manner:
    a. Injured rats will demonstrate higher latencies in the MWM, lower latencies in the PA task, explore the novel object less compared to the familiar object in the NOR task,

and show deficits in discrimination ability on all four scent discriminations in the Dig task compared to non-injured, sham rats.

2. Based upon differences in injury severity, it is hypothesized that mildly-injured rats will exhibit fewer performance deficits on all four tasks compared to rats with moderate-to-severe injuries. It is further hypothesized that the extent of the performance deficits will differ depending upon the behavioral task used such that:
    a. The MWM and Dig task, as a result of task difficulty, will be more sensitive at detecting performance deficits compared to either the PA or NOR task which rely heavily upon the exploratory behavior of the animal.
3. Based upon task protocol and task difficulty, it is hypothesized that some of the behavioral tasks will have an influence upon the secondary task it is paired with. It is further hypothesized that this influence will occur in the following manner:
    a. As a result of the task difficulty and the long testing protocol, rats assigned to the MWM or Dig task will show improved performance on the assigned secondary task. It is also believed that, as a result of the fear response elicited by the foot shock, rats assigned to the PA task will show a decrease in performance on the assigned secondary task.
4. Using a cresyl violet nissl stain to assess cavitation, it is hypothesized that mildly-injured rats will have less brain damage (higher brain volumes) compared to rats with moderate-to-severe injuries.

CHAPTER 2

METHODS

Animals

Seventy-two Sprague-Dawley rats, approximately three months of age with an average weight of 325 g (+/- 15 g), were included in this study. All experimental procedures described in this study were reviewed and approved by the Institutional Animal Care and Use Committee and the study was conducted in a facility certified by the American Association for the Accreditation of Laboratory Animal Care. Rats were housed singly in standard cages on a 12-hour light/dark cycle with *ad libitum* access to water. Rats were food restricted to 15-20 g of food per day and maintained at 85% of free feeding body weight during training and testing sessions. Training and testing were conducted during the light cycle. One week prior to the start of Dig training, rats were randomly assigned to one of six behavioral testing groups (see **Behavioral Testing**). Within each behavioral testing group, rats were randomly assigned to one of three experimental groups: mild TBI (mTBI), moderate-to-severe TBI (TBI), or intact sham (sham).

Surgery

Surgical procedures were performed under aseptic conditions according to previous studies (Hoane et al., 2003; Hoane, Lasley, & Akstulewicz, 2004; Hoane et al., 2005). Rats were anesthetized under a combination of Isofluorane (2-4%) and oxygen (0.8 L/min) and placed in a stereotaxic device. Body temperature was maintained at 37° C using a heated surgical stage. Rats were randomly assigned to three groups: mild bilateral, frontal CCI (mTBI; n = 24), moderate-to-severe bilateral, frontal CCI (TBI; n = 24), or intact sham (sham; n = 24). The procedure began with a midline incision through the skin and underlying fascia. A craniotomy measuring 6.0 mm in diameter was created using an electronic microdrill positioned at AP = +3.0, ML = 0.0 relative

to bregma. A drill bit was used and care was taken to prevent damage to the dura and cortex. The cortical region containing the frontal cortex was exposed. A 5.0 mm stainless steel impactor tip attached to an electromagnetic impactor (myneurolab.com) was used to induce the injury. The cortex of the mTBI group was impacted at a velocity of 1.5 m/s to a compression depth of 2.0 mm with a contact time of 0.5 seconds. The cortex of the TBI group was impacted at a velocity of 3.0 m/s to a compression depth of 2.5 mm with a contact time of 0.5 seconds. Following injury, bleeding was controlled and the incision sutured closed. Afterwards, the rats were placed in a heated recovery cage until locomotion returned. The sham group received anesthesia and an incision, but no craniotomy. All rats were placed on free feeding for four days following surgery.

Behavioral Testing

One week prior to dig training and 11 days prior to surgery, rats were food restricted to 15-20 g of food per day and maintained at 85% of free feeding body weight during training and testing sessions. Rats were then randomly assigned to one of six behavioral testing groups (Table 1). The experimental timeline consisted of six animal squads (n = 12 per squad) run over the course of six months. In order to avoid confounds in behavioral testing, all four behavioral tasks were accounted for by assigning rats, from each squad, to each behavioral task and running the tasks concurrently. When applicable, each testing day started in the morning with the Dig task, followed by the NOR task, followed by the MWM, and was completed in the late afternoon with the PA task (Figure 1).

Dig task.

The Dig task was utilized to assess cognitive behavior on days 7-47 post injury. Testing was conducted in a bank of chambers (30.5 x 25.4 x 23.2 cm) located in a dimly lit room. A piece of opaque Plexiglas (10 x 22 cm) with two circular holes (5.5 cm in diameter) to provide

locations for scent cups were placed in each chamber (Figure 2). The scent cups were constructed from PVC pipe end-caps (6 cm tall and 5.5 cm in diameter). The scented sands that were used in this task were selected from previous studies (Kaiser & Means, 2006; Martens et al., 2012a; Martens et al., 2012b) and consisted of cocoa, basil, cumin, and coffee. Each of the scented sands was mixed at a ratio of 1g of odorant to 110 g of unscented sand. The pairing of each scented sand and the order in which each scented sand pairing was presented was done randomly. Fruit loop cereal pieces were used as the reinforcer.

Dig training.

The first step of dig training consisted of habituation to the chamber and magazine training of the scent cups. During this step, the scent cups were only filled with the reinforcer. Rats were placed in the chambers and allowed to freely consume the reinforcer for 30 minutes. The first step was used to establish both scent cups as a source for reinforcement.

The second step consisted of training the rats to uncover the reinforcer from unscented sand. During this step, multiple reinforcers were buried in unscented sand in such a way that half protruded from the sand. As the rats consumed the visible reinforcers, additional ones were uncovered and consumed. To correct for side preference, manually uncovering the reinforcer in the non-preferred cup was sufficient to generate roughly equivalent digging across the two scent cups. The second step was used to establish digging behavior.

Once digging behavior was established, the third step consisted of training rats to recover a single reinforcer from the bottom of each scent cup. During this step the depth of the reinforcer was gradually increased from just under the surface of the sand to resting on the bottom of the scent cup. Rats were required to retrieve the reinforcer from the bottom of each scent cup. The third step was used to establish complete digging behavior.

Once digging was acquired, the final step was to familiarize rats with the discrimination testing trial structure. During this step rats were required to retrieve the reinforcer from the bottom of each scent cup within 30 seconds. Upon retrieving and consuming both reinforcers, or after 30 seconds had elapsed, the rats were placed into a separate holding cage. This continued for the number of trials necessary to meet criterion (recovering both reinforcers, within 30 seconds, eight consecutive times). Upon reaching criterion, rats were advanced to surgery.

Post-injury discrimination testing.

Rats assigned to the Dig task were tested seven days post injury on the following discriminations: simple discrimination 1 (cocoa scented sand [baited] vs. basil scented sand), reversal 1 (basil baited), simple discrimination 2 (cumin scented sand [baited] vs. coffee scented sand), and reversal 2 (coffee baited). During the post-injury discrimination testing rats were given 30 seconds (per trial) to dig in one of the scent cups. Correct choices were reinforced by consumption of the reinforcer followed by an ITI. Incorrect choices were punished by an immediate ITI. Rats were not allowed to correct for incorrect choices. The ITIs lasted 30 seconds and occurred in a separate holding cage. Rats received a clean Plexiglass/scent cup setup for each trial and the testing chambers were cleaned with 70% ethanol (EtOH) between each testing session to remove all olfactory cues. Rats received eight trials per session, one session per day. The criterion level was performance at 85% or greater for three consecutive days. Rats unable to reach criterion after 10 days were considered unable to learn/relearn the discrimination and moved on to the next scent.

Novel object recognition (NOR) task.

The NOR task was utilized to assess object recognition memory on days 7-12 post injury as originally described in a previous study (Ennaceur & Delacour, 1988; Ennaceur et al., 2005).

The testing arena consisted of a Plexiglas chamber (25.4 x 47 cm; San Diego Instruments [SDI], San Diego, CA) located in a dimly lit room (Figure 3). The objects used in this task consisted of glass bottles for the first day of testing and wooden shapes for the second day of testing (Figure 4). All objects were attached to the floor of the testing arena via Velcro to ensure that they would not be displaced by the rats. The arena and all objects were cleaned with 70% EtOH between each testing session to remove all olfactory cues.

Habituation phase.

On post-injury days 7-9 rats were allowed to freely explore the empty arena. The habituation phase consisted of three, ten minute sessions administered once per day.

Testing phase.

On post-injury days 10 and 12 rats were tested on the sample (T1) and choice (T2) trials of the NOR task. During T1, each rat was placed into the arena containing two identical, sample objects (tall glass bottles during testing day 10; wooden spheres during testing day 12). The objects were placed close (approximately 10 cm) to the two adjacent corners of the arena. The rats were allowed to explore the arena for three minutes before being returned to their home cage. After an ITI of 1 hour, T2 was administered. The procedure for T2 was identical to that of T1 except a novel object (short glass bottle during testing day 10; wooden cube during testing day 12) was substituted for one of the sample objects. The novel object was similar in material and color, yet discernibly different from the sample objects. The position of the novel object was counterbalanced. To avoid olfactory trails, the sample object presented during T2 was a duplicate of the sample object presented in T1.

For the purposes of the task, object exploration was defined as interaction with the object via sniffing, touching, or rearing.

Morris water maze (MWM).

The MWM was utilized to assess spatial learning and memory on days 11-17 post injury as described in previous studies (Hamm et al., 1996; Hoane, Tan, Pierce, Anderson, & Smith, 2006; Kaufman et al., 2010). The water maze consisted of a blue fiberglass tank (76 cm tall and 1.5 m in diameter) filled with water to a depth of 32 cm and maintained at 24° C and located in a dimly lit room (Figure 5). A clear Plexiglas platform (10 x 10 cm) was submerged 1 cm below the surface of the water. Video tracking system and SMART tracking software (SDI) were used to record the path length and latency of the animals to the submerged platform. For training and testing purposes, a trial was terminated when an animal either reached the submerged platform or when 90 seconds had elapsed. Rats unable to reach the platform after 90 seconds were guided by hand to the platform. Each animal was allowed to remain on the platform for 10 seconds and was then placed in a warm holding cage for 15 minutes prior to starting the next trial. There were two phases of testing: retrograde amnesia and working memory.

Retrograde amnesia task.

Four days prior to surgery rats assigned to the MWM were trained on the reference memory paradigm of the MWM. For this task, the clear Plexiglas platform was submerged in the center of the northwest quadrant of the water maze. Each rat was lowered in, facing the wall, from one of four randomly ordered release points (NE, SE, SW, NW). Rats received four trials per session (15-minute ITIs), one session per day for four consecutive days. On post-injury day 11 rats were tested on the retrograde amnesia paradigm of the MWM, which tests for the ability of the rat to remember the previously learned, fixed location. For the retrograde amnesia paradigm the rats received one session (four trials) of testing.

Working memory task.

On post-injury days 12-17 rats were tested on the working memory paradigm of the MWM. The procedure for this task was identical to that of the reference memory paradigm except the Plexiglas platform was placed in a randomized new location at the start of each testing day.

Passive avoidance (PA) task.

The PA task was utilized to assess non-spatial learning and memory on days 9-14 post injury as described in accordance to previously detailed methods (Hamm et al., 1993) with alterations in the number of testing days. The passive avoidance device consisted of two adjoining compartments, one illuminated (20.3 x 15.9 x 21.3 cm) and one darkened (20.3 x 15.9 x 21.3 cm), divided by a guillotine door (Med Associates Inc., St. Albans, VT; Figure 6). The floor of the compartments consisted of steel rods capable of delivering an electric footshock. The electric shock was delivered by a Programmable Animal Shocker (Med Associates Inc.). The device was cleaned with 70% EtOH between each testing session to remove all olfactory cues. A stop watch was used to record the amount of time spent in the illuminated compartment.

Procedure.

