ASSESSMENT OF BEHAVIORAL AND FMRI DIFFERENCES IN A MINIPIG MODEL OF PEDIATRIC CONCUSSION

By

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ABSTRACT

Traumatic brain injury (TBI) is a leading cause of death and disability among children and adolescents in the United States. An estimated 90% of head-injury-related emergency department visits result in a diagnosis of mild TBI (mTBI) also known as concussion. Historically ignored as a major public health concern, concussion can cause lasting neurocognitive changes that can persist for years or even decades; well beyond the typical 2-week clinical recovery period. Post-concussive syndrome (PCS) encompasses a constellation of cognitive and physiological symptoms that continue to occur weeks, months, or years after a concussion. In children and teenagers, these impairments can disrupt an individual's developmental trajectory, leading to underperformance in academics, poor integration into the workforce, and diminished quality of life in adulthood. Preclinical neuroscience has greatly improved our understanding of the consequences of head injury, however vast architectural differences between rodent and human brains has resulted in dismal translation of therapeutic strategies from the bench to the bedside. In recent decades, the domestic pig (sus scrofa) has attracted substantial attention as a highly promising model animal for studying age-specific responses to mechanical trauma due to striking similarities between pig and human brain anatomy, development, and neuroinflammatory response. To add to the growing body of work utilizing pigs for the study of brain injury, we have developed a model of pediatric concussion in juvenile Yucatan miniature pigs. We conduct an extensive battery of cognitive and behavioral assessments designed to reveal post-concussive complication in pigs. We also conduct clinically relevant live imaging procedures to better understand the effects concussion can have on brain connectivity and function. Results from this work show that pigs with concussion are at greater risk of developing symptoms of depression and anxiety when compared to healthy counterparts. Additionally, pigs with brain injury are more active in the open field than healthy

pigs, a potential maker of hyperactivity and ADHD-like symptoms. After injury, concussed pigs also exhibit deficits in memory and concentration reflective of the common learning deficiencies often reported in children with post-concussive complications. fMRI analysis revealed a trend towards abnormal response to visual stimulation 5 weeks post-injury in pigs with concussion. Preliminary work investigating the efficacy of non-invasive brain stimulation as a potential therapeutic option for PCS suggests that the delivery of repetitive transcranial magnetic stimulation (rTMS) may be beneficial for some symptoms, but not others. In pigs with brain injury, it was discovered that rTMS therapy minimizes symptoms of depression and improves memory and concentration. Imaging results in pigs who have received rTMS therapy show that rTMS potentially increases the sensitivity of pigs to tactile stimulation. The utilization of an animal model whose neuroanatomy closely resembles the human brain is critical to the development of therapeutic protocols that are effective and safe.

Dedicated to my Grandma Karolyn, my Husband Chris, and my Best Friend Katelyn.
Thank you for encouraging me to persevere and achieve my goals.

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TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION TO THE STUDY

1.1 Background

The field of Biomedical Engineering exists through the intertwining of engineering principals, biology, and medicine with the overarching goal of advancing healthcare through innovation and improve medical outcomes and augment the quality of life for patients. Traumatic brain injury (TBI) is a major public health concern currently affecting an estimated 5 million individuals in the United States alone (Wang et al. 2018). In the realm of medical research, TBI serves as a critical focal point for advancing neuroscientific understanding, therapeutic interventions, and diagnostic techniques. Investigating the intricacies of TBI can illuminate fundamental mechanisms of brain injury and recovery, paving the way for innovative treatments not only for TBI itself but also for other neurological conditions.

TBI is one of the leading causes of mortality and morbidity among all populations around the world. TBI-related disabilities can encompass a wide spectrum of physical, cognitive, emotional, and behavioral impairments, often necessitating comprehensive care and support. These disabilities may range from memory and attention deficits to motor impairments, and mood disturbances. The impact of TBI echoes throughout society, imposing significant economic burdens in terms of healthcare costs, rehabilitation services, and lost productivity. Families of individuals with TBI-related disabilities often bear the emotional and financial strains of caregiving and supporting their loved ones. Moreover, the prevalence of TBI-related disabilities emphasizes the urgent need for public health initiatives to raise awareness, enhance prevention strategies, and improve access to specialized care and rehabilitation services.

Of the nearly 3 million TBIs that occur annually in the United States, 90 percent of cases are considered to fall under the category of mild severity (Taylor et al. 2017). Mild TBI (mTBI) is

often referred to interchangeably with the term concussion (Mayer, Quinn, and Master 2017). Historically ignored as a major public health concern, concussion can cause lasting neurocognitive changes that can persist for years or even decades; well beyond the typical 14-day clinical recovery period. Post-concussive syndrome (PCS) describes the complex heterogenous collection of complications that patients often experience after brain injury including changes in learning, memory, cognitive processing, changes in mood, and sleep disturbances. These post-concussive complications can cause serious disruptions and distress in one's daily life.

Injury to the developing brain can be particularly devastating as post-concussive changes in executive functioning can throw an individual off their developmental trajectory. Due to physical differences such as weaker neck muscles and a larger head-to-body ratio, younger people may be more susceptible to concussive injuries (Baldwin, Breiding, and Dawn Comstock 2018; Pfister et al. 2016). There is also evidence suggesting that young people are at particular risk for more severe and longer lasting PCS considering rapid neurodevelopmental changes that occur in adolescence (Baldwin, Breiding, and Dawn Comstock 2018; Patel and Greydanus 2002; Patel, Shivdasani, and Baker 2005). Impairments in executive functioning can often go unnoticed for years until more complex cognitive and behavioral skills are needed to meet traditional developmental milestones (Mannix and Bazarian 2020; Davis and Purcell 2014). Delayed identification of individuals who are falling behind their peers can greatly impact overall quality of life outcomes as lower academic performance can lead to higher rates of grade retention and decreased job acquisition following high school. Nevertheless, research investigating post-injury changes specific to children and adolescents after mTBI is limited (Lumba-Brown et al. 2018).

In recent decades, there has been increased interest in understanding the effects of sports-related concussion among children and adolescents. In the United States, nearly 8 million young

people participate in high school athletics each year, and those who engage in contact sports are at increased risk of sustaining mTBI (Coronado et al. 2015). Brain injuries caused by external forces, such as sports-related concussions, remain challenging to model experimentally. Various anatomical, physiological, and biomechanical factors contribute to the type and severity of brain injuries. Some of most significant factors include brain size, tissue composition, skull thickness, age and developmental stage, as well as the energy, angle, and acceleration of the impact forces (Osier and Dixon 2016; Taylor et al. 2017). Brain injuries resulting from a singular event, or a series of events, can result in changes in cognition or potentially physiological or sensorimotor impairments that can be difficult to model in laboratory settings (Schretlen and Shapiro 2003; Conrad, Dilger, and Johnson 2012; Xiong, Mahmood, and Chopp 2013).

Preclinical neuroscience has increased and continues to increase human understanding of the cellular and molecular mechanisms surrounding TBI. Indeed, post-TBI cognitive and behavioral deficiencies can likely be attributed to the long-term anatomical and functional changes that occur at the cellular, neuronal and network levels following injury. These changes include the well-documented neuronal loss and white matter disruptions which occur throughout the brain (Wilde et al. 2006; Ewing-Cobbs et al. 2008; Li et al. 2014); cellular changes leading to increased seizure vulnerability (Arango et al. 2012; Matsumoto et al. 2013; Statler et al. 2009); inappropriate neuronal rewiring (Ip et al. 2002); and atypical neuronal responses (D'Ambrosio et al. 1998; Li et al. 2014; Schwarzbach et al. 2006). Decades of preclinical experimentation in rodents has yielded more than 40 promising neuroprotective agents which have been assessed in clinical trials, however all intervention strategies have failed in Phase II or Phase III clinical evaluation (Stein, Geddes, and Sribnick 2015; Sorby-Adams, Vink, and Turner 2018). The most widely accepted explanation for this dismal rate of clinical translation is the choice of animal model used (Vink

2018; Rosenfeld et al. 2012). While rodent models are cost efficient, well established, and easy to handle, their neuroanatomy differs from ours in several aspects considered paramount for studying the brain's overall response to injury (Vink 2018). In response, large animal species are emerging as promising models for understanding the effects of mechanical trauma in a human-like brain.

The most highly regarded large animal species used for preclinical neurotrauma research is the domestic pig (sus scrofa). The pig has gained considerable attention in recent years as a potentially ideal intermediary model for studying age-related responses to mechanical trauma. This attention is due in large part to the striking similarities between pig and human neuroanatomy, development, and neuroinflammatory response. The similarities between the human and porcine brain support the theory that young pigs may be the ideal animal model for studying the effects of mechanical injury on the immature brain.

The focus of this work was to develop a clinically relevant platform for studying the effects of pediatric concussion in a large animal model with the goal of developing novel treatment strategies that can improve the quality of life for patients. This chapter introduces the specific aims of this work including the rationale and expected results. I will complete this introduction by describing the organization of the rest of the dissertation.

1.2 Specific Aims

The body of this work has four central aims:

1. Development of Multimodal Cognitive and Behavioral Assessments for Use in Yucatan Minipigs
    - Rationale: Nonhuman animal subjects are unable to verbally express their feelings; thus, researchers must evaluate an animal's emotional state using methodologies that assess behavior, neurology, and physiology. Behavior is an essential parameter

for studying animal welfare, as animals will express changes in behavior that reflect changes in pathophysiology. In order for the minipig to be considered a suitable model for cognitive and biobehavioral research, various tests that can assess the cognitive functions of pigs must be validated. Many of the behavioral tests used to assess rehabilitation in rodents post-injury, such as challenge ladder and beam walking tests, are difficult to conduct in pigs due to limited physical capabilities. In order to determine whether pigs with mTBI would exhibit post-concussive symptoms, I first needed to establish methods for conducting behavioral testing in pigs that can inform us about behaviors which are clinically relevant for the study of TBI.

- Expected Results: In contrast to rats who are prey animals, in the open field test I hypothesized that pigs would not exhibit wall hugging behaviors and would more freely explore the behavior chamber. In the novel object test, I hypothesized that pigs, like rodents, would be more interested in a new object than a familiar object and thus spend more time interacting with the novel object. I hypothesized that the baited ball test could prove a valid measure of spatial learning in healthy pigs and that pigs would become better at completing the task with time and repetition.

2. Characterization of Post-Concussive Behavioral Symptoms in Brain Injured Minipigs

    - Rationale: Brain injury can result in profound changes in an individual's cognition, memory, mood, among other symptoms. The goal of behavioral testing is to design and implement experiments which can assess changes in various types of memory and learning. If pigs exhibit post-concussive-like cognitive and behavioral

differences, the pig may be a viable model animal for testing the efficacy of various neuroprotective and neuromodulatory therapies.

- Expected Results: I expected to see similarities to what is observed in human patients and other animal research subjects following injury. In the open field test, similarly to what is seen in rodent subjects, I hypothesized mTBI pigs would exhibit hyperactivity denoted by an increase in distance traveled during the 10-minute recording period. Research has shown that rats who sustain TBI are more curious about novel objects than non-injured animals, thus I hypothesized pigs who sustain TBI would also exhibit heightened exploration of the novel object compared to healthy controls. As the baited ball pit test likely reflects memory and task learning, I hypothesized that injured animals would take longer to retrieve successive food rewards. I anticipated that for memory-based tests, pigs with concussion would exhibit memory retention deficits and thus learn and perform tasks more slowly than non-injured peers.

3. Assessment of Functional Magnetic Resonance Imaging as a Non-Invasive Diagnostic Tool for Post-Concussive Syndrome

    - Rationale: Post-TBI cognitive and behavioral deficiencies can likely be attributed to the long-term anatomical and functional changes that occur at the cellular, neuronal and network levels following injury. Neuroimaging techniques are essential tools for understanding how the brain functions in vivo. Advancements in MRI technologies are giving researchers the opportunity to improve diagnostics and track outcomes by allowing for the development of highly sensitive methods which can visualize minute alterations in brain function and microstructure.