On post-injury day nine each rat was placed in the illuminated compartment. As soon as the rat crossed into the darkened compartment, the guillotine door was closed and the animal received an electric footshock (1.5 mA, 1 second). Following the footshock, the rat was removed from the apparatus and returned to its home cage.

On post-injury days 10-14 rats were tested for the retention of the PA response. Rats were placed in the illuminated compartment and the latency for the animal to cross into the darkened compartment was recorded. No shock was delivered during testing. Animals that did not

crossover into the darkened compartment were allowed to remain in the illuminated compartment for the full 5 minutes and assigned a latency of 300 seconds. Rats received one trial per session, one session per day for five days.

Histology

On day 31 post injury, rats were anesthetized with a lethal dose of sodium pentobarbital (Euthasol, Virbac Animal Health; 0.3 mL, i.p.). Once eye-blink and pedal responses disappeared, rats were transcardially perfused with ice cold 0.9% phosphate buffered saline (PBS), followed by 10% phosphate buffered formalin (PBF). After the brains were removed from the skull, they were post-fixed for one day in PBF. Brains were then placed in a 30% sucrose solution for three days. Following this, serial sections (40 µm thick) were sliced using a sliding microtome with an electronic freezing stage and collected in a phosphate buffer solution.

Cresyl-violet staining.

A series of slices transversing the lesion cavity (+0.50 to +4.20 mm relative to bregma) and matching sections from shams were mounted on gelatin-subbed slides for staining. The slices were rehydrated by sequential washes of xylene, 95% EtOH, 70% EtOH, 50% EtOH, and distilled water. The slices were then stained with cresyl-violet and dehydrated by sequential washes of distilled water, 50% EtOH, 70% EtOH, 95% EtOH, 95% EtOH + Glacial Acetic Acid, 100% EtOH, and xylene. The slices were then cover-slipped and prepared for light microscopy to examine the extent of the lesion.

Lesion analysis.

Five sections transversing the lesion cavity were selected for analysis (+0.50, +1.70, +2.70, +3.70, and +4.20 mm relative to bregma). The extent of the lesion was analyzed with an Olympus microscope (BX-51) and an Olympus 13.5-megapixel digital camera (DP-70). The

areas of the lesioned tissue were calculated using imaging software (Image Tool) according to previous studies (Hoane, Kaufman, Vitek, & McKenna, 2009; Hoane et al., 2008). For the injured animals, the total brain areas were calculated by adding the two hemispheres together. The Calvalieri method was then used to calculate the volume (Coggeshall, 1992). The number of sections (5) was then multiplied by the thickness (40 μm) and the average area of the brain sections. This subsequent volume was then compared to the sham group.

Data Analysis

Dig task.

The accuracy of the rats on each of the four scent discriminations was recorded. All analyses were run based on the assumptions of normality being met. A linear mixed effects model was used to assess rat performance as a function of Experimental Group (mTBI, TBI, sham) x Secondary Task (NOR, MWM, PA) x Discrimination (simple discrimination 1, reversal 1, simple discrimination 2, reversal 2) x Day x Trial (the latter two IVs were entered as continuous linear predictors). Planned comparisons were used to assess specific group differences. A significance level of p less than .05 was used for all statistical analyses.

Novel object recognition (NOR) task.

The percentage of time the rats explored the novel object was recorded for each testing day. All analyses were run based on the assumptions of normality being met. A linear mixed effects model was used to assess rat performance as a function of Experimental Group (mTBI, TBI, sham) x Secondary Task (Dig, MWM, PA) x Day (the latter IV was entered as a continuous linear predictor). Planned comparisons were used to assess specific group differences. A significance level of p less than .05 was used for all statistical analyses.

Morris water maze (MWM).

The latencies for the rats to reach the platform were recorded for each testing day. All analyses were run based on the assumptions of normality being met. A linear mixed effects model was used to assess rat performance as a function of Experimental Group (mTBI, TBI, sham) x Secondary Task (Dig, NOR, PA) x Testing (Pre-injury Testing, Post-injury Testing) x Day (the latter IV was entered as a continuous linear predictor). Planned comparisons were used to assess specific group differences. A significance level of p less than .05 was used for all statistical analyses.

Passive avoidance (PA) task.

The latencies for the rats to enter into the darkened compartment were recorded for each testing day. All analyses were run based on the assumptions of normality being met. A linear mixed effects model was used to assess rat performance as a function of Experimental Group (mTBI, TBI, sham) x Secondary Task (Dig, NOR, MWM). Planned comparisons were used to assess specific group differences. A significance level of p less than .05 was used for all statistical analyses.

Lesion analysis.

All analyses were run based on the assumptions of normality being met. The brain volume of each rat was analyzed using a one-way between subjects ANOVA. Planned comparisons were used to assess specific group differences. A significance level of p less than .05 was used for all statistical analyses.

CHAPTER 3

RESULTS

Of the 72 rats used in this study, eight were excluded from analyses based upon a predetermined anatomical criterion (absence of sufficient bilaterality of the craniotomy). The distribution of the remaining 64 rats by both behavioral task and experimental group are shown in Table 2.

Behavioral Results

Dig task.

The accuracy of the rats on each of the four scent discriminations was recorded. A linear mixed effects model was used to assess rat performance as a function of Experimental Group (mTBI, TBI, sham) x Secondary Task (NOR, MWM, PA) x Discrimination (simple discrimination 1, reversal 1, simple discrimination 2, reversal 2) x Day x Trial (the latter two IVs were entered as continuous linear predictors). The fixed effects from the model are shown in Table 3. The effects of Discrimination, Day, and Trial were allowed to randomly vary across subjects, capturing both within-subject dependencies and individual differences (Gelman & Hill, 2006).

There were significant main effects of Experimental Group, $F(2, 22.73) = 5.33$, $p = .013$, Discrimination, $F(3, 71.75) = 11.41$, $p < .0001$, Day, $F(1, 23.5) = 147.55$, $p < .0001$, and Trial, $F(1, 65.62) = 39.29$, $p < .0001$. There were also significant interactions of Experimental Group*Day, $F(2, 23.53) = 7.01$, $p = .004$, Discrimination*Day, $F(3, 63.39) = 6.32$, $p < .0008$, Day*Trial, $F(1, 153.8) = 15.12$, $p = .0001$, and Secondary Task*Day*Trial, $F(2, 112.6) = 3.32$, $p = .040$.

For the main effect of Experimental Group, planned comparisons indicated that, overall, the sham group (*LSM* = 0.81) was significantly more accurate on the scent discriminations compared to the TBI group (*LSM* = 0.62), $p < .05$. Additionally, the mTBI group (*LSM* = 0.77) was significantly more accurate on the scent discriminations compared to the TBI group, $p < .05$. No differences in accuracy occurred between the sham and mTBI groups, $p > .05$. For the main effect of Discrimination, planned comparisons indicated that rats were significantly more accurate on simple discrimination 2 (*LSM* = 0.88) and significantly less accurate on reversal 1 (*LSM* = 0.58), $p < .05$. No differences in accuracy occurred on either simple discrimination 1 (*LSM* = 0.73) or reversal 2 (*LSM* = 0.77), $p > .05$ (Figure 7). For the main effect of Day, the positive slope (slope = .101, *SE* = .008) indicated that overall accuracy increased across testing days. The largest improvement in accuracy occurred from testing days 1-5 (Figure 8). For the main effect of Trial, the positive slope (slope = .017, *SE* = .003) indicated that accuracy increased within each testing day; however, the effect was relatively minor (Figure 9).

For the Experimental Group*Day interaction, the improvement in accuracy across testing days was equal for both the sham (slope = .123) and mTBI groups (slope = .124), SE for the slope difference was .012. The similarities in accuracy improvement occurred from testing days 1-5. No significant improvement in accuracy across testing days occurred for the TBI group (slope = .056) (Figure 8). For the Discrimination*Day interaction, the improvement in accuracy across testing days was largest on reversal 1 (slope = .127) followed by reversal 2 (slope = .114), SE for the slope difference was .008. A significant improvement in accuracy across testing days also occurred on simple discrimination 1 (slope = .089, *SE* = .008) and simple discrimination 2 (slope = .074, *SE* = .008) (Figure 8). For the Day*Trial interaction, a significant improvement in accuracy occurred across trials across testing days (slope = .096, *SE* = .001); however, the effect

was minor with the largest improvement in accuracy occurring early on during testing (Figure 10). For the Secondary Task*Day*Trial interaction, rats also assigned to the NOR task (slope = .124) had a significant improvement in accuracy across trials across testing days compared to rats also assigned to either the MWM (slope = .105) or the PA task (slope = .074), SE for the slope difference was .002 (Figure 11).

Novel object recognition (NOR) task.

The percentage of time the rats explored the novel object was recorded for each testing day. A linear mixed effects model was used to assess rat performance as a function of Experimental Group (mTBI, TBI, sham) x Secondary Task (Dig, MWM, PA) x Day (the latter IV was entered as a continuous linear predictor). The fixed effects from the model are shown in Table 4. The effect of Day was allowed to randomly vary across subjects, capturing both within-subject dependencies and individual differences.

There was a significant main effect of Day, $F(1, 18.27) = 17.95$, $p = .0005$. No other effects were significant.

For the main effect of Day, the positive slope (slope = .100, *SE* = .024) indicated that exploration of the novel object was greater on post-injury testing day 12 compared to post-injury testing day 10 (Figure 12).

The type of interaction that occurred with the novel object (number of sniffs, touches, and rears) was recorded for each testing day. A linear mixed effects model was used to assess rat performance as a function of Experimental Group (mTBI, TBI, sham) x Secondary Task (Dig, MWM, PA) x Day (the latter IV was entered as a continuous linear predictor). The effect of Day was allowed to randomly vary across subjects, capturing both within-subject dependencies and individual differences.

There was a significant main effect of Day for both time spent sniffing, $F(1, 22.09) = 6.85$, $p = .016$, and time spent rearing toward the novel object, $F(1, 16.75) = 4.73$, $p = .044$. Time spent touching the novel object was not significant, $F(1, 19.44) = 1.89$, $p = .184$, (Figure 13). No other effects were significant.

Morris water maze (MWM).

An analysis was performed on post-injury rat swim speed (distance divided by latency) to examine consistency between the two measures recorded. Prior to analyzing, a log transformation was performed on the latency and distance data points in order to correct for skewness in the distribution of the data. A linear mixed effects model was used to assess rat performance as a function of Experimental Group (mTBI, TBI, sham). No experimental group differences were found, $F(2, 23) = 1.45$, $p = .255$, indicating that motor deficits were not a significant factor in latency scores. As a result of reliability between the two measures, remaining MWM analyses used latency as the dependent variable.

The latencies for the rats to reach the platform were recorded for each testing day. Prior to analyses, a log transformation was performed on the latency data points in order to correct for skewness in the distribution of the data. A linear mixed effects model was used to assess rat performance as a function of Experimental Group (mTBI, TBI, sham) x Secondary Task (Dig, NOR, PA) x Testing (Pre-injury Testing, Post-injury Testing) x Day (the latter IV was entered as a continuous linear predictor). The fixed effects from the model are shown in Table 5. The effects of Testing and Day were allowed to randomly vary across subjects, capturing both within-subject dependencies and individual differences.

There were significant main effects of Experimental Group, $F(2, 26.86) = 7.07$, $p = .003$, Testing, $F(1, 27.36) = 8.91$, $p = .001$, and Day, $F(1, 36.75) = 34.76$, $p < .0001$. There was also a significant interaction of Testing*Day, $F(1, 32.72) = 16.95$, $p = .0002$.

For the main effect of Experimental Group, a planned comparison indicated that, overall, the sham group (*LSM* = 13.39) took significantly less time to reach the platform compared to the TBI group (*LSM* = 22.32), $t(26.86) = 14.13$, $p = .001$. No differences in time occurred between the sham and mTBI groups (*LSM* = 17.03), $t(26.86) = 3.13$, $p = .088$, or the mTBI and TBI groups, $t(26.86) = 3.60$, $p = .069$, (Figure 14). For the main effect of Testing, rats took significantly less time to reach the platform during pre-injury testing (*LSM* = 14.42) compared to post-injury testing (*LSM* = 20.52), $F(1, 27.36) = 8.91$, $p = .001$ (Figure 14). For the main effect of Day, the negative slope (slope = -.127, *SE* = .022) indicated that performance of the rats improved across each testing day. The improvement in performance was greatest pre-injury (Figure 14).

For the Testing*Day interaction, the improvement in performance across testing days was larger pre-injury (slope = -.238) compared to post-injury (slope = -.015), *SE* for the slope difference was .027 (Figure 14).

Passive avoidance (PA) task.

The latencies for the rats to enter into the darkened compartment were recorded for each testing day. Prior to analyses, a log transformation was performed on the latency data points in order to correct for skewness in the distribution of the data. A linear mixed effects model was used to assess rat performance as a function of Experimental Group (mTBI, TBI, sham) x Secondary Task (Dig, NOR, MWM). The fixed effects from the model are shown in Table 6.

There were significant main effects of Experimental Group, $F(2, 24) = 12.03$, $p = .0002$, and Secondary Task, $F(2, 24) = 3.68$, $p = .040$.

For the main effect of Experimental Group, a planned comparison indicated that, overall, the sham group (*LSM* = 133.96) took significantly more time to enter the darkened compartment compared to both the mTBI (*LSM* = 43.35), $t(24) = 7.63$, $p = .011$, and TBI groups (*LSM* = 19.25), $t(24) = 23.73$, $p < .0001$. No differences in time occurred between the mTBI and TBI groups, $t(24) = 3.76$, $p = .064$, (Figure 15). For the main effect of Secondary Task, a planned comparison indicated that rats also assigned to the Dig task (*LSM* = 89.83) took significantly more time to enter the darkened compartment compared to rats also assigned to the MWM (*LSM* = 31.07), $t(24) = 6.75$, $p = .016$ (Figure 16). No differences in time occurred between rats also assigned to the NOR task (*LSM* = 40.07), $t(24) = 3.91$, $p = .059$.

The same linear mixed effects model was used to assess the fear response of each rat as a function of Urination (presence of urine, absence of urine) x Defecation (amount of fecal boli).

Urination and defecation.

Regarding urination, a significant interaction of Experimental Group*Day, $F(2, 24) = 4.64$, $p = .020$, occurred. No other effects were significant.

For the Experimental Group*Day interaction, across testing days, urine production for the sham group decreased (slope = -.103) while the mTBI (slope = .007) and TBI groups (slope = .000) had no change, SE for the slope difference was .024, (Figure 17).

No difference in the total number of fecal boli produced occurred.

Histological Results

Lesion analysis.

A one-way between subjects ANOVA was used to assess differences in brain volume between rats perfused on post-injury day 18 compared to post-injury day 47. No differences in brain volume occurred between the mTBI rats perfused on post-injury day 18 and those perfused on post-injury day 47, $F(1, 18) = 0.08$, $p = .788$. No differences in brain volume also occurred between the TBI rats perfused on post-injury day 18 and those perfused on post-injury day 47, $F(1, 18) = 0.03$, $p = .860$. As a result of these findings, remaining analyses used a combination of both post-injury brains.

The brain volume was calculated for each rat and analyzed using a one-way between subjects ANOVA. The analysis showed a significant main effect of Experimental Group, $F(2, 61) = 60.93$, $p < .001$. A planned comparison indicated that both the mTBI ($M = 9.94$), $t(61) = 8.74$, $p < .001$, and TBI groups ($M = 9.55$), $t(61) = 9.98$, $p < .001$, had significant reductions in total brain volume compared to the sham group ($M = 12.70$). No differences in lesion size occurred between the mTBI and TBI groups, $t(61) = 1.19$, $p = .241$, (Figure 18). Representative histological images are shown in Figure 19.

Relationship between Behavioral Performance and Brain Volume

The findings that differences in behavioral performance occurred between the mTBI and TBI rats on the Dig task without there being any differences in brain volume prompted further analyses. Thus, the relationship between performance of the mTBI and TBI rats on the Dig task and their respective brain volumes was assessed.

Dig task.

A Pearson's correlation was run to determine the relationship between brain volume and accuracy on each of the four scent discriminations (simple discrimination 1, reversal 1, simple discrimination 2, reversal 2) between mTBI and TBI rats. A positive correlation occurred on reversal 2, $r = .522$, $p = .022$, indicating that rats with less brain damage (higher brain volumes) performed more accurately on the final discrimination compared to rats with more brain damage (lower brain volumes) (Table 7). No relationship between brain volume and accuracy occurred on any other discrimination.

CHAPTER 4

DISCUSSION

Four primary research hypotheses were made regarding the current study. (1) Performance deficits on the four tasks (Dig, novel object recognition [NOR], Morris water maze [MWM], passive avoidance [PA]) would occur in frontally-injured rats compared to non-injured, sham rats. (2) Mildly-injured (mTBI) rats would exhibit fewer performance deficits on all four tasks compared to moderate-to-severely-injured (TBI) rats. (3) Rats assigned to either the MWM or Dig task would show improved performance on the assigned secondary task while rats assigned to the PA task would show a decrease in performance on the assigned secondary task. (4) MTBI rats would have less brain damage (higher brain volumes) compared to TBI rats. Of the four primary research hypotheses, three were partially supported by the findings of this study. The following section will first summarize and then address these findings.

Summary of the Findings

Hypothesis 1.

It was hypothesized that performance deficits on the four tasks would occur in frontally-injured rats compared to non-injured, sham rats. The results from the study indicated that an injury effect was shown in three of the four tasks (Dig, MWM, PA).

On the Dig task, sham rats were significantly more accurate on the scent discriminations compared to TBI rats. Sham rats also demonstrated improved performance across testing compared to TBI rats. This improvement was most transparent during the reversal performance (52% accuracy during reversal 1, 74% accuracy during reversal 2).

On the MWM, sham rats were significantly faster at locating the platform throughout testing compared to TBI rats.

On the PA task, sham rats took significantly more time to enter the darkened compartment compared to both mTBI and TBI rats. Sham rats also demonstrated significant decreases in urine production across testing compared to both mTBI and TBI rats.

Hypothesis 2.

It was hypothesized that mTBI rats would exhibit fewer performance deficits on all four tasks compared to TBI rats. The results from the study indicated that only one of the four tasks was sensitive enough to detect differences in injury severity.

On the Dig task, mTBI rats were significantly more accurate on the scent discriminations compared to TBI rats. MTBI rats also demonstrated improved performance across testing compared to TBI rats.

Hypothesis 3.

It was hypothesized that rats assigned to the MWM or the Dig task would show improved performance on an assigned secondary task while rats assigned to the PA task would show decreased performance on an assigned secondary task. The results from the study indicated that this effect occurred, but only in one of the hypothesized three tasks.

On the Dig task, rats also assigned to the NOR task had a significant improvement in accuracy across trials across testing days compared to rats also assigned to the MWM or PA task.

On the PA task, it was believed that rats also assigned to the Dig task took significantly more time to enter the darkened compartment compared to rats also assigned to the MWM. In graphing out the data, however, it appears that this finding was the result of a floor effect caused by the limited variability of the rats also assigned to the MWM (Figure 16).

Hypothesis 4.

It was hypothesized that mTBI rats would have less brain damage (higher brain volumes) compared to TBI rats. Interestingly, no differences in lesion size occurred between the mTBI and TBI rats (Figure 18).

Discussion of the Findings

The injury that was administered in this study resulted in cell death and tissue loss to a range of medial prefrontal cortical (mPFC) regions including the cingulate, prelimbic, and infralimbic cortex (Figure 20). As a direct result, input from various subcortical regions (e.g., amygdala, hippocampal formation) and neuromodulatory systems was also lost. As evident by the vast amount of research on the mPFC, damage to any of these regions can lead to impairments in learning, memory, and inhibition (Dalley, Mar, Economidou, & Robbins, 2008; Euston, Gruber, & McNaughton, 2012; Kolb, 1984, 1990). What follows is a discussion of the findings of this study, specifically how impairments in learning, memory, and inhibition caused by damage to regions of the mPFC can best explain the performance of the frontally-injured rats on the four behavioral tasks.

Performance by the frontally-injured rats on the Dig task was varied. The results indicate that mTBI rats were significantly more accurate than TBI rats on the scent discriminations. The distribution of data for each mTBI rat supports this finding (Figure 7). As a group, the mTBI rats performed similarly well on the Dig task. The results also indicate that TBI rats made no improvements in accuracy across testing days and performed at chance level for three of the four scent discriminations (54% accuracy on simple discrimination 1, 55% accuracy on reversal 1, 58% accuracy on reversal 2). This indicates that TBI rats were unable to learn the changing contingencies of the different discriminations and, instead, adopted a bias toward one side (i.e.,

only dug in the right or left scent cup for each session) ensuring reinforcement half of the time. However, the distribution of data for each TBI rat indicates learning slowly occurred (54% accuracy on simple discrimination 1, 68% accuracy on simple discrimination 2) for nearly half of the rats (Figure 7). For the few rats that were unable to learn the changing contingencies of the Dig task, the positive correlation between performance and brain volume (Table 7) indicates that these rats had more severe brain damage affecting both the dorsal (cingulate) and ventral mPFC (prelimbic cortex, infralimbic cortex). While damage to the dorsal mPFC did not appear to influence performance on the Dig task as evident by mTBI rat performance, damage to the ventral mPFC may have as damage to this region has been shown to produce impairments in both reinforcement learning and response inhibition. Research has shown that dopaminergic signals from the ventral tegmental area to the ventral mPFC implements reward learning by signaling whenever behavior should be redirected from a previously reinforcing stimulus to one in which a more valuable goal can be achieved. This signal prompts the exploration of the more valuable goal while interference of this signaling has been shown to lead to perseverative behavior (i.e., exploitation) (Cohen, McClure, & Yu, 2007; Montague, Hyman, & Cohen, 2004). Research has also shown that lesions of the ventral mPFC leads to increases in both impulsive choice as assessed by delay discounting (i.e., preference for smaller, immediate over larger, delayed rewards) and increases in premature responding accompanied by fast response latencies as assessed by reaction time tasks (Chudasama et al., 2003; Churchwell, Morris, Heurtelou, & Kesner, 2009; Narayanan, Horst, & Laubach, 2006). For the rats that performed at chance on the scent discriminations of the Dig task, it is unclear whether impairments in reinforcement learning, impairments in behavioral inhibition, or a third variable accounted for the performance deficits. Interference of the signal from the ventral tegmental area due to ventral mPFC cell loss

would account for the perseverative behavior (i.e., side bias) in these rats. However, as these rats were not allowed to correct for incorrect choices and as the scent cups were generally approached from the same direction, impairments in behavioral inhibition (i.e., rats consistently digging in the first scent cup that was approached) would also account for the behavior. Without a specific task to rule out impulsivity, it is difficult to assert that either impairment attributed to the rats' behavior. However, further support would be lent to the findings of the Dig task by assessing the extent to which either impairment contributed to the deficits also see on the NOR and PA tasks.

Performance by the rats on the NOR task was comparable. The results indicate that all rats spent more time exploring the novel object on post-injury testing day 12 (Figure 12). These findings support the notion that regions of the mPFC are not important for the discrimination of novel or familiar objects (Barker, Bird, Alexander, & Warburton, 2007; Cross, Brown, Aggleton, & Warburton, 2012; Nelson, Cooper, Thur, Marsden, & Cassaday, 2011). These findings also indicate something reinforcing about the properties of the object used on that testing day, a wooden cube (Figure 4). Unlike the other objects used in this task, the wooden cube had a solid base, large surface area, and accessible corners constructed from wood making it potentially reinforcing for animals that commonly explore environments through climbing. This was in contrast to the smaller base and smooth surface area of the other objects used in the task (glass containers, wooden sphere). Of particular interest with this task is that rats assigned to both the NOR and Dig task performed more accurately on the scent discriminations of the Dig task (Figure 11). It is unclear if this indicates that exploration on the NOR task by the rats led to increased exploration (and subsequent accuracy) on the Dig task, if frontally-injured TBI rats assigned to both NOR and Dig tasks had less damage to the ventral mPFC leading to

less overall behavioral deficits, or if some aspect of the other secondary tasks (MWM, PA) led to a decrease in accuracy on the Dig task. What is clear is that behavior on the NOR task appears to influence behavior on the Dig task and vice versa. If the influence of the NOR task on the Dig task is positive (i.e., performance on NOR improves performance on Dig), it could indicate a potential therapeutic tool to improve performance on the Dig task as well as other decision-making tasks.