Specifically, fMRI techniques can be implemented to identify changes in neural activation indicative of cellular damage or metabolic complications.

- Expected Results: The Pelled Lab has previously demonstrated that TBI in rats induces neuronal hypoactivity and decreases in long-term potentiation. Based on these findings, I hypothesized that pigs with mTBI will exhibit a decrease in fMRI responses to stimulation in comparison to sham animals.

4. Establish Protocols for the Delivery and Assessment of Non-Invasive Transcranial Magnetic Stimulation as a Therapy for Post-Concussive Symptoms in Yucatan Minipigs
    - Rationale: The use of large animal species to investigate the rehabilitative potential of various treatment options may hold the key to increasing the successful translation of preclinical outcomes to the clinic. Previous work from the Pelled Lab shows that rTMS treatment increased the expression of genes involved in long-term potentiation and that rats showed significant improvement in a battery of cognitive and sensorimotor behavioral tests.
    - Expected Results: I hypothesized that pigs with brain injury, when treated with rTMS therapy would perform more similarly on behavioral assessments to sham animals when compared to pigs with mTBI without therapy.

1.3 Organization of the Dissertation

Chapter 2 of this dissertation provides a comprehensive and detailed review of literature relevant to understanding the various topics involved in this work, the state of the field of neurotrauma research, and the need to develop and characterize a large animal model of brain injury. The chapter will first provide details regarding the incidence of TBI, followed by post-concussive complications, and the significance of pediatric concussion. The chapter then goes on

to describe the history and current state of preclinical neurotrauma research, as well as the ever-increasing use of pigs for the study of brain injury. Background information is also provided regarding neuroimaging techniques and the implementation of advanced neuroimaging strategies within the field of neurotrauma. Lastly, the chapter will cover information about the need for non-invasive treatment strategies and give additional details about previous work using transcranial magnetic stimulation. Chapter 3 describes the methods used in the development of this research. Here, I provide all pertinent information regarding the use of animals in this study, as well as how I designed and implemented various strategies to assess cognitive and behavioral differences in pigs with and without concussion. Chapter 4 details the results of my work. The first section of this chapter describes behavior in healthy pigs throughout maturation. The second section provides details regarding the effects of anesthesia on the BOLD fMRI signal in healthy pigs. The third section is where I provide the results from my main objective: characterizing post concussive changes in fMRI and behavior in minipigs. And finally, the fourth section details preliminary result from my efforts to establish protocols for delivering non-invasive repetitive transcranial magnetic stimulation in pigs with concussion. In Chapter 5, I summarize the results of my work and describe the implications of my findings. I also compare the results of my efforts to those published by other researchers. I also provide details regarding the limitations of this study as well as some potential future research directions.

CHAPTER 2: REVIEW OF THE LITERATURE

2.1 Traumatic Brain Injury

Traumatic brain injury (TBI) is a significant public health concern, and one of the leading causes of morbidity and mortality among all populations worldwide. The Center for Disease Control and Prevention (CDC) defines TBI as any "bump, blow, or jolt to the head" which results in altered function of the brain (Lumba-Brown et al. 2018). In the United States alone, an estimated 3 million patients go to the emergency room each year for TBI-related injuries (Taylor et al. 2017). The overall impact of TBI is profound and far reaching. As a leading cause of morbidity and mortality worldwide, TBI exacts a major toll on affected individuals and their families. The immediate impact of TBI can lead to impairments in motor function, sensory perception, and cognitive abilities, drastically altering an individual's daily life (Mangeot et al. 2002; Kinder et al. 2019; Schretlen and Shapiro 2003). Furthermore, the potential for long-term cognitive deficits and emotional disturbances underscores the gravity of TBI's enduring effects. Beyond the personal sphere, the societal burden of TBI extends to increased healthcare expenditures, diminished workforce productivity, and strained healthcare systems (Yeates et al. 2004). The staggering economic costs of TBI, arising from medical treatments, rehabilitation, and lost productivity, further emphasize its significance as a public health challenge. An estimated 5 million individuals currently live with TBI-related disabilities in the United States, and the prevalence of TBI can be a great socioeconomic burden for patients and their families (Runge 1993; Wang et al. 2018). The annual economic impact of TBI in the United States was estimated at nearly $77 billion in 2010, encompassing both direct and indirect costs (Humphreys et al. 2013). This can be especially burdensome for individuals and families as the National Institutes of Health (NIH) estimates the

cost of a single hospitalization can range from $2,130 to $401,808. The costs associated with post injury care and rehabilitative therapy sessions are also staggering.

TBI is a heterogenous, multifaceted condition which is produced through a combination of primary injury and secondary injury mechanisms (Borgens and Liu-Snyder 2012). The primary injury refers to the initial mechanical insult to the head and brain. This primary injury is typically induced either through the head coming in rapid contact with an object, or through rotational acceleration forces. The most common cause of TBI in the United States is falling (Coronado et al. 2015; Taylor et al. 2017). Nearly half of all TBI cases (47 percent) are the result of falls. Falls are the leading cause of brain injury for infants, children, and elderly patients. Accounting for 15 percent of all TBIs is being struck by or against an object such as being hit by falling debris or forcefully bumping heads with another person. It is the second most prevalent cause of TBI among the general population and accounts for 20 percent of TBIs in children under 15 years of age. The third leading cause of TBI, accounting for 14 percent of injuries, is motor vehicle crashes including those involving bicycles and trains. Motor vehicle crashes account for the largest percentage of TBI-related deaths. Assaults are the next most prevalent cause of brain injury, accounting for 10 percent of TBIs, typically occurring in children aged 0 to 14, and elderly individuals over the age of 65. The remaining 15 percent of injuries fall under the categories of unknown and other. The other category consists of any injury that does not fit into the previous categories. This includes injuries resulting from explosions, electrocution, wielding flash burn, and animal attacks.

The secondary injury of TBI refers to a metabolic cascade of complex pathological processes which can occur within the first several hours to days following primary injury. While the primary injury inflicts immediate mechanical damage to brain tissue, secondary injury mechanisms exacerbate this initial damage and contribute significantly to the overall extent of

brain injury (Llado et al. 2004; Kilbaugh et al. 2011). Secondary injury encompasses a wide range of processes, including inflammation, oxidative stress, excitotoxicity, mitochondrial dysfunction, and disruption of the blood-brain barrier. These processes trigger a series of damaging events, including the release of pro-inflammatory cytokines, swelling of brain cells, and the accumulation of toxic byproducts. Secondary injury can lead to reduced cerebral blood flow, increased intracranial pressure, and a protracted state of neuroinflammation, all of which can further harm neurons and other brain cells (Konsman 2022; Liy et al. 2021). Understanding and targeting secondary injury mechanisms is a key focus within TBI management as interventions aimed at mitigating these processes hold the potential to limit the extent of brain damage and improve patient outcomes. Researchers and clinicians continue to investigate strategies to modulate secondary injury pathways and develop neuroprotective treatments for individuals with TBI.

The severity of TBI can range from mild, moderate, and severe (Tenovuo et al. 2021). Accurate classification of injury severity is crucial for guiding clinical decisions and optimizing patient care. The Glasgow Coma Scale (GCS) is the most commonly used assessment tool for determining impaired consciousness and severity of brain injury. Developed at the University of Glasgow Medical School by Sir Graham Teasdale and Bryan Jennett in 1974, the GCS assigns numerical scores to three key components of a patient's neurological response: eye opening, verbal response, and motor response. Each component is scored on a scale from 1 to 4 or 1 to 6, with higher scores indicating a more favorable response. The GCS scores for each component are then summed to obtain the total GCS score, which can classify an injury as severe (3-8), moderate (9-12), and mild (13-15). This standardized assessment enables healthcare providers to gauge the severity of a patient’s neurological impairment and monitor changes quickly and objectively over time. The GCS is invaluable in triage decisions, treatment planning, and prognostication.

Of the number of patients who receive medical consultation for their injuries, an estimated 70-90% of diagnoses fall under mild severity (Lumba-Brown et al. 2018; Silverberg et al. 2020; Coronado et al. 2015; Taylor et al. 2017). Mild TBI (mTBI), often referred to as concussion, represents a complex neurological condition characterized by transient, albeit significant, disruption of brain function following head trauma. Understanding the exact number of individuals who experience mTBI is challenging as even though the prevalence of concussion in our society may be high, it is an often overlooked and severely underreported condition (Silverberg et al. 2020). Official diagnosis for mTBI typically relies upon initial GCS score of 13-15, loss of consciousness for less than half an hour, and post-traumatic amnesia lasting less than a day. Neurological imaging procedures in these patients yield normal results, with no indication of gross anatomical damage using computerized tomography (CT) and magnetic resonance imaging (MRI) (Beauchamp et al. 2011). Neuropsychological assessments, including cognitive and behavioral evaluations, play a crucial role in detecting subtle cognitive impairments, mood disturbances, and post-concussive symptoms that may persist after the injury.

2.2 Post Concussive Syndrome

Approximately 90 percent of concussion symptoms are considered to be transient, with symptoms typically resolving spontaneously within the clinically recommended 10-to-14-day recovery period (McCrory et al. 2009). Although the majority of patients are able to recover from their injuries within this time period, an estimated 20 percent of individuals with concussion will continue to experience lasting debilitating symptoms for quite some time beyond the typical acute recovery period (Wilmoth et al. 2019; Fullerton et al. 2019; Baldwin, Breiding, and Dawn Comstock 2018; Rivara et al. 2012). Some patients report a continuation of symptoms anywhere from several weeks to years post injury. Post Concussive Symptom (PCS) is the term used to

describe the condition in which patients experience these lasting symptoms of mTBI long past the 14-day clinical recovery.

PCS is characterized by a constellation of persistent and diverse symptoms that can have a taxing effect on individuals and cause serious disruptions in daily life. These symptoms encompass a wide range of domains, including cognitive, physical, emotional, and interpersonal aspects (Yeates et al. 2004; Wilmoth et al. 2019). Cognitive complications may involve difficulties with memory, concentration, and attention, hindering daily tasks and work performance. Physical symptoms can include headaches, dizziness, fatigue, changes in vision, and disturbances in sleep patterns. Emotionally, PCS may contribute to mood swings, irritability, anxiety, and depression, exacerbating the psychological burden on individuals already coping with the aftermath of concussion. The precise underlying mechanisms of PCS remain under investigation, but they likely involve a complex interplay of neuroinflammatory processes, alterations in neurotransmitter levels, and disrupted neural circuitry. While the exact prevalence of PCS varies, it underscores the need for specialized and multidisciplinary approaches to assessment and management.

Treatment strategies for PCS are multifaceted and may encompass cognitive rehabilitation, physical therapy, psychotherapy, and pharmacological interventions targeting specific symptoms. Moreover, early intervention and tailored rehabilitation programs hold promise in mitigating the long-term effects of PCS (Ponsford et al. 2001). The recognition and study of PCS have evolved over time, shedding light on the importance of distinguishing it from the acute phase of concussion recovery. Advancements in research are not only enhancing our understanding of the condition but also contributing to the development of evidence-based guidelines for effective management. As our comprehension of PCS deepens, the aim is to refine diagnostic criteria, identify predictive factors, and implement interventions that can alleviate the profound impact PCS has on individuals'

quality of life, providing them with the necessary tools to navigate the challenges of post-concussive symptoms and regain optimal well-being.

2.3 Pediatric Concussion

Biological considerations such as large head to body ratio and weak neck musculature make children and adolescents particularly susceptible to mTBI (Baldwin, Breiding, and Dawn Comstock 2018; Pfister et al. 2016). In the United States, there are an estimated 7 million students which participate in high school athletics each year (McCrory et al. 2013). Injury to the developing brain can have lasting consequences on one's quality of life as changes in executive functioning can throw an individual off of their developmental trajectory (Baldwin, Breiding, and Dawn Comstock 2018; Patel and Greydanus 2002; Patel, Shivdasani, and Baker 2005). Slow cognitive processing and difficulty concentrating can lead to poorer performance in academics, which can severely limit career outcomes in adulthood. The prevalence of pediatric concussion is a growing concern, with an increasing number of cases being reported in recent years. Participating in sports and recreational activities, as well as accidental falls, are common mechanisms leading to pediatric concussion overall.

The developing brain is particularly vulnerable to the disruptive forces of concussion, which can manifest in a range of short- and long-term consequences. In the immediate aftermath of a concussion, young people may experience typical signs of PCS including headache, dizziness, nausea, and cognitive impairments. However, what makes pediatric concussion unique is its potential to disrupt crucial cognitive, emotional, and social milestones during critical periods of growth and development (Mannix and Bazarian 2020; Davis and Purcell 2014). Moreover, multiple concussions sustained during childhood can heighten the risk of persistent symptoms, cognitive deficits, and even long-term neurodegenerative conditions. These factors have prompted

heightened awareness of the importance of proper concussion management and return-to-play protocols in youth sports. Early diagnosis, appropriate rest, gradual reintegration into school and activities, and targeted interventions such as cognitive rehabilitation are crucial in mitigating the impact of pediatric concussion. Comprehensive support from healthcare professionals, educators, parents, and coaches is pivotal in ensuring the optimal recovery and future well-being of children and adolescents following concussion, as well as minimizing the potential cognitive and emotional ramifications that may persist into adulthood.

2.4 Preclinical Research Studies

Preclinical research studies are an essential step in better understanding the biological and psychological nuances surrounding various neurological disorders and developing more effective treatment strategies. Laboratory animal models are central to the preclinical neuroscience process, providing a valuable platform with which researchers can uncover the underlying mechanisms of brain function and dysfunction in a living system. The ability to control experimental variables, perform invasive procedures, conduct genetic manipulations, collect brain tissues, and analyze biological samples are some of the distinct advantages of using animal models (Huang et al. 2020). Throughout history, neuroscience researchers have used a wide variety of animal species to model diseases and neurological conditions (Bovenkerk and Kaldewaij 2015). The most common and well-characterized model animals used in laboratory neuroscience research are rodents, specifically mice and rats (Ellenbroek and Youn 2016).

Research using rodent models has greatly contributed to the understanding of fundamental neurological processes. The use of rodent models has uncovered information surrounding neuronal function, neurotransmission, synaptic plasticity, and brain connectivity (Shin et al. 2018). Rodents, particularly mice, are preferred models for genetic manipulation due to short reproductive cycles

and well-characterized genomes. Researchers can selectively introduce or delete specific genes in rodent models to study their effects on brain development, behavior, and disease susceptibility. These genetic manipulations have provided crucial insights into the roles of specific genes in neural function and have helped identify potential targets for drug development. In behavioral neuroscience, rats are widely regarded as excellent animal models due to their behavioral complexity and adaptability. Using various behavioral paradigms, researchers can study various aspects of cognition, memory, learning, emotion, and motor function. These studies can help us to understand the underlying complex behaviors underlying various neurological disorders and contribute to our understanding of neural injury and disease states.

Rodents have long been used to model a plethora of neurological and psychiatric disorders, including neurodegenerative disease, neural injury, and psychiatric conditions such as drug addiction, anxiety, and depression (Hughson et al. 2019). The replication of these disordered phenotypes and disease states allows researchers the ability to investigate the mechanisms underlying these conditions, study the progression of the diseases, and develop and test new and exciting therapeutic intervention strategies. Preclinical research using rodent models of disease continues to be an invaluable step in the development and evaluation of therapies and treatments. Many studies which have used rodents to investigate various treatments such as pharmaceutical interventions or gene therapies have shown great promise. Humans and rodents share many genetic and physiological similarities, thus the use of rodents in preclinical research allows for rigorous testing of potential therapies before those interventions are tested in human trials.

Preclinical rodent neuroscience research has contributed greatly towards the development of potentially promising therapies and treatments for many neurological disorders, including TBI. Research has revealed that although some potential treatment strategies for TBI can show

incredible promise in rats and mice, an astonishingly high percentage of these treatments do not work in humans, failing in clinical trials (O'Collins et al. 2006; Stein, Geddes, and Sribnick 2015). One of the more common explanations for this abysmal rate of translation articulated among researchers within the field of neurotrauma are the considerable anatomical and physiological dissimilarities between the brains of rodents and humans (Sorby-Adams, Vink, and Turner 2018). Rodents and other small animals have lissencephalic brains, with smooth cortices and low gray to white matter ratio (Sorby-Adams, Vink, and Turner 2018). The lack of robust white matter regions in a model organism is less than ideal for the study of neurological disorder or diseases which involve regions of white matter such as diffuse TBI or Multiple Sclerosis (Todea et al. 2020). The reaction of the rodent brain to mechanical stress is also different from what is experienced by the human brain as in gyrencephalic human brains, mechanical stress is forced down into the base of the sulci, closer to the white matter regions of the brain subjecting these regions to potential damage; smooth cortices on the other hand result in a more even distribution of mechanical stress across the surface of the brain (Cloots et al. 2008).

Researchers within the neurotrauma community have proposed that large animals are a better fit for studying the effects of mechanical insult to the brain than rodents and other small animal species. One such species, the domestic pig (sus scrofa) has attracted considerable attention within the field of neuroscience due to striking similarities between pig and human brain anatomy, physiology, and development (Dobbing and Sands 1979; Sorby-Adams, Vink, and Turner 2018; Conrad, Dilger, and Johnson 2012; Gieling et al. 2011). Pigs are surprisingly intelligent, easily motivated, and can perform a wide array of behaviors, making them an excellent model for interdisciplinary translational research (Netzley et al. 2021; Haigh, Chou, and O'Driscoll 2020; Kornum and Knudsen 2011; Kornum et al. 2007). The most notable advantages of using the pig

as a model species for neuroscience research is the large brain size and gyrencephalic anatomy (Sauleau et al. 2009). There are many similarities between the pig and human brain including gyrencephalic anatomical structure with comparable grey to white matter ratio, analogous cortical organization, and regional distribution of neurotransmitter systems (Ryan et al. 2018; Henry et al. 1996). Pigs have a similar brain size and overall structure to humans, such as complimentary cortical folding, connectivity and white matter tracts, as well as subcortical structures (Dickerson and Dobbing 1967; Holm and West 1994). One of the most compelling reasons researchers have interest in the pig for neuroscience research is similarity in neural development which allows for the study of the underlying processes associated with neurodevelopmental disorders (Jelsing et al. 2006). Mammals, including pigs, undergo a period of rapid brain growth sometime surrounding birth; in pigs and humans, this developmental spurt happens during the perinatal period which spans from late gestation through early infancy (Dobbing and Sands 1979; Ryan et al. 2018). Pigs are also an excellent model for studying human-relevant behaviors which enables intense study of complex cognitive phenomena such as learning, memory, and social behavior which can be impaired in humans with neurological disorders (C.C. Croney 2003; de Jong et al. 2000; Kornum and Knudsen 2011). Moreover, pig metabolism and general physiology are more analogous to humans than rats and mice, enabling a more rigorous investigation of responses to pharmacological treatments (Howard et al. 2018; Wolf et al. 2014). Collectively, these considerations have elevated the pig as an attractive model species for the study of human neurological conditions and brain injury with the potential to enhance our understanding of complex neurological disorders. These factors can help facilitate the safe and successful translation of preclinical findings to the frontlines of human clinical care.

In translational neuroscience, the pig is most used in the study of traumatic brain injury (TBI). The pig has been used as a model for various input methods in TBI research, each replicating various key aspects of brain injury. The most common input method for implementing TBI in pigs is controlled cortical impact (CCI) (Osier, Korpon, and Dixon 2015). CCI requires a craniotomy which is conducted to expose a specific region of the brain, such as the frontal cortex or parietal cortex. An electromagnetic or spring-loaded impactor device is typically used to deliver CCI to the exposed dura mater or neural tissue. The controlled aspect of this injury relies upon the precise alignment of the impactor, ensuring accurate and consistent brain injury. Parameters such as impact depth, velocity, and dwell time can be adjusted to meet research needs and control the extent of damage (Pareja et al. 2016). After impact, the skull is typically closed by adding plating or some protective covering. Although CCI is a well-characterized method for implementing TBI in pigs (Simchick et al. 2021; Baker et al. 2019; Kinder et al. 2019; Manley et al. 2006; Pareja et al. 2016; Wang et al. 2023), the majority of human TBIs are closed-head injuries (Ginsburg and Huff 2023). Craniotomy-based models more closely model open head injuries such as gunshot wounds or impalement, which likely cannot fully replicate the mechanisms and consequences of closed-head injuries, particularly because CCI requires that the subject's head be secured in a stereotaxic frame, preventing head motion during impact.

In contrast, models of rotational-acceleration utilize rotational forces to induce brain injury in pigs (Cullen et al. 2016; Mayer et al. 2022; Mayer et al. 2021; McNamara et al. 2020; O'Donnell et al. 2023). The procedures involve the implementation of a specialized device which allows for controlled, rapid, rotational movement (Cullen et al. 2016). Various methods can be used to achieve rotational acceleration, such as a rotational platform, pendulum, or a custom-built device. The severity of the injury can be controlled by altering the rotational force and duration. During this

procedure, the head undergoes rapid angular acceleration, which leads to the deformation of brain tissue and neuronal shearing, mimicking the rotational forces endured by humans during closed-head TBI. This injury is particularly useful for modeling motor vehicle accidents, sports-related head injuries, and abusive head trauma in infants.

Less commonly used than the previous models mentioned, pigs are also used to investigate the consequences of blast-induced brain injury. (Chen et al. 2017; Kallakuri et al. 2017; Cralley et al. 2022). The blast injury model uses an explosive blast or compressed gas to generate a concussive shockwave. This model is designed to replicate the sequela associated with blast related TBI, often experienced by members of the military while in active combat. This type of injury often involves complex mechanisms surrounding the injury such as the initial blast wave, secondary injury caused by debris, and additional injuries stemming from body displacement. The blast model is crucial for the unique study of blast-related brain injury, uncovering the effects of shockwaves on neural tissues and the subsequent neuroinflammation and deficits in cognition.

2.5 Significance of Behavior in Preclinical Research

Cognitive and behavioral assessment within preclinical neuroscience research provides a phenotypic bridge between basic science and real clinical outcomes. The investigation of behavior and cognition is essential for better understanding gross neuronal function and modeling human brain disorders. A large number of psychiatric and neurological disorders manifest with various cognitive and behavioral differences. The observation and quantification of behavioral responses in animal models of disease and disorders allow researchers to study various nuances involved in brain functioning such as the perception of the senses, changes in learning and memory, social behavior, motor control, and emotional processing. Pigs are considered to be cognitively complex and thus exhibit a vast array of behaviors and mental acumen relevant for the study of human

cognition. Impressively, pigs have excellent learning capabilities, spatial memory, problem-solving skills, and intricate social behavior. As a potential model species for biobehavioral research, it is imperative that researchers implement reliable methodology for assessing pig behavior. Comon techniques include direct observation, video recording, and analysis using various software and artificial intelligence which allows for precise and accurate analysis of behavioral phenomena. As technology advances, more precise and sophisticated behavioral assessment methods are being developed, which can greatly ease the difficulties associated with pig behavior research.

Cognitive impairment is a common consequence of brain injury. Changes in executive functioning can include problems with learning and memory, attention, and processing speed.

Memory impairments are one of the more common sequela experienced by patients with TBI (Kim et al. 2009). Several types of memory can be affected by brain injury and specific behavioral tests can be implemented to assess deficits for the various types of memory.