Performance by the frontally-injured rats on the PA task was also comparable. The results indicate that both mTBI and TBI rats entered the darkened compartment significantly faster on post-injury testing days 10-14 compared to non-injured, sham rats (Figure 15). These findings indicate similarities in the behavior of the frontally-injured rats on the PA task implicating impairments in response inhibition, fear learning, or both. A study assessing behavior after moderate-to-severe fluid percussion injury to the right parietal cortex found that, compared to non-injured rats, injured rats performed significantly worse on a PA task, yet significantly better on an active avoidance task (Hogg et al., 1998). The researchers concluded that decreases in freezing behavior by the injured rats prompted performance on both tasks. Research has also shown that memory for fearful events are disrupted after damage to or inactivation of regions of the mPFC (anterior cingulate, prelimbic cortex) due to the interference of dopaminergic signals from the amygdala to the mPFC (Lauzon, Bishop, & Laviolette, 2009). These impairments have been shown to occur during stimulus-shock (tone, odor) or context-shock (passive avoidance) pairings and do not appear to be affected by delay (Bissiere et al., 2008; Blum, Hebert, & Dash, 2006; Corcoran & Quirk, 2007; Lauzon et al., 2009; Riekkinen, Kuitunen, & Riekkinen, 1995). Impairments in fear learning also do not appear to be dependent on infralimbic cortex involvement (Morgan, Romanski, & LeDoux, 1993; Quirk, Russo, Barron, & Lebron, 2000). If

decreases in behavioral inhibition influenced TBI rat performance on the Dig task, it seems likely that it would also influence performance on the PA task. Combining impulsive behavior with impairments in fear learning (i.e., ability to remember the footshock) would explain the consistently short latencies seen in this group. However, impulsive behavior does not easily explain performance by mTBI rats given their performance on the Dig task. However, the speed at which the majority of mTBI rats entered the darkened compartment compared to sham rats does indicate some level of diminishing fear response on the part of the mTBI rats. Perhaps entry into the darkened compartment on testing day 10 by the mTBI rats without receiving a footshock reinforced entry into the darkened compartment on the subsequent testing days. As deficits in fear learning have been shown to occur with damage to the anterior cingulate (Bissiere et al., 2008; Blum et al., 2006; Riekkinen et al., 1995), the diminishing fear response by mTBI rats (i.e., faster entry into the darkened compartment) holds true.

In the final task, performance by the frontally-injured rats on the MWM was varied. The results indicate that, while no differences occurred with mTBI rats, TBI rats took significantly longer to locate the hidden platform compared to sham rats. Of interest with these findings is that the frontally-injured rats performed consistently throughout testing regardless of the task used (retrograde amnesia vs. working memory). For example, throughout testing it took TBI rats a mean of 55-65 seconds to locate the platform while mTBI rats located it within a mean of 25-35 seconds. This was consistent across both retrograde amnesia (used to assess remembering) and working memory (used to assess learning) tasks indicating that, within each group, the frontally-injured rats may have relied on the same strategy to locate the platform. Research has shown that dense projections connecting the hippocampus to the dorsal (cingulate) and ventral (prelimbic cortex) mPFC implements behavioral flexibility such that, after injury to the mPFC, rats have

difficulty shifting their behavior away from a previously correct goal resulting in perseverative behavior (de Bruin, Sanchez-Santed, Heinsbroek, Donker, & Postmes, 1994; Euston et al., 2012; Gemmell & O'Mara 1999; Jay & Witter, 1991; McDonald, King, Foong, Rizos, & Hong, 2008; Montague et al., 2004; St-Laurent, Petrides, & Sziklas, 2009; Van Groen & Wyss, 1990). In one study, rats were given multiple trials per session to locate a hidden platform that was relocated at the start of each session. While non-injured rats were able to swim directly to the platform by the second trial, mPFC-injured rats were not. However, with continued practice at each location the mPFC-injured rats were eventually able to swim directly to the platform. The findings of the study indicated that damage to the mPFC impaired the behavioral flexibility of these rats, not their ability to navigate spatially (McDonald et al., 2008). Regarding the current study, consistent, within group performance by the frontally-injured rats on both the retrograde amnesia and working memory tasks is consistent with the notion that damage to the mPFC resulted in impairments in the behavioral flexibility of these rats. Overall, the finding that impairments in behavioral flexibility best explain TBI rat performance deficits on three of the four behavioral tasks (Dig, PA, MWM) suggesting that mPFC-related deficits have the ability to permeate multiple behavioral tasks regardless of what the task is believed to assess.

Four behavioral tasks were used in this study to assess cognitive functioning following frontal TBI. Each task was selected based on 1) its use as an assessment measure of cognitive behavior and 2) its prevalence in the TBI field. Prior to running the study, it was assumed each task would pinpoint a specific impairment after frontal TBI (spatial and non-spatial learning and memory, object recognition, decision-making). It is now believed that the tasks were not assessing specific impairments, but one or two impairments (behavioral flexibility/reinforcement learning, behavioral inhibition) that pervaded throughout three of the four tasks. The research

used to support this theory consisted primarily of lesions studies focusing on specific regions of the mPFC. Unfortunately, the mPFC is a highly complex region that contains multiple structures involved in numerous executive functions. The sheer amount of damage produced by the TBI in this study may have resulted in global impairments that were oversimplified through the use of lesion studies. Thus, in order to get a better understanding of how effective behavioral tasks are in the assessment of frontal TBI for the purpose of therapy development, it is imperative that more research be conducted using frontal TBI models.

CHAPTER 5

CONCLUSION

The purpose of this study was to better understand the effectiveness of behavioral tasks used in the TBI field for the assessment of frontal injury. The results of the study indicated that impairments in behavioral flexibility/reinforcement learning and behavioral inhibition resulted in performance deficits on each of the four tasks (behavioral flexibility/reinforcement learning on Dig, PA, and MWM; behavioral inhibition on Dig and PA). However, the extent of brain damage that occurred in this study was significant and the reliance on lesion work to support the findings may have resulted in an oversimplification of the impairments. Unfortunately, the past decade has seen little advancement in the TBI field with regard to rodent frontal brain injury. Until more research is conducted that can better explain the results of this study, the source of the deficits seen on the four behavioral tasks must be inferred through lesion work.

What do the results of this study mean for human frontal TBI? First, the results of this study indicate that deficits in learning and inhibition are major problems after injury. The effect size for each of the affected behavioral tasks was large, explaining 13-29% of the overall variance (Dig task $\eta\rho^2 = .24$, PA task $\eta\rho^2 = .13$, MWM $\eta\rho^2 = .29$). Second, the results of this study may also be applied to humans. Research indicates that deficits in learning and inhibition are commonly reported to occur in humans after TBI. Regarding behavioral inhibition, Draper, Ponsford, and colleagues found that impairments in response inhibition can occur up to 10 years post-TBI in individuals with a wide range of injury severities. Using the same database, these researchers also found that a significant relationship between injury severity and cognitive impairment exists (Draper & Ponsford, 2008; Ponsford, Draper, & Schonberger, 2008). Regarding reinforcement learning, Schlund and colleagues found that TBI reduced the sensitivity

to consequences and reinforcement contingencies. In one study, individuals with brain injury made less adaptive choices and earned significantly less money on a task that required the pressing of a response key under a series of concurrent response-reinforcer contingencies that periodically delivered money for responding and not responding (Schlund & Pace, 2000). In another study, individuals with brain injuries failed to discriminate among significant contingencies on a delay discounting task (Schlund, 2002). Regarding fear learning, Bryant (2010) theorizes that overwhelming stress and fear felt at the time of injury may increase ones susceptibility for developing posttraumatic stress and other emotional disorders such as anxiety and depression. This theory is of utmost importance with regard to combat TBI.

Research findings like the ones listed above are thorough in listing the types of impairments seen after frontal TBI. Unfortunately, they do little to recommend treatment. In replicating what appear to be similar impairments, this study presents an opportunity for treatment development. While this study can do nothing to alleviate the biomolecular and physiological changes that occur following TBI, through the implementation of behavioral and pharmacotherapies, this study can help to improve recovery of function following frontal injury. However, before potential therapies can be considered, the findings from this study must be replicated. In doing so a few alterations to the study design should be considered.

1. Future studies should include an even “milder” injury. The cell death and tissue loss that resulted from the “mild” TBI was determined to be more moderate damage than mild. Including a fourth group (sham, mild, moderate, severe) into future studies would help to clarify why significant differences occurred between sham and mTBI rats on the MWM, but not on the Dig and PA tasks.

2. Future studies should also consider the strengths and weaknesses of different rodent strains available. In certain cases, frontal TBI can lead to rodent temperament changes (e.g., extreme fear, aggression, hyperactivity) making the calm temperament and easy going demeanor of Sprague-Dawleys (SDs) excellent research subjects. Post-injury, SDs are easier to work with compared to some of the more active strains. However, when conducting cognitive research, it has been suggested that Long-Evans (LEs) perform better (Andrews, Jansen, Linders, Princen, & Broekkamp, 1995; Tonkiss, Shultz, & Galler, 1992). Long-Evan rats have been shown to be more accurate on the MWM and are able to discriminate novel from known objects on two-choice object discrimination tests. Unfortunately, research assessing strain differences after TBI has not been as straightforward. After FPI, no performance differences occurred between SDs and LEs on the MWM working memory task and between SDs and Fischer 344s on the MWM reference memory task (Reid et al., 2010; Tan, Quigley, Smith, & Hoane, 2008). Since strain selection does not appear to be a significant predictor of cognitive ability post-TBI, a strain should be selected based upon temperament and the ability to recover well after surgery.
3. Future studies using the same behavioral tasks should also consider making specific task changes.
    a. Regarding the Dig task, rats should be tested for the same number of days instead of being moved on after reaching a predetermined criterion. The variability in the number of testing days on the Dig task not only limited the way in which the data could be analyzed, but it altered the environment of the Dig rats such that some were run for a short amount of time while others were run much longer.

b. Regarding the MWM, more days should be included in the retrograde amnesia task. According to McDonald and colleagues (2008), practice on the MWM was shown to improve the behavioral flexibility of frontally-lesioned rats in the water maze. Adding one or two more testing days to the retrograde amnesia task would enable the researcher to see if practice improves performance.
c. Regarding the PA task, a voltage less than 1.5 mA should be considered. The intensity of the 1.5 mA footshock was such that the majority of non-injured, sham rats froze in the illuminated compartment for the entirety of testing. A reduction in voltage would result in more varied behavior from the sham group and more interesting results for the researcher.
d. An impulsivity measure should be included. Results on a number of the tasks suggested an increase in impulsive behavior by the frontally-injured rats. However, without an assessment measure it is difficult to know for sure whether this occurred in the current study.

After replicating the findings from this study, different behavioral and pharmacotherapies can be administered. Some interesting avenues to explore include 1) Assessing the significant interaction between the NOR task and the Dig task. If the influence of object exploration on simple discrimination is positive (i.e., performance on NOR improves performance on Dig), it could indicate a potential therapeutic tool for individuals who have impairments in decision-making such as those with frontal TBI. 2) Assessing if and how continued practice on the MWM improves behavioral flexibility after mPFC-injury. 3) Assessing how salience might improve decision-making ability. If increased exploration of the wooden cube was a result of its salient features, it could indicate a potential therapeutic tool for individuals who have impairments in

deciding which environmental contingencies to attend to and which to ignore such as those with attention deficit disorders. 4) Assessing whether dopaminergic drug administration is able to counteract dysfunction after frontal TBI through the upregulation of receptors in regions around the damaged tissue. Dopaminergic pharmacotherapies may help to alleviate impairments in decision-making and reinforcement learning that occurs as a result of dopamine dysfunction. Only through exploration of these and other research questions will we have the ability to treat cognitive dysfunction after frontal TBI. The results from the current study indicate that the majority of these behavioral tasks have the potential to assess cognitive impairment after TBI. However, these results are only a beginning. More work is needed before we can fully understand the efficacy of each of these tasks as behavioral assessment measures for cognitive functioning after TBI and even more work before we can use these tasks to help those in need.