The most general forms of memory are long-term memory, short-term memory, and working memory (Cowan 2008; Chan et al. 2018). Long-term memory refers to any memory that we are able to retain and recall after 30 seconds. The majority of memory falls under the category of long-term memory. Long-term memory can be divided into two primary categories: implicit memory and explicit memory. Implicit memory are memories that tend to form unconsciously such as motor skills used for walking and riding a bike. Explicit memories tend to be things that we deliberately take the time to form and recall, such as dates and phone numbers. Explicit memories can be either episodic, such as remembering a certain event, or semantic as in general facts about a topic.

There are a wide variety of behavioral tasks which can assess specific aspects of long-term memory in animals. Recognition memory specifically refers to the ability of an individual to identify a familiar stimulus or situation (Chan et al. 2018). This is the form of memory that allows humans to easily recognize faces. Novel object recognition tasks or novel choice placement tasks are used to assess the ability of animal subjects to remember a familiar object or location as well as willingness to explore a new stimulus. In these tests, the subject is presented with a previously explored stimulus and a never-before-seen stimulus. Typically, the total duration of time or the percentage of time spent engaging with the familiar versus the new stimulus are recorded.

Spatial memory refers to the cognitive process that enables an individual to recall the location of an object or where in space multiple objects are located in relation to one another (Shrager et al. 2007). Spatial memory is essential for orienting oneself and navigating within the environment. It allows us to perform simple tasks, avoid dangerous areas, and remember where we left our keys. Several behavioral tests are commonly used to assess spatial memory in animals such as maze tasks. Tests such as a T-maze, or radial arm maze tend to involve placing an animal in one part of the maze and incentivizing them to explore by baiting one of the arms with a food reward. The animal must remember where they have been previously in order to efficiently locate the reward.

Short-term memory, sometimes referred to as primary or active memory, is the cognitive process involved in remembering small amounts of information for a breif period of time, typically 30 seconds or less (Norris 2017). Miller's law, formulated by Princeton psychology professor George Miller, is the theory that the average human can store about 7 pieces of information within their short-term memory (Miller 1994). More recent studies have shown that people are more reliably able to recall about 4 pieces of information (Cowan 2001). Common behavior tasks that

assess short-term memory are distraction tasks. Typically, these types of tests involve showing a subject one or more objects, then presenting them with a variety of objects and the subject must either identify the singular object presented previously or identify the new object that was not part of the previously shown grouping.

Working memory refers to memory when used to plan and implement specific tasks (Manktelow et al. 2017). This problem-solving form of memory is often used to assess overall intellect (Salthouse and Pink 2008). There is debate within the neuropsychological community whether working memory is a distinct form of memory or if it is synonymous with short-term memory. Many theorists do consider working memory to be its own distinct form of memory as it allows for the retrieval and manipulation of long-term information while incorporating newly acquired short-term information. The vast majority of behavioral testing relies heavily on working memory as the ability to concentrate and maintain attention is dependent upon working memory.

2.6 Imaging for Traumatic Brain Injury

Neuroimaging is a critical tool used in translational research which often provides in-depth assessment of the anatomical structure, function, and connectivity of the brain (Yen, Lin, and Chiang 2023). Studies which implement neuroimaging techniques can advance understanding of research findings and accelerate the translation of preclinical methods to the clinic. Advanced neuroimaging techniques including magnetic resonance imaging (MRI), functional MRI (fMRI), and positron emission tomography (PET), are used to study the brain in a living organism. These remarkable techniques help researchers to visualize abnormalities in the brain and investigate changes associated with specific neurological and psychiatric disorders, advancing diagnostic strategies and providing information crucial for treatment planning and tracking treatment responses (Beauchamp et al. 2011; Chou et al. 2016; Kaiser et al. 2021). A common goal of

neuroimaging is the identification of biomarkers which can objectively indicate the presence of disease, monitor its progression, and illustrate treatment outcomes. Neuroscience researchers can use modern neuroimaging techniques to better understand the fundamental processes surrounding neurological disorders, determine how effective a treatment may be, and design precise, individual treatment strategies.

MRI is a widely used modality in modern medical imaging, offering a non-invasive and versatile method to visualize the internal structures of the body with remarkable clarity and detail. MRI utilizes the principals of nuclear magnetic resonance to generate highly detailed cross-sectional images by exploiting the interaction of hydrogen nuclei within the body's tissues with a strong magnetic field and radiofrequency pulses. Conventional MRI sequences, such as T1-weighted, T2-weighted, and fluid-attenuated inversion recovery (FLAIR) images, provide valuable anatomical information and enable detection of hemorrhages, contusions, and diffuse axonal injuries. Diffusion-weighted imaging (DWI) reveals microstructural changes by assessing water diffusion patterns, offering insights into axonal injury and white matter integrity. Susceptibility-weighted imaging (SWI) enhances sensitivity to microbleeds and iron deposition, particularly relevant for detecting TBI-related microvascular damage. Functional MRI (fMRI) enables visualization of brain activity and connectivity alterations, shedding light on neural network disruptions following TBI (Duhaime et al. 2003; Grate et al. 2003). Advanced quantitative techniques, such as diffusion tensor imaging (DTI) and magnetic resonance spectroscopy (MRS), provide quantitative metrics that reflect tissue integrity and metabolic changes. With advances in technology and the advent of cutting-edge sequences and techniques, MRI continues to revolutionize medical diagnosis, research, and treatment planning, offering a comprehensive and personalized approach to healthcare that addresses diverse clinical needs.

Blood oxygenation level dependent (BOLD) fMRI is a method for non-invasively measuring neural activity and the connection between brain regions by capitalizing on the body's natural hemodynamic response. Briefly, when there is activity in the brain, energy is consumed. The body responds to this consumption of energy by dilating blood vessels and directing more blood flow to the area, delivering more oxygen and nutrients needed to generate more energy. The BOLD signal is generated by comparing the magnetic spin of deoxygenated and oxygenated blood enabling the identification of regions with disrupted functional connectivity post-concussion. It provides insights into neurocognitive dysfunction, as deviations in activation patterns during cognitive tasks can serve as markers of injury. fMRI is routinely used in the clinic for presurgical mapping, and in clinical and preclinical research for studying brain function in health and disease states (Silva et al. 2018; Simchick et al. 2021). Studies which use fMRI can include task-evoked response experiments and resting-state in the absence of a stimulus.

Functional Magnetic Resonance Imaging (fMRI) represents a groundbreaking innovation in neuroimaging, enabling the non-invasive investigation of brain function and connectivity with unparalleled precision (Simchick et al. 2019). By capitalizing on the hemodynamic response to neural activity, fMRI captures changes in blood oxygenation levels to create intricate maps of brain regions engaged during various cognitive, sensory, and motor tasks. This revolutionary technique has revolutionized our understanding of brain organization and dynamics, uncovering networks responsible for perception, memory, emotion, and higher-order cognition. Through task-based fMRI, researchers and clinicians gain insights into brain activation patterns associated with specific behaviors or stimuli. Furthermore, resting-state fMRI unveils intrinsic connectivity networks, shedding light on the brain's intricate web of interactions even in the absence of explicit tasks. fMRI's ability to visualize brain function and connectivity has profound implications,

enabling the study of healthy brain processes, revealing aberrant patterns in neurological and psychiatric disorders, and guiding treatment strategies. As a dynamic tool at the forefront of neuroscientific research, fMRI continues to open new frontiers in our quest to decipher the complexities of the human brain and improve our understanding of cognitive and emotional processes, thereby shaping the landscape of neuroscience and clinical practice.

Concussion presents a unique diagnostic challenge due to the absence of detectable gross anatomical abnormalities on conventional imaging modalities such as CT and MRI (Beauchamp et al. 2011). Unlike moderate and severe TBI that often reveal evident structural damage, concussions primarily manifest as functional disturbances within the brain's neural networks. This elusive nature of concussion has spurred interest in advanced neuroimaging techniques, particularly fMRI as a potentially valuable diagnostic tool. In addition to anatomical MRI, fMRI can help to identify individuals at risk for more severe and longer lasting post-concussive symptoms by revealing the intricate neural perturbations underlying concussion and informing individualized treatment strategies for optimal recovery (O'Neill et al. 2017; Cousins, Blencowe, and Blazeby 2019).

Imaging procedures and experiments that are conducted in animal models or young children oftentimes must be done under anesthetic sedation to prevent the subject from moving during scans. There is a vast body of evidence which has indicated that different anesthetic agents can have vastly different effects on the BOLD fMRI signal, and hence impact the interpretation of the results (Ogawa et al. 1990). These anesthetic agents likely affect mechanisms of neurovascular coupling through changes in blood volume, blood flow and blood deoxyhemoglobin. Thus, it is important to identify anesthetic protocols which induce the most robust and reproducible fMRI responses (Kratzer et al. 2017; Mapelli et al. 2021; Moody et al. 2021; Slupe and Kirsch 2018).

For decades, sevoflurane had been the standard for the maintenance of general anesthesia in pediatric surgical patients due to rapid induction, ease of administration, and lack of foul odor (Goa, Noble, and Spencer 1999). While recovery from sevoflurane tends to be quick, recent studies have reported that many pediatric patients experience agitation and nausea upon emergence (Park et al. 2019; Cravero et al. 2003; Uezono et al. 2000). As an alternative, clinicians have begun to prefer using intravenous propofol for the maintenance of general anesthesia (Uezono et al. 2000; Bryan et al. 2009). Both propofol and sevoflurane are GABAA receptor agonists, although the two agents interact with different subunits of the receptor (Garcia, Kolesky, and Jenkins 2010). Evidence suggests that sevoflurane and other volatile anesthetics interact with the α subunits of the GABAA receptor, whereas propofol is primarily associated with β subunits (Garcia, Kolesky, and Jenkins 2010). While the mechanisms behind sevoflurane-induced versus propofol-induced unconsciousness are believed to be similar, fMRI studies conducted in rodents provide evidence that different anesthetic agents result in varied BOLD responses (Masamoto and Kanno 2012; Aksenov et al. 2015).

Large animal models, pigs included, provide a clinically relevant platform to study how different anesthetic agents affect physiology and fMRI signals. To date, the majority of imaging studies conducted in pigs administer volatile anesthetics such as sevoflurane for the maintenance of general anesthesia (Duhaime et al. 2003; Simchick et al. 2019). These studies tend to be acute in nature, and the animals are not recovered following imaging procedures. Given what is known regarding emergence complications in humans following sevoflurane anesthesia, volatile anesthetics may be less than ideal for use in long-term studies. Additionally, as propofol becomes more commonly used in pediatric patients, the need to conduct preclinical research using propofol anesthesia increases.

The remarkable similarities between pig and human biology make pigs a highly attractive animal species for neuroimaging studies. The anatomical and physiological similarities of the pig and human brains as well as the large body size of the pig allows for the utilization of clinically available techniques and equipment with which to study neurological conditions. The pig's brain is also analogous to humans in neural function. Studies have demonstrated that the resting state neural networks of pigs are particularly homologous to humans (Simchick et al. 2019). The similarities between pigs and humans can help progress our understanding of how various disease states alter the communication between different regions of the brain. In this regard, pigs are an excellent model species for testing the effect of intervention strategies on neuronal networks.

2.7 Non-Invasive Therapeutic Strategies

In general, neurological disorders represent a significant and growing global health challenge. Traditional treatments for many neurological disorders often involve invasive interventions, such as surgery or implantation of medical devices, which come with inherent risks and limitations. Introduction of medical devices into the body carries serous risks such as infection, bleeding, or damage to healthy tissues (Cloft 2011). Invasive procedures in the brain or spinal cord can lead to complications like post-operative infections, cognitive impairments, or physical disabilities. These surgeries often necessitate lengthy recovery periods, during which patients may experience discomfort, pain, or limitations in their daily activities. Invasive procedures often require extended hospital stays, specialized equipment, and skilled medical personnel, making them prohibitively expensive. Additionally, many individuals with neurological disorders may not be suitable candidates for invasive procedures due to various reasons, such as their overall health, age, or the severity of their condition.