Table 1.

Behavioral testing groups.

Behavioral Testing Group	Experimental Group	Behavioral Task 1	Behavioral Task 2
1	mTBI *n* = 4 TBI *n* = 4 Sham *n* = 4	Dig	NOR
2	mTBI *n* = 4 TBI *n* = 4 Sham *n* = 4	Dig	MWM
3	mTBI *n* = 4 TBI *n* = 4 Sham *n* = 4	Dig	PA
4	mTBI *n* = 4 TBI *n* = 4 Sham *n* = 4	NOR	MWM
5	mTBI *n* = 4 TBI *n* = 4 Sham *n* = 4	NOR	PA
6	mTBI *n* = 4 TBI *n* = 4 Sham *n* = 4	MWM	PA

Table 2.

Distribution of rats for analyses.

Task	*N*	mTBI (*n*)	TBI (*n*)	Sham (*n*)
Dig	31	10	9	12
NOR	32	10	10	12
MWM	32	10	10	12
PA	33	10	11	12

Table 3.

Dig task fixed effects.

Effect	F-test	p-value
Exp. Group	5.33	.013*
Secondary Task	2.38	.115
Exp. Group*Secondary Task	1.26	.315
Discrimination	11.41	<.0001*
Exp. Group*Discrimination	1.42	.220
Secondary Task*Discrimination	0.09	.997
Exp. Group*Secondary Task*Discrimination	0.95	.500
Day	147.55	<.0001*
Exp. Group*Day	7.01	.004*
Secondary Task*Day	3.18	.060
Exp. Group*Secondary Task*Day	1.75	.174
Discrimination*Day	6.32	.0008*
Exp. Group*Discrimination*Day	1.36	.243
Secondary Task*Discrimination*Day	1.20	.319
Exp. Group*Secondary Task*Discrimination*Day	1.00	.461
Trial	39.29	<.0001*
Exp. Group*Trial	1.15	.323
Secondary Task*Trial	1.58	.216
Exp. Group*Secondary Task*Trial	0.71	.587
Discrimination*Trial	1.10	.349
Exp. Group*Discrimination*Trial	1.45	.201
Secondary Task*Discrimination*Trial	0.63	.705
Exp. Group*Secondary Task*Discrimination*Trial	0.81	.641
Day*Trial	15.12	.0001*
Exp. Group*Day*Trial	0.82	.444
Secondary Task*Day*Trial	3.32	.040*
Exp. Group*Secondary Task*Day*Trial	1.04	.391
Discrimination*Day*Trial	1.63	.182
Exp. Group*Discrimination*Day*Trial	1.42	.209
Secondary Task*Discrimination*Day*Trial	0.51	.798
Exp. Group*Secondary Task*Discrimination*Day*Trial	0.63	.816

Table 4.

Novel object recognition task fixed effects.

Effect	F-test	p-value
Exp. Group	1.93	.174
Secondary Task	0.07	.932
Exp. Group*Secondary Task	1.02	.424
Day	17.95	.0005*
Exp. Group*Day	1.96	.169
Secondary Task*Day	0.28	.763
Exp. Group*Secondary Task*Day	0.16	.957

Table 5.

Morris water maze fixed effects.

Effect	F-test	p-value
Exp. Group	7.07	.003*
Secondary Task	1.03	.372
Exp. Group*Secondary Task	1.42	.254
Testing	8.91	.001*
Exp. Group*Testing	2.84	.076
Secondary Task*Testing	0.53	.593
Exp. Group*Secondary Task*Testing	0.27	.895
Day	34.76	<.0001*
Exp. Group*Day	0.36	.698
Secondary Task*Day	2.64	.085
Exp. Group*Secondary Task*Day	3.15	.055
Testing*Day	16.95	.0002*
Exp. Group*Testing*Day	0.94	.400
Secondary Task*Testing*Day	0.68	.512
Exp. Group*Secondary Task*Testing*Day	1.83	.147

Table 6.

Passive avoidance task fixed effects.

Effect	F-test	p-value
Exp. Group	12.03	.0002*
Secondary Task	3.68	.040*
Exp. Group*Secondary Task	1.36	.276

Table 7.

Dig task performance and brain volume (mTBI vs. TBI rats) correlation table.

Discrimination	Pearson Correlation	p-value
Simple Discrimination 1	.350	.142
Reversal 1	-.042	.863
Simple Discrimination 2	.236	.332
Reversal 2	.522	.022*

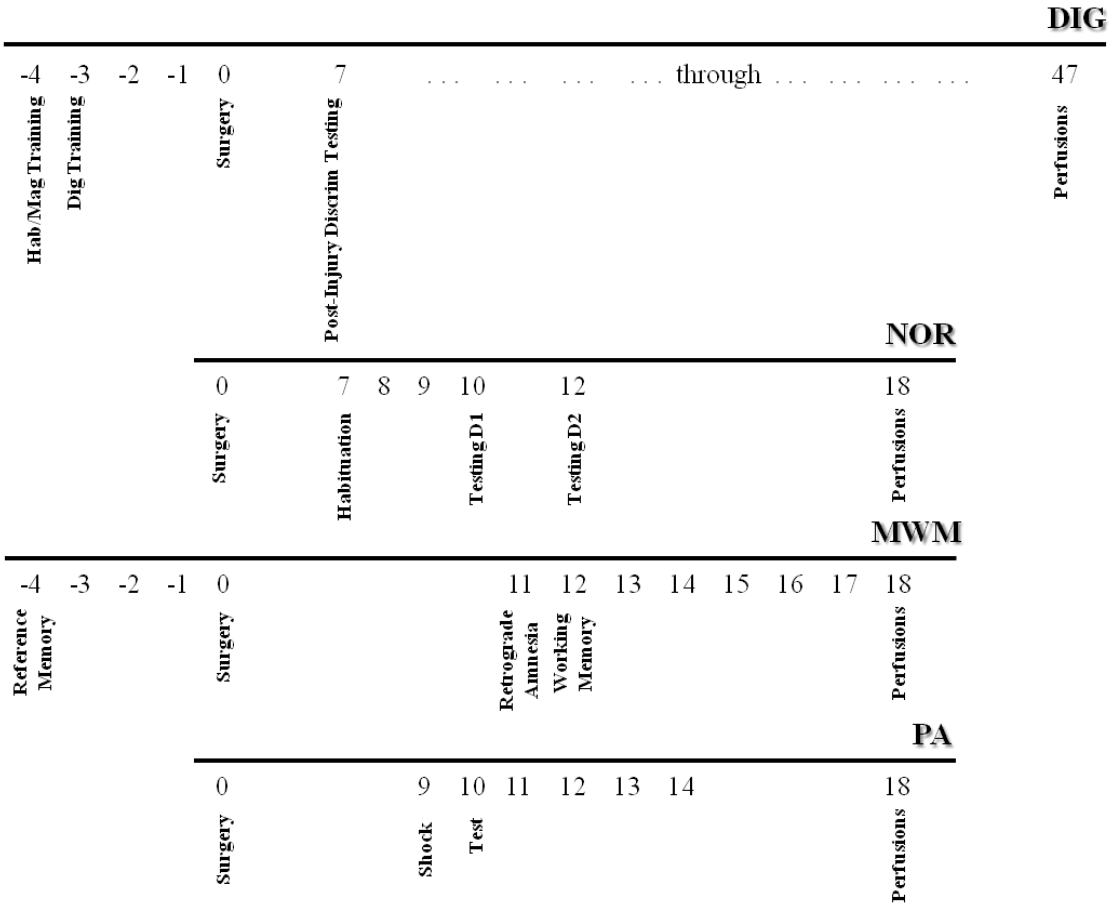


Figure 1. Experimental timeline. The figure shows the experimental timeline for all four behavioral tasks.

Figure 2. Dig chamber. The image shows the testing chamber for the Dig task. A piece of Plexiglass with inserted scent cups, to allow for presentation of the reinforcer, is shown.

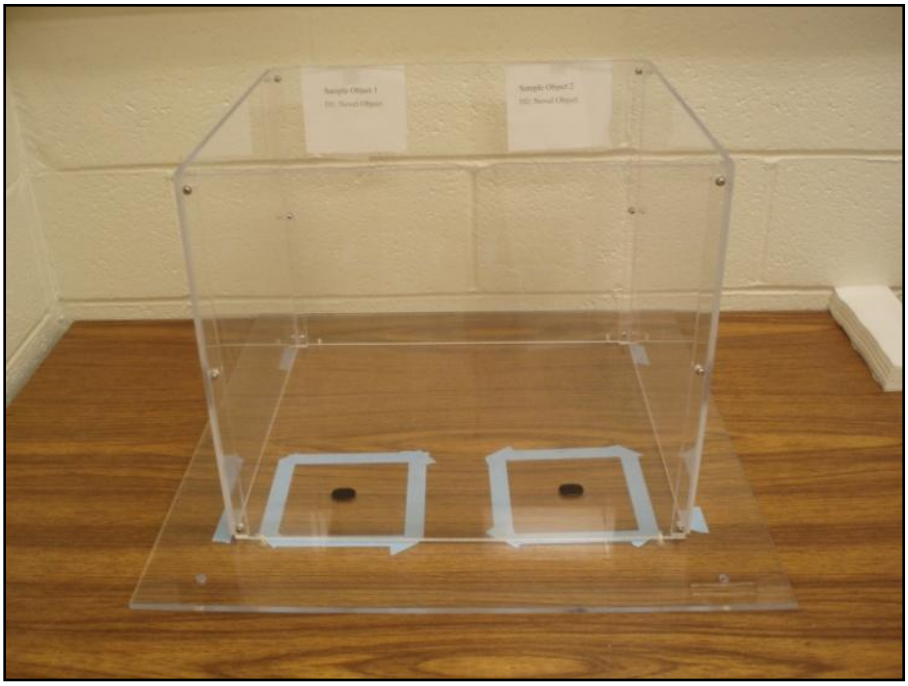


Figure 3. Novel object recognition testing arena. The image shows the testing arena for the novel object recognition task. The blue squares signify the placement of the objects during testing.

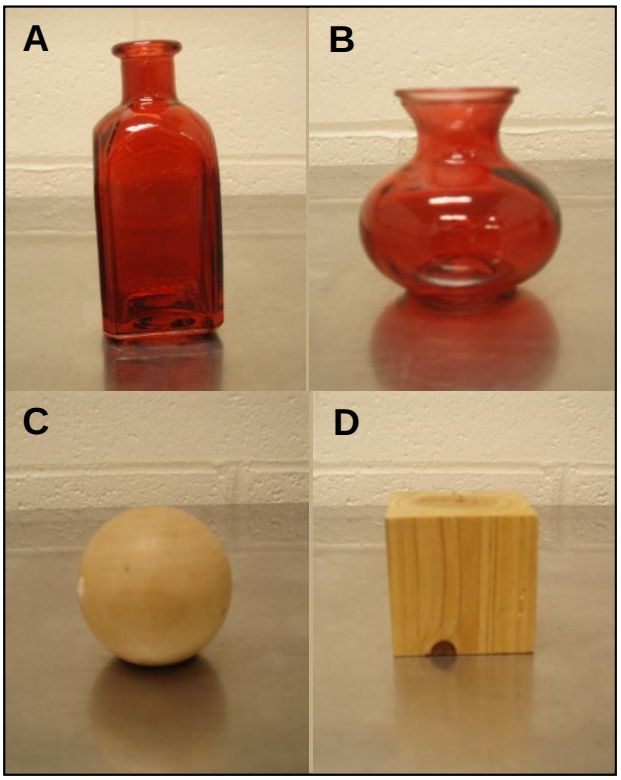


Figure 4. Novel object recognition objects. The images show the objects used in the novel object recognition task. (A) The sample object used on testing day 10. (B) The novel object used on testing day 10. (C) The sample object used on testing day 12. (D) The novel object used on testing day 12.