Over the past decade, the Pelled lab has been working to develop non-invasive neuromodulatory therapies for the treatment of neural injury. Our lab has previously demonstrated (Lu et al. 2015; Shin et al. 2018) that TBI induced by controlled cortical impact (CCI) (Dixon et al. 1999; Adelson et al. 1998; Pullela et al. 2006; Scafidi et al. 2010; Robertson, Saraswati, and Fiskum 2007; Prins, Povlishock, and Phillips 2003; Kline et al. 2002) in young rats leads to neuronal hypoactivity and interferes with long term potentiation (LTP), one of the most instrumental mechanisms of plasticity, in neurons located in the non-injured primary somatosensory cortex (S1). Nevertheless, TBI is believed to elicit a short, post-injury "critical period" time frame during which neural plasticity is heightened (Overman and Carmichael 2014; Nahmani and Turrigiano 2014; Zeiler et al. 2015; Ng et al. 2015). Taking advantage of this post-injury plasticity, we have reported that the use of high-frequency, repetitive transcranial magnetic stimulation (rTMS), a non-invasive neural stimulation method which has been shown to produce long-lasting effects (Shin and Pelled 2017), facilitated recovery in a rat model of pediatric TBI. Specifically, we found that rTMS treatment increased the expression of calcium calmodulin kinase II (CaMKII), a protein involved in LTP (Lu et al. 2015) and that rats receiving the therapy showed significant improvement in a battery of cognitive and sensorimotor behavioral tests. rTMS is currently FDA approved for the treatment of depression and has been studied as a possible treatment for a myriad of other disorders including schizophrenia (Schulz, Gerloff, and Hummel 2013), pain (Leo and Latif 2007; Lefaucheur et al. 2004; Nardone et al. 2014), stroke (Schulz, Gerloff, and Hummel 2013; Long et al. 2018; Volz et al. 2016), and neurodegenerative disease (Bocci et al. 2016; Spagnolo et al. 2013; Ferbert et al. 1992). Harnessing the brain's innate post-injury plasticity mechanisms using rTMS may very well revolutionize long-term post-injury rehabilitative care in the clinic.

In conclusion, developing non-invasive therapies for neurological disorders is essential to enhance patient safety, reduce healthcare costs, improve accessibility to treatments, and uphold ethical principles. These therapies have the potential to revolutionize the field of neurology, offering hope and relief to millions of people affected by these debilitating conditions.

CHAPTER 3: RESEARCH METHODS

3.1 Subjects and ACUC

Experimental procedures reported herein were approved by the Michigan State University Institutional Animal Care and Use Committee and all were conducted in compliance with National Institutes of Health Animal Research Advisory Committee guidelines.

A total of 22 Yucatan minipigs (Premier BioSource) were used throughout this study (14 female, 8 castrated male). Pigs were same sex group housed at room temperature (~27 ºC) in an enriched environment at the Michigan State University Research Containment Facility (AAALAC approved, BSL3). Pigs were maintained on a twice daily feeding schedule with unrestricted access to water and a 12-hour (7:00-19:00) light cycle.

A summary of respective contributions made by the members of the research team is provided in **Table 3.1**. The table encompasses the creation of the impact device, surgical procedures, imaging procedures, therapy procedures, and behavioral experiments.

3.2 Statistical Analysis

Graphical representation and statistical analysis were conducted using GraphPad Prism 10. Analysis of behavioral videos were conducted manually via watching videos and recording the time of key behaviors in Microsoft Excel. Behavioral results for TBI pigs presented in this dissertation were prepared by normalization of weekly scores to baseline measurements taken 1 week prior to injury. Behavioral results were reviewed by MSU biostatistician Dr. Ana Vazquez.

Summary of Contributions		
Procedure	**Personnel**	**Contributions**
Device	Ricardo Mejia-Alvarez	Design of Impact Device, Construction of Impact Device
	Galit Pelled	Design of Impact Device
	Alesa Netzley	Design of Impact Device
	Alex Vu	Construction of Impact Device
Surgery	Aimee Colbath	Incision, Sutures
	Jane Manfredi	Incision, Sutures
	Kirk Munoz	General Anesthesia
	Deb Papared	Sedation, Intubation, Catheterization
	Thorolf Hoesler	Sedation, Intubation, Catheterization, Operation of Impact Device
	Galit Pelled	Operation of Impact Device
	Alesa Netzley	Preparation of Impact Device, Post-Surgical Monitoring
Imaging	Jane Manfredi	Eye Sutures
	Kirk Munoz	General Anesthesia
	Deb Papared	Sedation, Intubation, Catheterization
	Thorolf Hoesler	Sedation, Intubation, Catheterization
	Alexis Willis-Redfern	Operation of Magnet, Data Acquisition
	Jie Huang	Experimental Design, Quality Control
	Galit Pelled	Experimental Design, Delivery of Stimulus
	Alesa Netzley	Delivery of Stimulus, Data Analysis
TMS	Galit Pelled	Experimental Design, Delivery of Stimulus
	Alesa Netzley	Delivery of Stimulus
Behavior	Alesa Netzley	Experimental Design, Data Acquisition, Data Analysis
	Galit Pelled	Experimental Design
	Ana Vazquez	Statistical Guidance
	Ryan Hunt	Data Acquisition, Data Analysis
	Sanjida Islam	Data Analysis

Table 3.1: Summary of Contributions. List of personnel involved in this work and their respective contributions to experimental procedures. This list encompasses contributions in the development of the impact device, surgical procedures, imaging procedures, delivery of therapeutic stimulus, and behavioral experiments.

3.3 Surgical Procedure

Surgical procedures took place at the Michigan State University College of Veterinary Medicine in the Brinker Surgical Suite. 4 of the 22 pigs used in this study were naiive control animals who did not undergo any invasive procedures. 2 pigs served as trial mTBI subjects. For

the purposes of this study, 16 pigs were randomly assigned to experimental groups: mTBI (n=6), Treatment (n=4), and Sham control (n=6).

Pigs were sedated using an intramuscular injection of midazolam (0.4 mg/kg) and butorphanol (0.4 mg/kg). Anesthesia was induced using isoflurane (5%) in oxygen using a face mask and pigs were orotracheally intubated. General anesthesia was maintained using isoflurane throughout procedures. Once at a surgical plane of anesthesia, an elliptical incision was made, exposing the coronal and sagittal sutures. The periosteum was elevated just posterior to the junction of the right coronal and sagittal suture in the right parietal bone. Following impact, the periosteum and skin were closed in separate layers, and pigs were admitted as patients at the veterinary hospital for 24-hour monitoring.

The impact procedure itself involved the usage of a custom engineered electromagnetically driven impact device which utilized CCI-like highly controlled and reproducible instrumentation to deliver a weight-drop like injury to the closed skull. The device itself was controlled using LinMot ® software which is used to program all elements of the impact including velocity, acceleration, impact depth, dwell time, and retraction rate. The device was outfitted with interchangeable stainless steel impactor tips which were sterilized between subjects. A 3D-printed clear polymer rubber-like disk was placed on the skull to protect from damage. The device was set to deliver a 40 g-force impact, with 15 g of force experienced by the head of the pig.

3.4 Imaging Procedure

Imaging procedures were conducted at the Michigan State University College of Veterinary Medicine Animal Hospital in the dedicated large animal imaging bay.

Pigs were sedated via intramuscular injection of midazolam (0.4 mg/kg) and butorphanol (0.4 mg/kg). Anesthesia was induced using sevoflurane (5%) in oxygen using a face mask. Pigs

were orotracheally intubated, and an intravenous catheter (25G BD PrecisionGlide needle, Franklin Lakes, NJ) was placed in the lateral ear vein. MR-compatible eyelid retractors were used to keep the pigs' eyelids open during imaging procedures. The conjunctivae were temporarily sutured to maintain central placement of the pupil of the eye. Tropicamide (1%), phenylephrine HCl (10%) and atropine (1%) eye drops were instilled on the cornea to dilate the pupils. The eyes were lubricated using Optixcare eye lubricant (Aventix Animal Health, Burlington, ON). General anesthesia was maintained using either inhaled sevoflurane (5%) in oxygen via face mask, or intravenous propofol via continuous rate of infusion (2-20 mg/kg/hr) using the ear catheter.

Imaging procedures were conducted using a Siemens MAGNETOM Espree 1.5T scanner with a 6-channel head/neck coil. Pigs were secured in the supine position. Two fiber optic cables (Thor Labs, Newton NJ) were led alongside the bed and secured to the head coil so the lights would shine directly into the dilated pupil.

T1-weighted and T2-weighted anatomical images and visual-evoked fMRI measurements were acquired with the following parameters; Ax T2: TE/TR 70/10440ms, NEX=2, FOV=140mm, FA=150°, voxel size = 0.7x0.5x2.0 mm3, 40 slices, orientation= transversal, phase encoded direction = R>>L, Matrix 256. Ax T2 Flair: TE/TR=82/6520 ms, NEX=2, FOV=140mm, FA=150°, voxel size = 0.7x0.5x2.0 mm3, 30 slices, TI=2129.6 ms orientation= transversal, phase encoded direction= R>>L, TD=0.0 ms, Base resolution 256, Phase resolution 80%. T1 3D TE/TR=3.02/2400 ms, NEX=2, FOV=250 mm, FA=8°, voxel size = 1.0x1.0x1.0 mm3, slab=1, TI=854 ms, orientation=transversal, phase encoded direction= R>>L, base resolution= 256, phase resolution= 100%. GE-EPI: TE/TR=37/2000 ms, NEX=1, FOV 172 mm, FA=90°, voxel size 2.7x2.7x2.7 mm3, 38 slices, orientation= transversal, phase encoded direction= R>>L, TD=0.0 ms, Base resolution 64, Phase resolution 100% and 120 volume images.

During stimulation-evoked fMRI, pigs were subjected to a 20-seconds-on/20-seconds-off block stimulus paradigm over the course of 160 seconds. During tactile stimulation, both hind limbs of the pig were simultaneously manually pinched and scratched from the heel of the hoof to the calcaneus. During visual stimulation, the lights were dimmed and brightened in quick succession to implement a flashing stimulus.

Analysis of task-based fMRI data was completed using the Analysis of Functional NeuroImages (AFNI) suite using an Ubuntu Linux GPU. Anatomical images were opened in AFNI and a mask of the brain was manually drawn for each individual pig. The new brain mask was used to skull strip both the anatomical and functional datasets via the 3dcalc program. The afni_proc.py script was used to correlate functional signal with the 20-seconds-on/20-seconds-off stimulation paradigm. Normalization of the resulting datasets was conducted by 3dQwarp to a pig brain template published by Fil et al. (Fil et al. 2021). This brain template was used to manually draw several regions of interest (ROI): Left Cortex, Right Cortex, Left Occipital Lobe, Right Occipital Lobe, Left Somatosensory cortex, Right somatosensory cortex. Coordinates were determined using pig brain atlases by Felix et al. (Felix et al. 1999) and Sauleau et al. (Sauleau et al. 2009). Using the AFNI graphical user interface, the finalized anatomical dataset was set as the underlay, and functional datasets set as the overlay. The threshold of the functional dataset was set to 0.05, negative values and clusters of voxels less than 5 were excluded. The total remaining number of voxels were recorded throughout the whole brain, the cortex, and the regions of interest for both visual and tactile stimulation.

3.5 rTMS Procedure

Delivery of repetitive transcranial magnetic stimulation was conducted at the Michigan State University Research Containment Facility in a space adjacent to the housing room and

behavior suite. Prior to surgical procedures, pigs in the treatment group were trained to tolerate restraint in a swine sling (Lomir Biomedical, Quebec CA). Beginning 3 days post-injury, pigs were restrained in the sling for 10 minutes while receiving rTMS treatment. Treatment was delivered using a standard 70mm figure 8 human coil (Magstim, Whitland UK) at 40% current in 20 trains of 4 seconds on 26 seconds off.

3.6 Behavioral Assessment

Behavioral testing was conducted at the University Research Containment Facility in a dedicated Pig Behavior Suite adjacent to the housing room. In preparation for testing, pigs were trained to associate the sound of a training clicker with a treat. Clicker training was used to train pigs to follow a lead stick with a red ball at the end for easy handling. Many of the behavioral methods presented here were originally published in Netzley et al (Netzley et al. 2021).

The majority of behavioral experiments took place in an open behavior chamber measuring 1.83m x 1.83m. The structure consisted of walls made of PVC board which measured 1m in height. A small camera (Omron Sentech) was suspended overhead to record activity and behavior. The concrete floor of the chamber was cleaned with water between subjects. Each behavioral test was conducted once per week.

Open Field Test

In the Open Field Test, pigs were brought individually to the behavioral chamber. The pig was allowed to spontaneously explore the chamber for 10 minutes while locomotor activity was recorded by the camera suspended overhead.

Motion tracking and analysis were conducted using DeepLabCut. The software was first trained to identify various body parts of the pig in multiple poses. This trained model was then used to analyze all open field recordings, and coordinate data for specified body parts was

extracted. Coordinate data was imported to RStudio where the distance traveled by body parts of interest was calculated using a custom-written series of code. RStudio was further used to generate heat maps to illustrate where the pig spent most of their time during the course of the test.

Additional analyses were conducted to determine the amount of time pigs spend trying to escape the chamber. These analyses were conducted by manually scoring videos and recording the time (in seconds) that pigs were engaging in escape-attempt behaviors. These behaviors included rooting at the bottom or pushing against the walls of the chamber and jumping or rearing up against the walls. The data were recorded in Microsoft Excel.

Novel Object Recognition Test

The Novel Object Recognition Test was conducted in two parts: the habituation phase, and the test phase. In the habituation phase, pigs were led individually to the behavioral chamber and exposed to two identical objects. Pigs were allowed to freely explore the objects for the 10-minute testing period and all activity was recorded by the camera suspended overhead. Each pig was then returned to the housing room for an inter-trial interval of 15 minutes for naiive pigs, and 1 hour for experimental pigs. Following this interval, the pig was again led to the behavioral chamber and presented with one familiar object from the habituation phase and one new/novel object that had not been seen before. Again, the pig was allowed to freely explore the objects for 10 minutes while activity was recorded by the overhead camera, after which pigs were returned to the housing room.

Analyses of the data were conducted by watching the recorded videos and manually recording behavior in Microsoft Excel. The time in which pigs spent interacting with both the familiar and novel objects was recorded.

Baited Ball Pit Test

Preparation for the Baited Ball Pit Test involved the placement of a plastic wading pool (91.44 x 91.44 x 17.53 cm) in the behavioral chamber and the filling of the pool with colorful plastic balls (5.59 cm diameter). Six apple slices were buried under the balls with five placed equidistant around the perimeter of the pool and one apple slice in the center. Following preparations, pigs were individually led to the behavioral chamber where they were expected to utilize their natural rooting behaviors to search for the food rewards. Overhead and handheld cameras were used to record activity as the pigs searched for and retrieved apple slices. The test was completed when all six apple slices were located, averaging less than 5 minutes in duration.

Analysis of this test was conducted manually by watching the videos and recording the timestamp at which each apple slice was retrieved using Microsoft Excel. The latency between successful retrievals was calculated, and a mean latency for each trial was determined.

Shape Discrimination Test

A custom-built apparatus was designed to implement a short-term-memory-based shape discrimination task. The apparatus was constructed using PVC board and painted black to generate contrast with the white shapes. The structure of the apparatus was designed in a u-shape measuring 167.64 cm in length, 60.96 cm for the depth of the wings, and 76.2 cm in height. A liftable panel measuring 167.64 cm x 30.48 cm was secured to the front of the apparatus via door hinges. Two circles and two stars were printed on conventional printer paper, cut out, and laminated to be used in this task. Velcro patches were affixed to the back of the shapes with corresponding patches attached to the apparatus; one in the center of the liftable panel, and two on the main board equidistant from the edges and each other so the shapes would be hidden under the liftable panel.

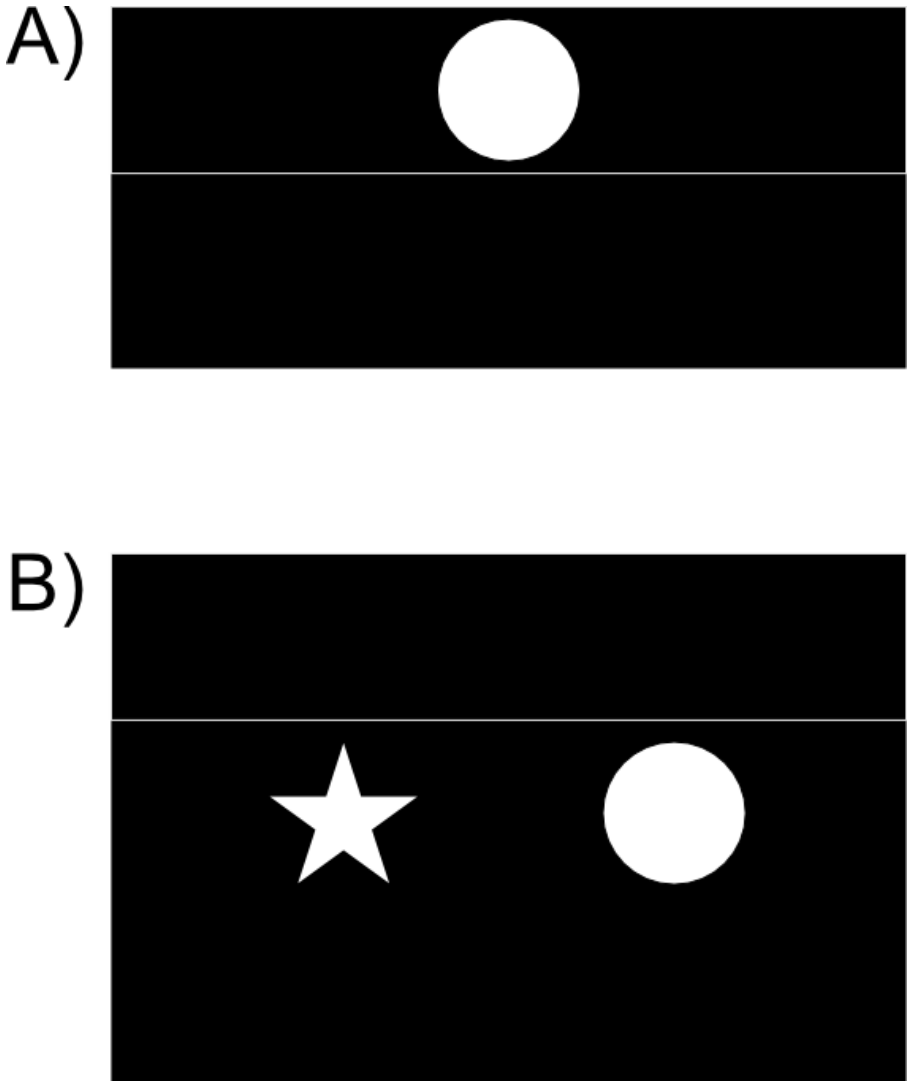

Figure 3.1: Schematic of the set up for the shape discrimination test. **a)** Visual representation of the panel down showing only the target shape. **b)** Visual representation with the panel up, revealing the target and distractor shape.

In preparation for this test, the apparatus was placed in the behavior chamber against the wall opposite the door and one handler was stationed behind the apparatus equipped with food rewards. A target shape (either star or circle) was attached to the center of the liftable panel, and both the star and circle were attached to the main board, hidden behind the aforementioned panel. A second handler would then lead individual pigs into the behavior chamber. Upon nose contact with the target shape presented, the pig would be rewarded with a click from the second handler and a treat from the first. This nose-touch-reward paradigm was repeated two more times, for a

total of 3 contacts with the target shape. Immediately following the delivery of the third reward, the second handler would gently turn the pig around while the first handler would lift the panel, revealing two shapes. The pig was then released and expected to identify the target shape that was previously shown from the two shapes then presented. A correct choice and nose touch of the desired shape was again rewarded with a click and treat, whereas an incorrect choice was neither rewarded nor punished. Again, the pig was expected to make 3 nose contacts, which were rewarded by a click and treat.

Following this first trial of the test, the second handler would lead the pig out of the chamber, while the first handler would swap the locations of the two shapes under the panel. The pig was again led into the chamber and presented with the same target shape. The trial continued and the pig was expected to identify the target shape in its new location. The pig was led out of the chamber again, and the first handler would then swap the target shape. The trials continued so the pig could experience target circle left, target circle right, target star left, and target star right. The circle was always presented as the target shape first, but directionality of the shapes under the panel were always random. Activity was recorded via overhead video.

This test was scored manually by watching videos and recording the time to first touch of the correct shape, total time to complete 3 touches, and the number of incorrect responses.

Color Discrimination Test

Another custom-built apparatus was designed for the color discrimination task. The apparatus was built using PVC board to hold small, lidded boxes (25.4 cm x 30.48 cm x 15.24 cm) in place. The main portion of the apparatus measured 167.64 cm x 35.56 cm. The back edge of the board was raised using a 167.64 cm x 25.4 cm panel, creating a wedged surface on which boxes could be placed. In order to secure the boxes in place, small pieces of PVC board were attached

perpendicular to the main board to holster three boxes. Six vertical pieces (12 cm x 6 cm) were attached from left to right at measurement 25.4 cm, 53.34 cm, 68.58 cm, 96.52 cm, 111.76 cm, and 139.7 cm. Three horizontal pieces (10 cm x 6 cm) were attached at measurements 25.4 cm, 68.58 cm, and 111.76 cm.

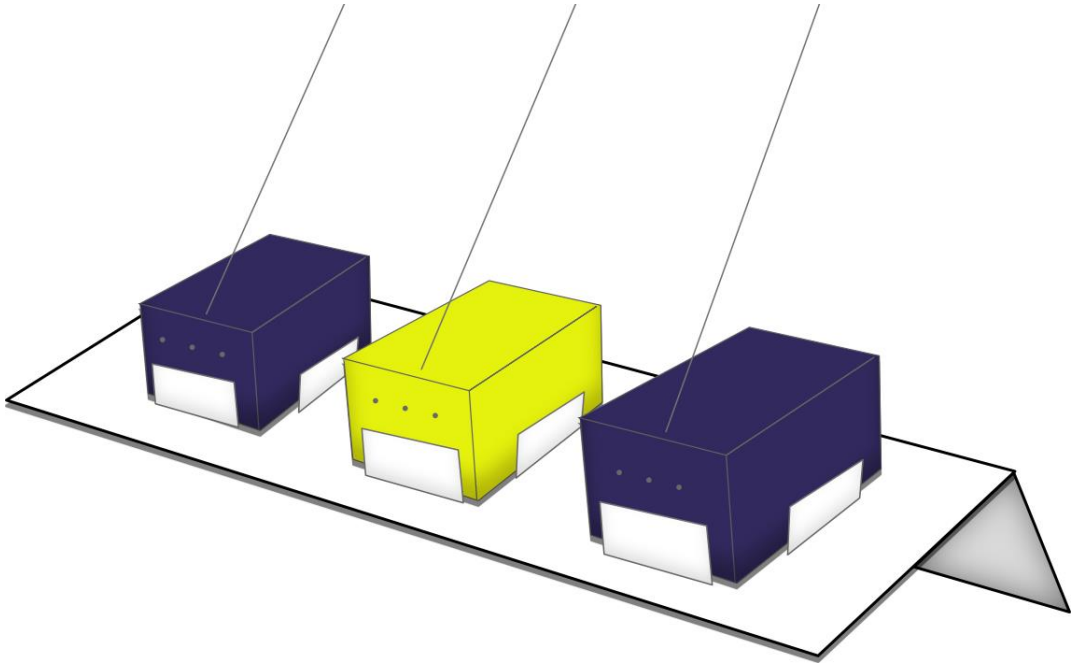

Figure 3.2: Illustration of the set up for the color-discrimination task showing 3 boxes aligned on an angled platform with strings attached to the top of boxes to allow lids to be opened and grant pigs access to reward.