Figure 5. Morris water maze. The image shows the blue fiberglass tank used for the Morris water maze. The clear Plexiglas platform, submerged below the surface of the water, is pictured in the lower left.

Figure 6. Passive avoidance chamber. The image shows the chamber used for the passive avoidance task. The metal handle of the guillotine door, separating the illuminated from the darkened compartment, is shown protruding from the top of the chamber.

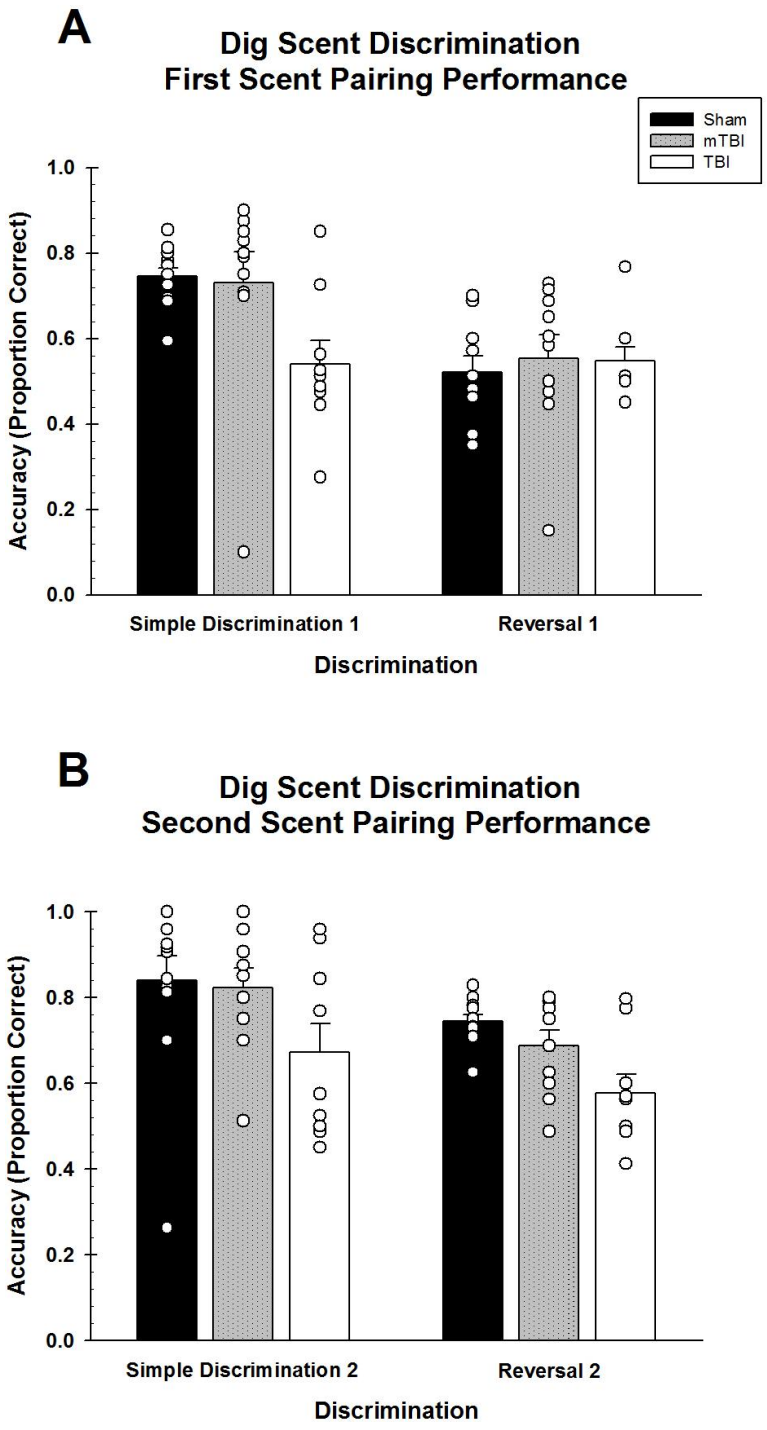


Figure 7. Dig scent discrimination performance. The graphs show the accuracy scores for all three experimental groups for each of the four scent discriminations with the white dots representing data points for individual rats in each group. As a whole, Dig rats were significantly

more accurate on simple discrimination 2 and significantly less accurate on reversal 1. By experimental group, sham and mTBI rats were significantly more accurate on the scent discriminations compared to TBI rats.

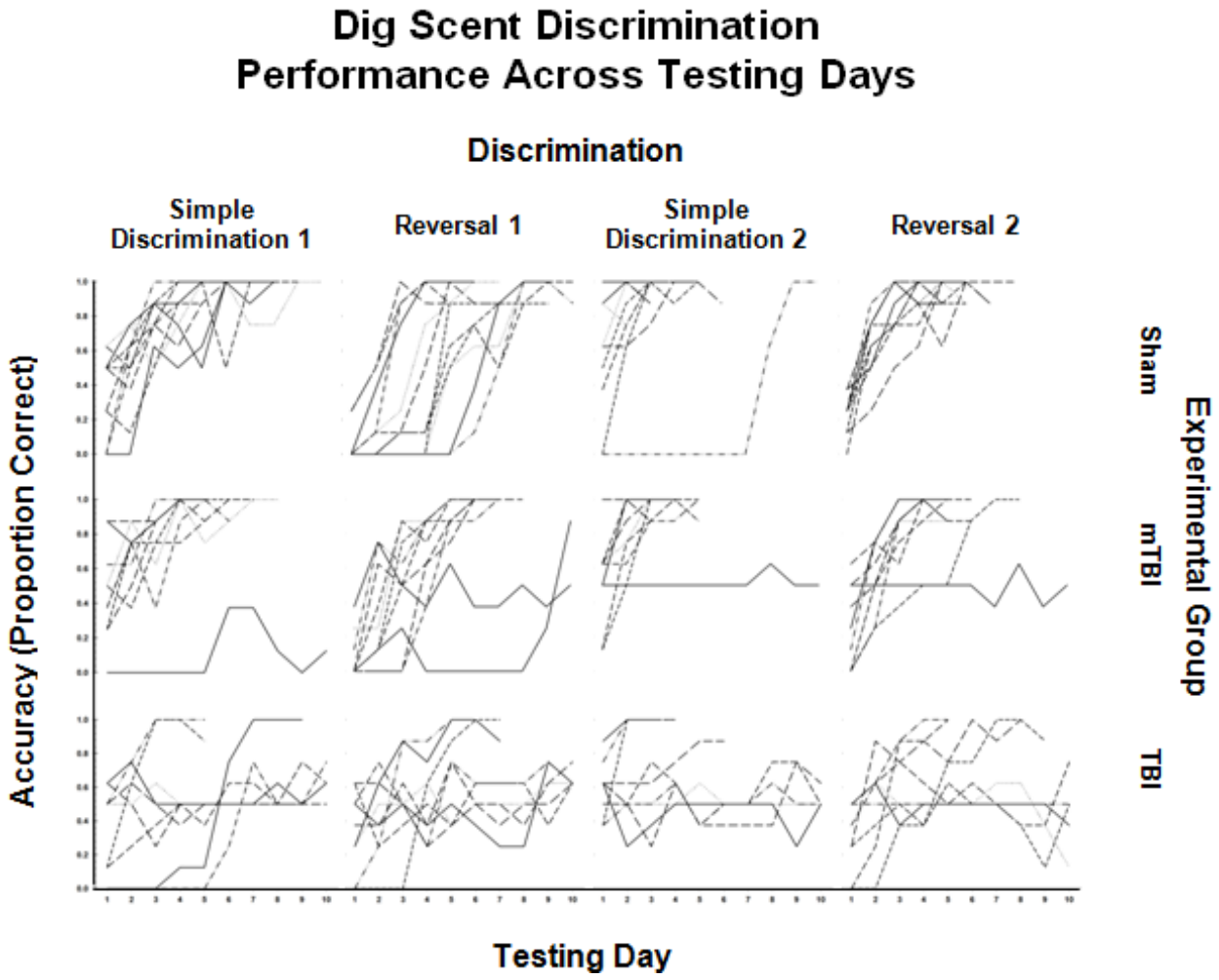


Figure 8. Dig scent discrimination performance across testing days. The graph shows the performance of individual Dig rats on each of the four scent discriminations across each day of testing. The improvement in accuracy across testing days was equal for the sham and mTBI groups, with the largest improvement in accuracy occurring on reversal 1followed by reversal 2. No significant improvement in accuracy occurred for the TBI group.

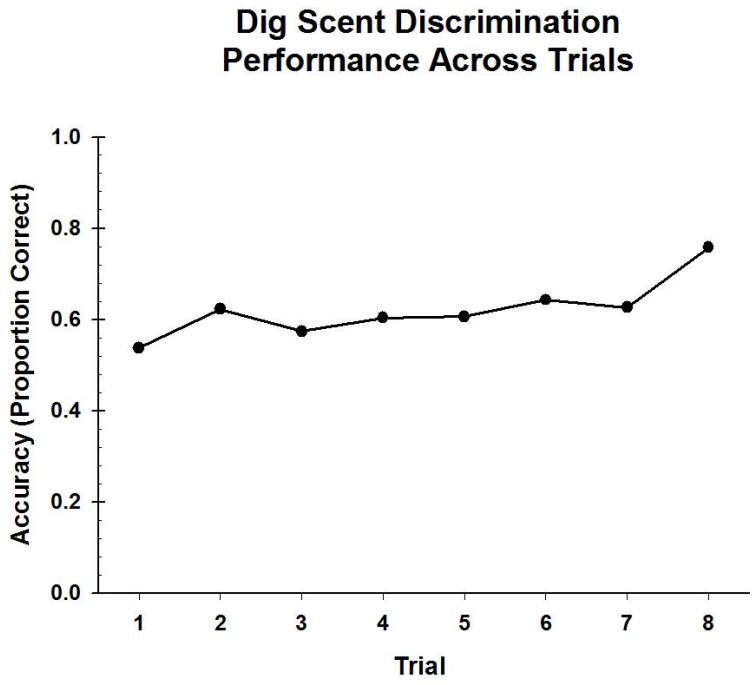


Figure 9. Dig scent discrimination performance across trials. The graph shows the accuracy scores for Dig rats across each trial of testing. A slight improvement in accuracy occurred across testing trials.

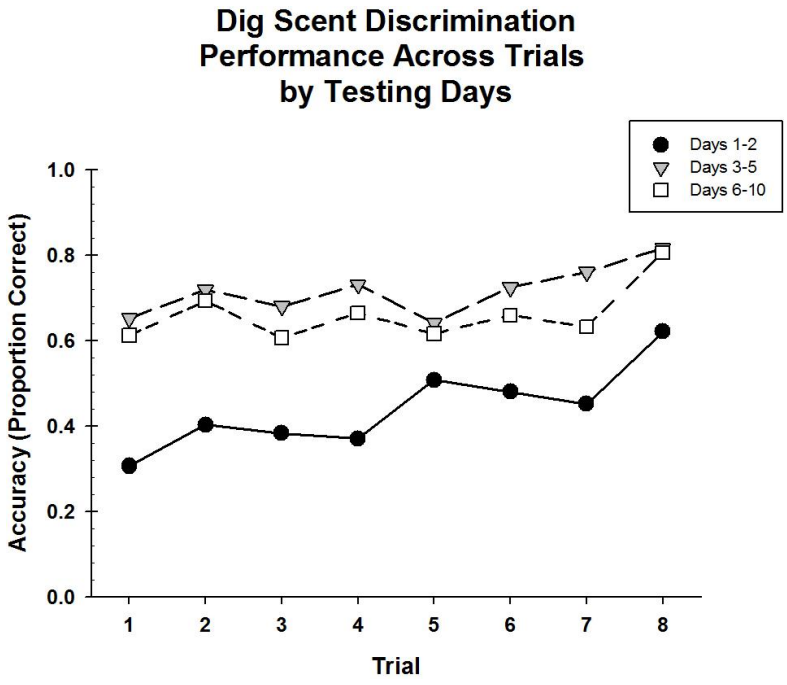
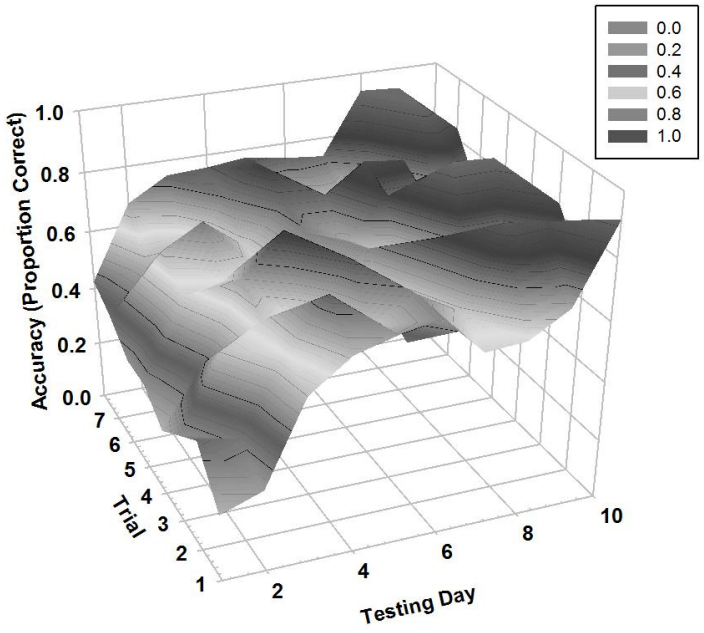


Figure 10. Dig scent discrimination performance across trials by testing days. The graph shows the accuracy scores for Dig rats across trials during a range of testing days. The largest improvement in accuracy occurred early on during testing.