Preparation for this test involved placing the apparatus against the back wall of the behavior chamber opposite the door. An assortment of treats was placed inside two blue and one yellow box. The back edges of the boxes were sealed so they could only be opened from the front. A pull string was attached to the front end of the lids of the boxes, and scent holes were drilled into the front of the basin. The three boxes were placed in random order in the apparatus and the two blue boxes were sealed so only the yellow box could be opened using the pull string. The three pull strings were draped over the edge of the back of the behavior chamber and weighed down lightly

via the attachment of metal washers. One handler was positioned behind the back wall of the behavior chamber to operate the pull strings.

During the test, a second handler would lead individual pigs into the behavior chamber where the pig would be allowed to approach a box in an attempt to eat the food inside. Upon approach of the yellow box, the second handler would reward the pig with a click and the first handler would then pull the string, allowing the pig access to the food inside. No reward or punishment was given for incorrect approaches. Following this first trial, the second handler would remove the pig from the chamber and reposition the boxes so the yellow box was in a new location on the apparatus. The pig would be allowed into the chamber a second time, and upon approach of the yellow box rewarded with a click and access to food. This process was repeated one final time so there were three trials total, and the yellow box had been placed in all three positions.

Analyses of this test were conducted manually. Pigs were scored based on how quickly from time of entry into the chamber they successfully identified the yellow box, and how many times the pig approached the incorrect blue boxes.

Food Aggression Test

This test was implemented only during experimental cohorts of four pigs. All four pigs were led into the behavior chamber and presented with a single bowl of food measuring 24 cm in diameter. The four pigs were allowed to freely attempt to gain access to the food, though only two pigs were able to fit their snouts into the bowl at a time. The test continued until all of the food had been eaten, averaging less than 5 minutes. Video was recorded using a handheld camera.

This test was scored manually by scoring the amount of time in seconds that the pigs spent with their head in the bowl and then dividing by the total duration of the test in order to determine

the percentage of time. Additional analysis was conducted to count the number of individual aggressive events perpetrated by each pig. These events included head swipes and bites.

Activity Tracking

Activity tracking took place withing the pigs' home room in order to determine natural daily activity. No other tasks were completed on activity tracking days. Pigs were outfitted with either conventional fitness tracking devices (Fitbit®) affixed to miniature swine harnesses or Fi® Smart Dog Collars. The pigs wore the tracking devices for 8 continuous hours. Step data was extracted from either the Fitbit® app or the Fi® app.

Sleep Analysis

Continuous 24-hour video monitoring was conducted in the housing room using wide angel cameras (Amazon Cloud Cam©, WYZE Cam©). Times that pigs were asleep or awake were recorded by manually reviewing videos and noting times in Microsoft Excel.

CHAPTER 4: RESULTS

4.1 Development of Cognitive and Behavioral Assessments for Use in Pigs

In order to confidently prepare a battery of behavioral assessments with the potential to identify subtle changes post-injury, I first needed to determine if it would be possible to conduct traditional behavioral testing in pigs. Therefore, I adapted several traditional behavioral assessments for use in pigs and measured outcomes from 18 weeks of age to 37 weeks of age. The results shown in section 4.1 were originally published in Netzley et al (Netzley et al. 2021).

The open field test was used as a neuropsychological screening tool for executive function, anxiety, willingness to explore a new environment and locomotion. In this study, pigs were placed in a 1.83 m x 1.83 m open chamber and their activity was recorded using overhead cameras for 10 minutes, once per week. We used deep-learning artificial intelligence (AI) software, DeepLabCut (Mathis et al. 2018; Hunt et al. 2021), to track pig locomotor activity in the open field arena.

Figure 4.1 shows the distance travelled by pigs (n=4) in the open field from 18 weeks of age to 36 weeks of age. The results indicate that on average the pigs walked 217± 20 m, but between the age of 27-29 weeks old there was a marked increase in walking (396 ± 19 m). RStudio analysis of coordinate data extracted from DeepLabCut allowed for the calculation of the spatial distribution of the pigs' movement and this analysis revealed pigs spend much of their time near the door/entrance to the arena.

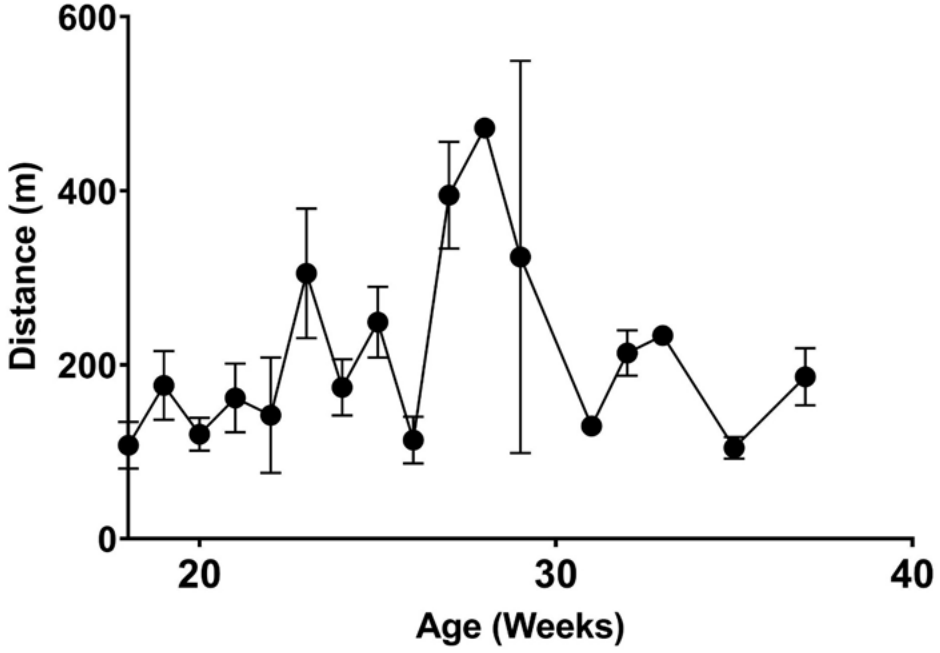

Figure 4.1: Locomotor Activity in the Open Field. The total average distance minipigs traveled in the open field arena. Data show mean and SEM (n=4). A Pearson correlation analysis between age and distance traveled yields an r of 0.149 and a p-value of 0.567.

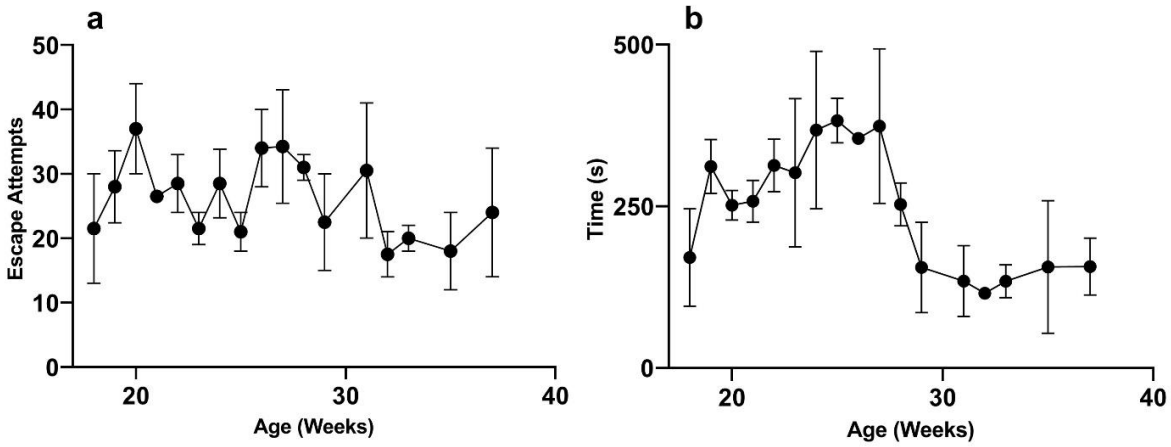

Figure 4.2: Escape Attempts in the Open Field. **a)** Number of times pigs actively attempted to escape the arena during the open field test. Escape attempts consisted of pushing on the walls of the arena with the snout or by rearing on the back two legs. Reported data are mean and SEM (n=4). A Pearson correlation analysis shows no correlation between age and escape attempts p=0.137 **b)** Cumulative duration of seconds pigs spent actively attempting to escape the arena. A non-linear regression analysis yields a Gaussian best fit line with an r-squared value of 0.326.

Figure 4.2 illustrates attempts by the pigs to escape the arena defined as any behavior where the pigs reared or pushed on the walls or door of the chamber. **Figure 4.2a** illustrates the number of individual events where pigs would exhibit this behavior, whereas **4.2b** shows the cumulative seconds pigs spent engaged in escape behaviors. **Figure 4.3** shows heatmap representations of pig location within the chamber. **Figure 4.3a** is a composite heatmap of 4 pigs during the open field test across all weeks. These results confirm observations that pigs spend much of the test period in the area immediately surrounding the door (within 30 cm). Further analysis shows that pigs' spatial distribution differs with age. **Figure 4.3b** is a heatmap representation of one pig at 18 weeks of age, whereas **4.3c** shows the heatmap for the same pig at 36 weeks of age. When pigs are younger, they are less inclined to explore the arena. These data are indicative that young pigs may be more anxious than adults.