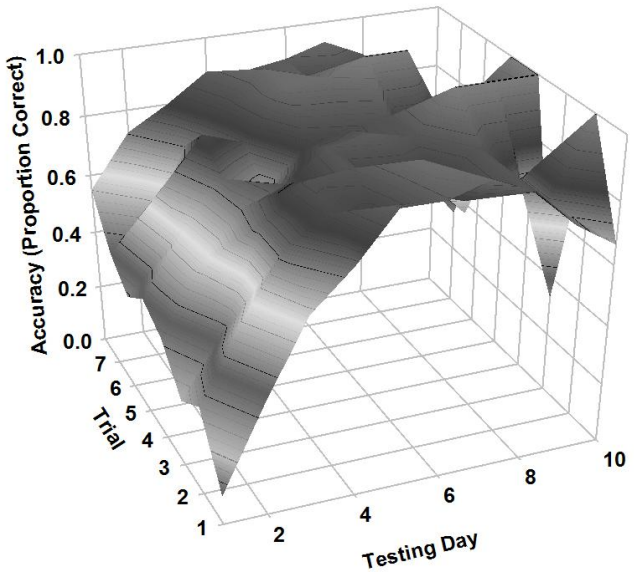
A

Dig Scent Discrimination Performance Across Trials Across Testing Days by Secondary Task (MWM)



B

Dig Scent Discrimination Performance Across Trials Across Testing Days by Secondary Task (NOR)



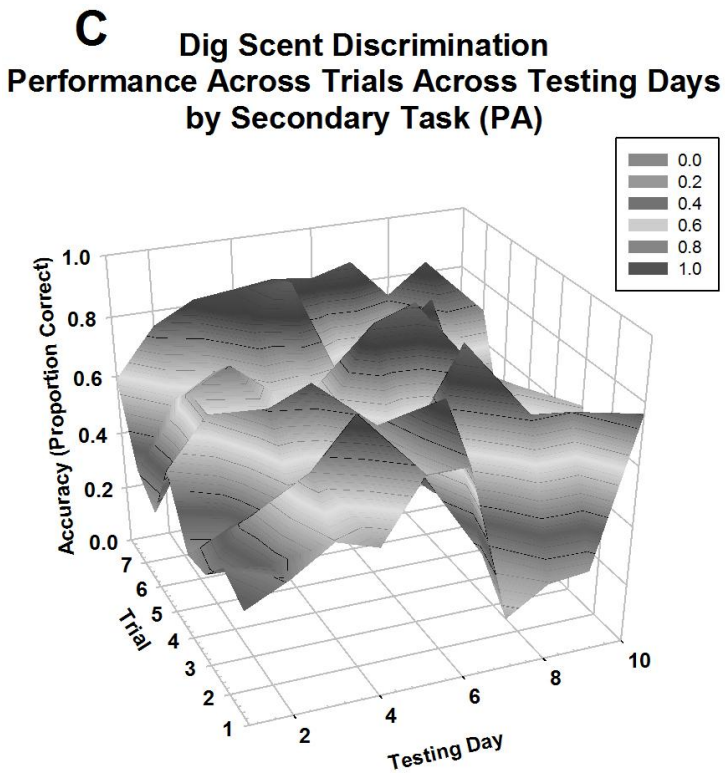


Figure 11. Dig scent discrimination performance across trials across testing days by secondary task. The graph shows the accuracy scores for Dig rats on each of the secondary tasks across trials across testing days. Rats also assigned to the NOR task had a significant improvement in accuracy across trials across testing days compared to rats also assigned to the MWM or PA task.

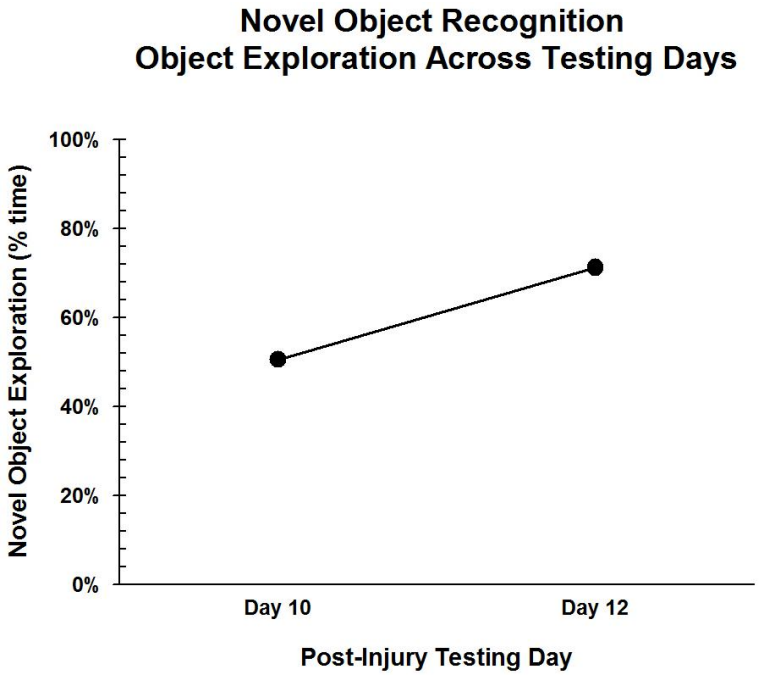


Figure 12. Novel object exploration across testing days. The graph shows the percentage of time the novel object was explored by NOR rats on each post-injury testing day. On post-injury testing day 12, the novel object was explored significantly longer compared to on post-injury testing day 10.

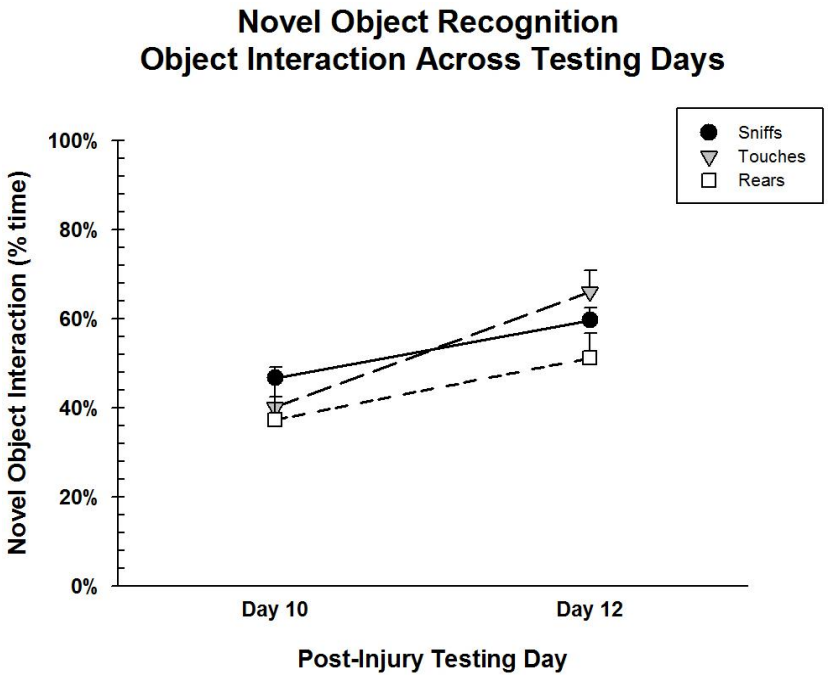


Figure 13. Novel object interaction across testing days. The graph shows the type and amount of interaction that occurred with the novel object by NOR rats on each post-injury testing day. On post-injury testing day 12, more time was spent sniffing and rearing toward the novel object compared to on post-injury testing day 10.

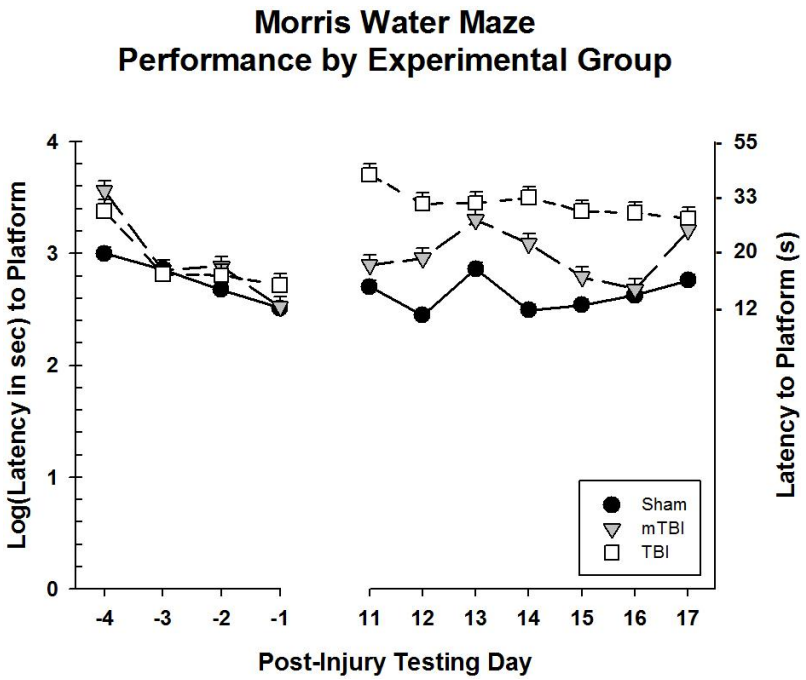


Figure 14. Morris water maze performance by experimental group. The graph shows the log latency scores for all three experimental groups across each day of MWM testing. Overall, the sham group took significantly less time to locate the platform compared to the TBI group. No differences occurred between the sham and mTBI groups or the mTBI and TBI groups.

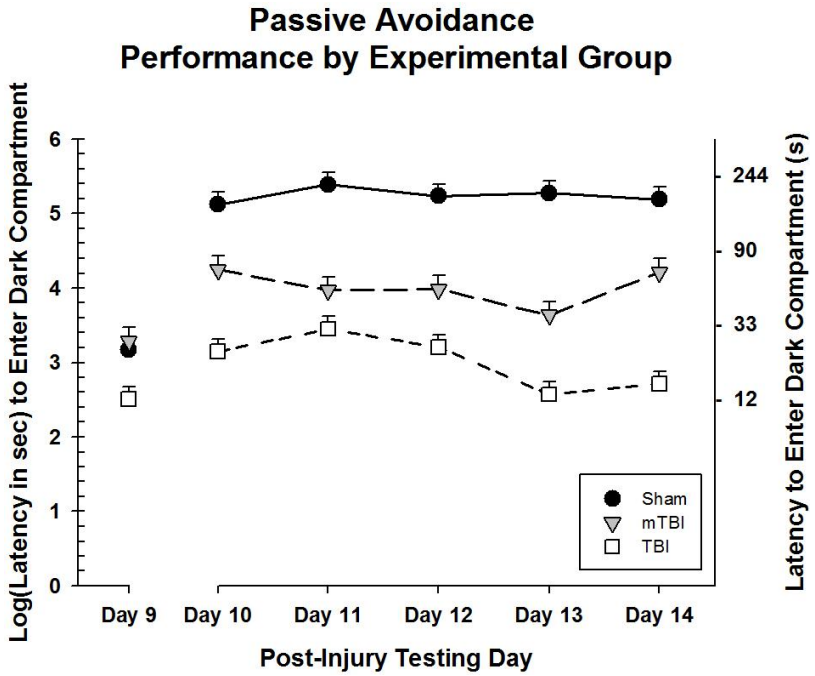


Figure 15. Passive avoidance performance by experimental group. The graph shows the log latency scores for all three experimental groups across each day of PA testing. Overall, the sham group took significantly more time to enter the darkened compartment compared to both the mTBI and TBI groups. No differences occurred between the mTBI and TBI groups.

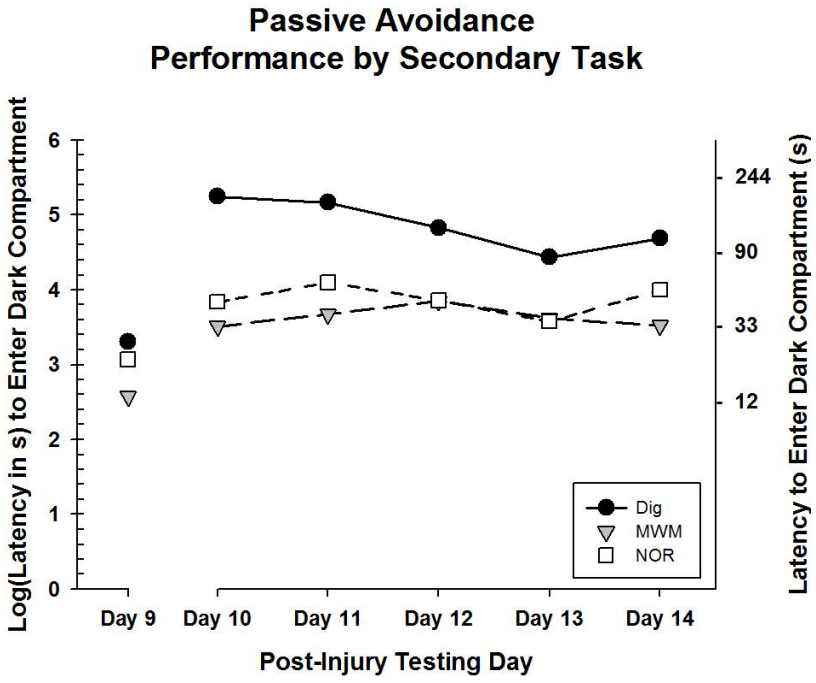


Figure 16. Passive avoidance performance by secondary task. The graph shows the log latency scores for PA rats on each of the secondary tasks across testing days. Rats also assigned to the Dig task had significantly longer latency scores compared to rats also assigned to the MWM.

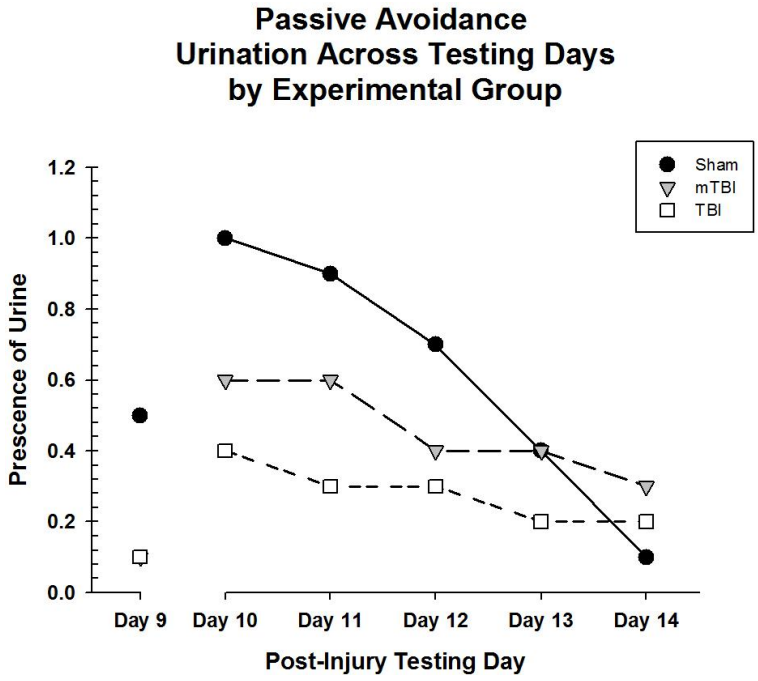


Figure 17. Passive avoidance urination across testing days by experimental group. The graph shows urination by all three experimental groups across each day of PA testing. The sham group had a greater decrease in urine production over the testing days compared to either the mTBI or TBI groups.

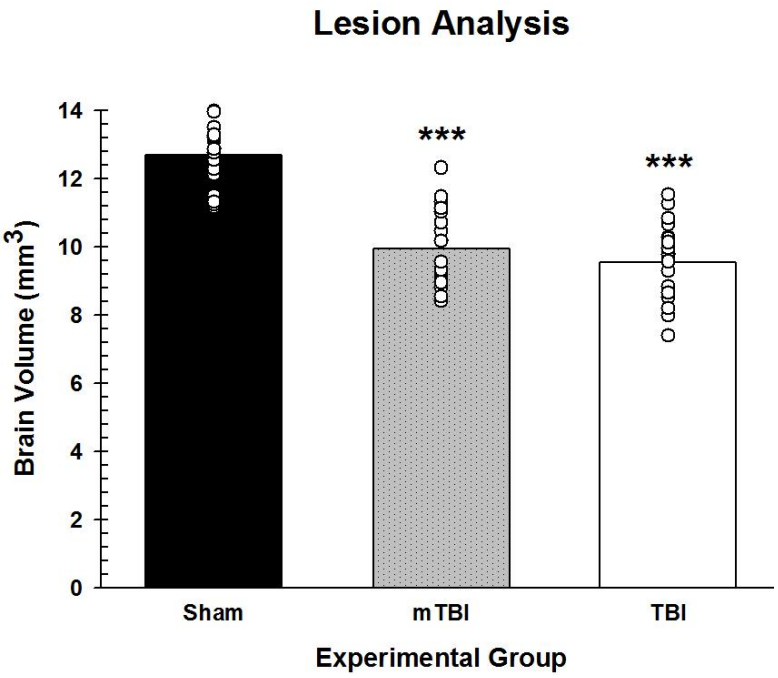


Figure 18. Lesion analysis. The graph shows the mean (±SEM) brain volume between the three experimental groups with the white dots representing data points for individual rats in each group. Both the mTBI and TBI groups had significant reductions in total brain volume compared to the sham group (*** $p < 0.001$). No differences in brain tissue volume occurred between the mTBI and TBI groups.

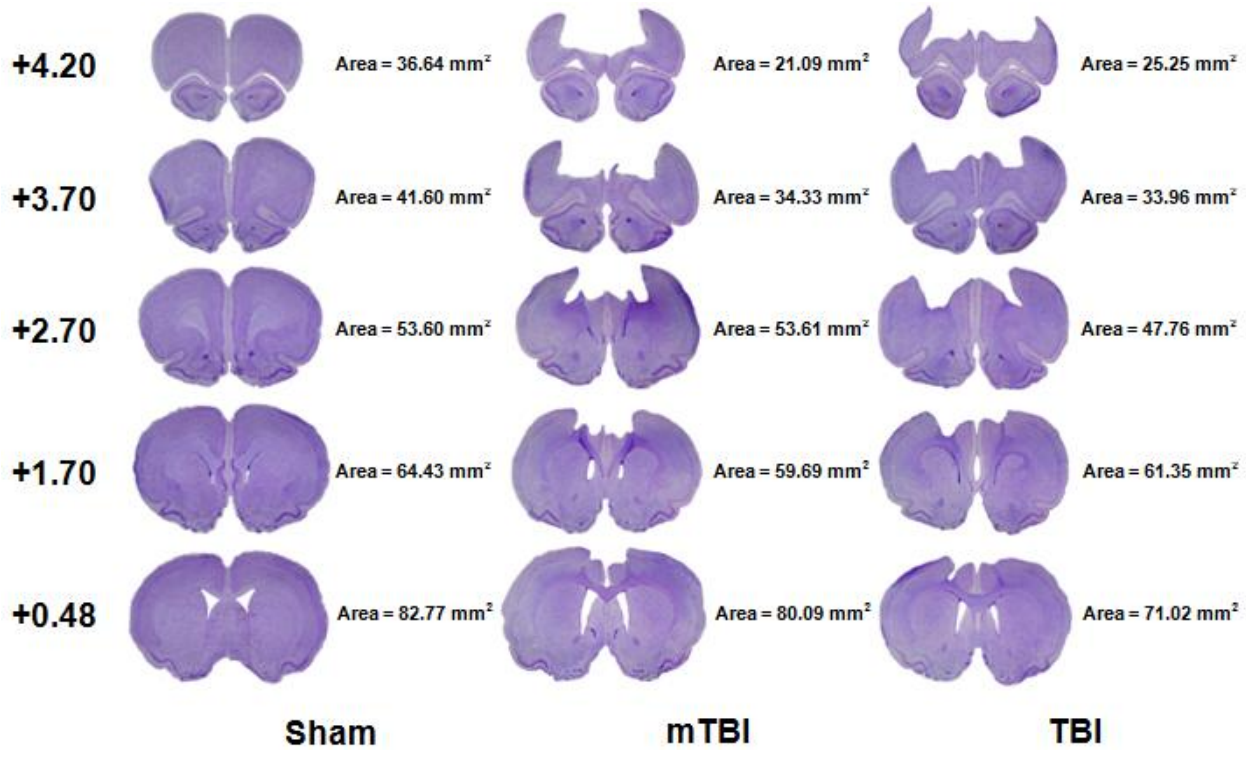


Figure 19. Histology plate. Shown are representative cresyl violet images of sections (+0.48, +1.70, +2.70, +3.70, and +4.20 mm relative to bregma) from a representative sham, mTBI, and TBI brain. The area (mm^2) of each section is included.

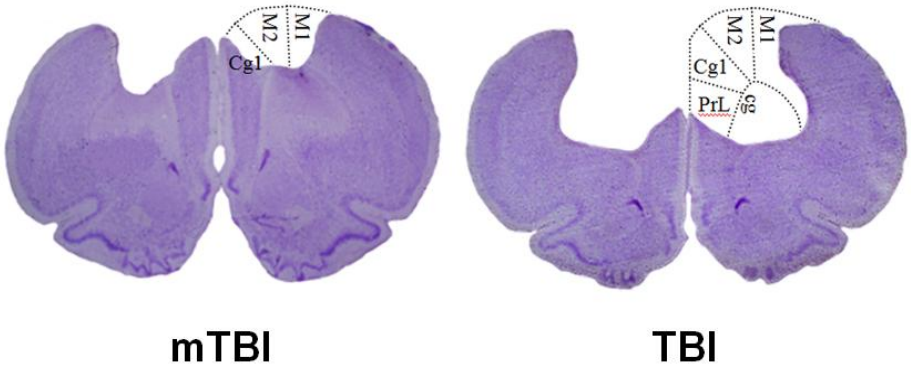


Figure 20. Severity of damage to the rat brain. Shown are images of one section (+2.70 mm relative to bregma) from an mTBI and TBI brain illustrating the range of cell death that occurred to structures of the prefrontal cortex. Note: M1 (primary motor cortex); M2 (secondary motor cortex); Cg1 (cingulate cortex area 1); PrL (prelimbiccortex); Cg (cingulum).

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