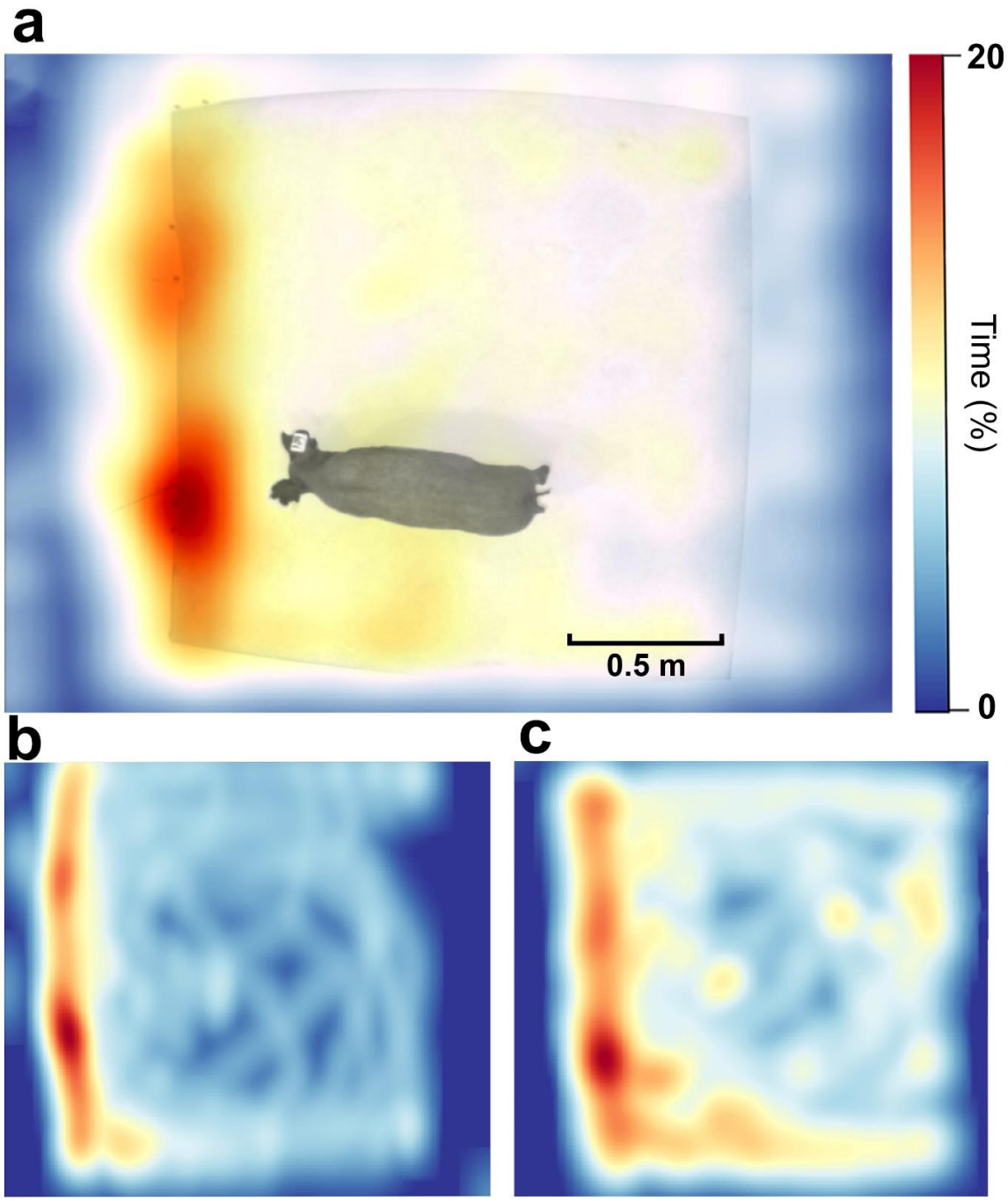

Figure 4.3: Heatmap representation of pig activity in the open field arena. **a)** Composite heatmap of pig location from 18-36 weeks. **b)** Representative heatmap of a single pig at 18 weeks of age. **c)** Representative heatmap of a single pig at 36 weeks of age. Images were generated in RStudio 1.3.1056 using data extracted from DeepLabCut ver 2.2

Measurements of learning and memory, anxiety and depression were performed using the novel object recognition test (Haigh, Chou, and O'Driscoll 2020; Antunes and Biala 2012). In the test phase of this assessment, a pig was presented with one familiar object, and one novel object. The quantity and duration of contacts with both the familiar and novel objects were recorded. The test took place once per week from 19 weeks to 36 weeks. **Figure 4.4** shows results from the test phase of the novel object recognition task. **Figure 4a** illustrates the cumulative duration of contact with the familiar and novel objects. Throughout development, pigs display a significant preference for exploring an unknown, new object. Statistical analysis shows that an unpaired t-test between contacts with the novel object and familiar object yields a p-value less than 0.0001. These results indicate that the difference between contacts with the objects is not the result of chance. **4b** shows the percentage of time the pigs interacted with the novel and familiar objects out of the total time pigs interacted with objects. These results further confirm that healthy pigs prefer interacting with the novel object.

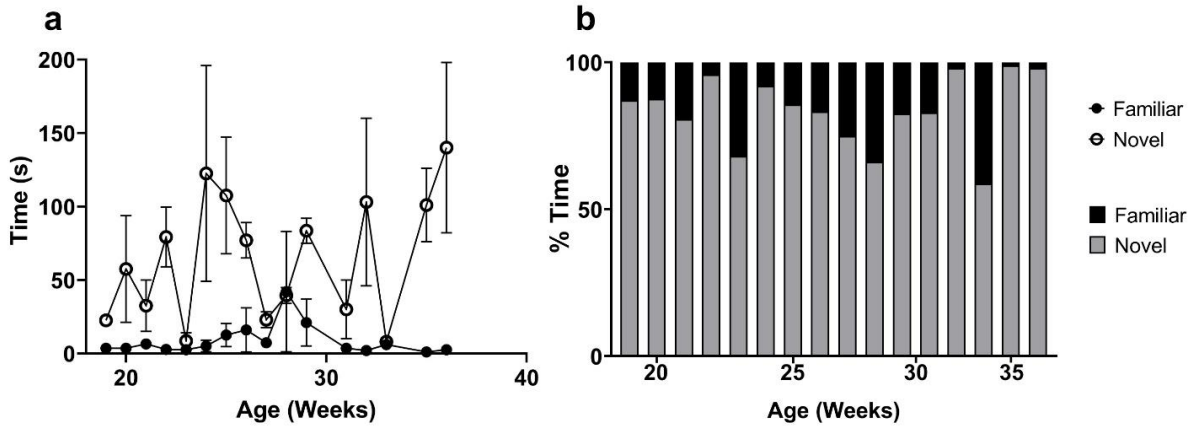

Figure 4.4: Novel Object Recognition. **a)** Cumulative duration of contact with objects during the novel object recognition task. Interactions with the familiar object are represented with solid black circles, whereas the interactions with the novel object are represented by open circles. Pigs preferred to interact with a previously unseen novel object than with an object with which they had been previously habituated. An unpaired T-test yields a two-tailed p-value of 0.0002, and an r-squared value of 0.41. **b)** Average percentage of time pigs spent interacting with objects, split between interactions with the familiar object (black) and the novel object (gray). Pigs consistently interacted with the novel object more than the familiar object. Analysis via unpaired T-test gives a two tailed p-value of less than 0.0001 and an R-squared value of 0.89.

The baited ball pit is a novel assessment of my own creation designed to test executive function, processing speed and spatial learning and memory while utilizing the natural rooting behavior of the pig. In this test, the pig must find six apple slices that are hidden in the ball pit. The slices were always hidden at the same location and the time the pig required to retrieve each of the six slices was determined. **Figure 4.5** demonstrates that throughout development the pig became increasingly faster at identifying the hidden objects. On the first week that the test was administered at age 19 weeks, the pigs completed it within 26.5 ± 0.23 s, but increased their speed at finding the object by 100% when they reached 36 weeks old (7.8 ± 0.09 s; n=4).

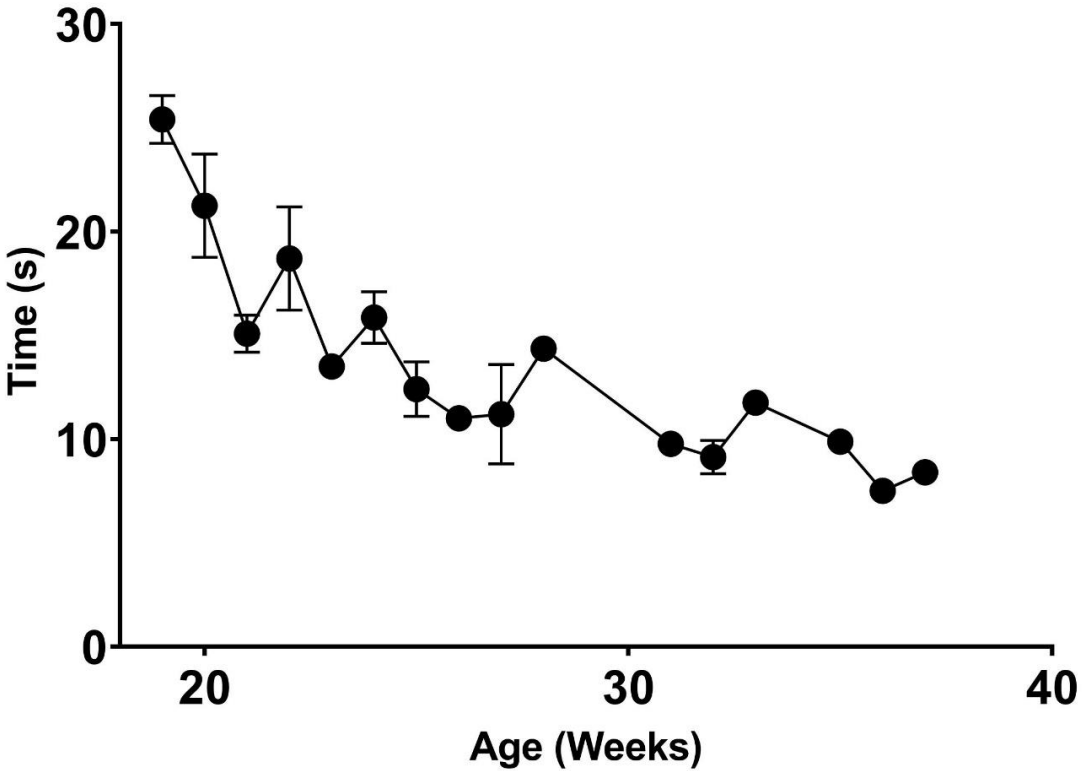

Figure 4.5: Latency between successful food reward retrievals in the ball pit. Pigs exhibit increased rate of successes with age. As pigs are exposed to the test, they become better at finding apple slices. A simple linear regression yields a slope of -0.77, and R-squared value of 0.59 and a p-value less than 0.0001.

The pigs' natural circadian rhythms were assessed in 5-month-old pigs. We combined results from activity tracker and night-vision video recording to determine the pigs wake and sleep cycles. **Figure 4.6** shows that pigs are most active between the hours of 12 pm and 4 pm. Pigs are fed at 8 am and 3 pm. **Figure 4.7** shows that pigs have consistent sleep cycles, waking around 7 am and sleeping around 11 pm, sleeping for an average of 8.7 hours (± 0.2 h).

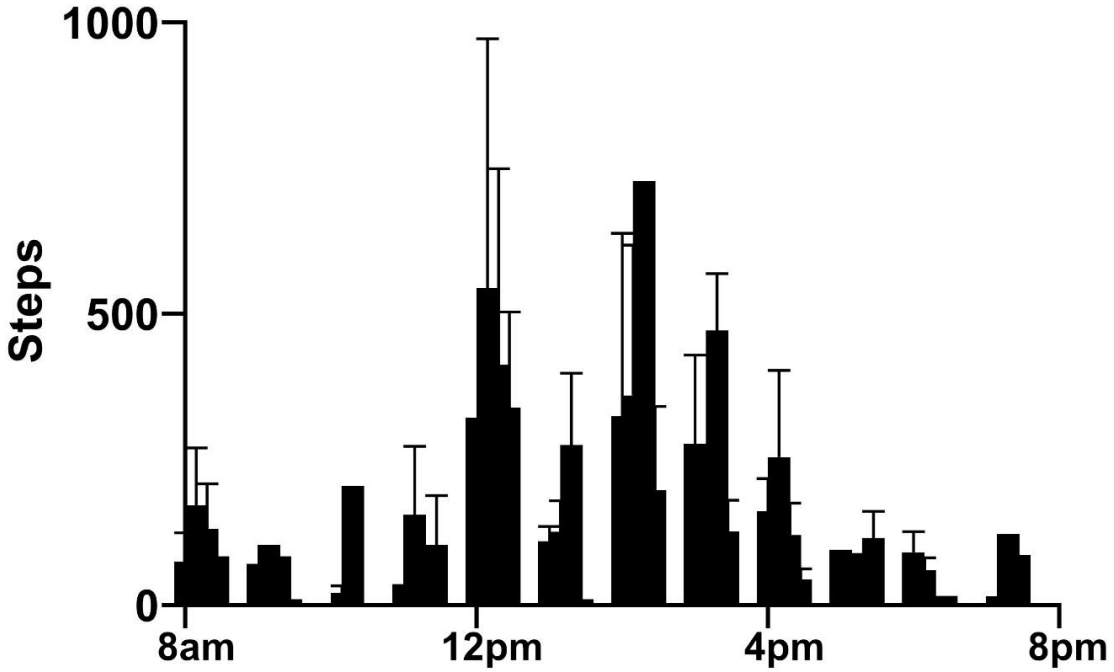

Figure 4.6: Activity tracking/Fitbit step counting. 12-hour (8:00 am-8:00 pm) graph showing steps recorded by an activity tracker (Fitbit) on pigs averaged over 5 days, broken into 15-minute bins. Pigs were most active in the middle of the day, between noon and 4 pm.

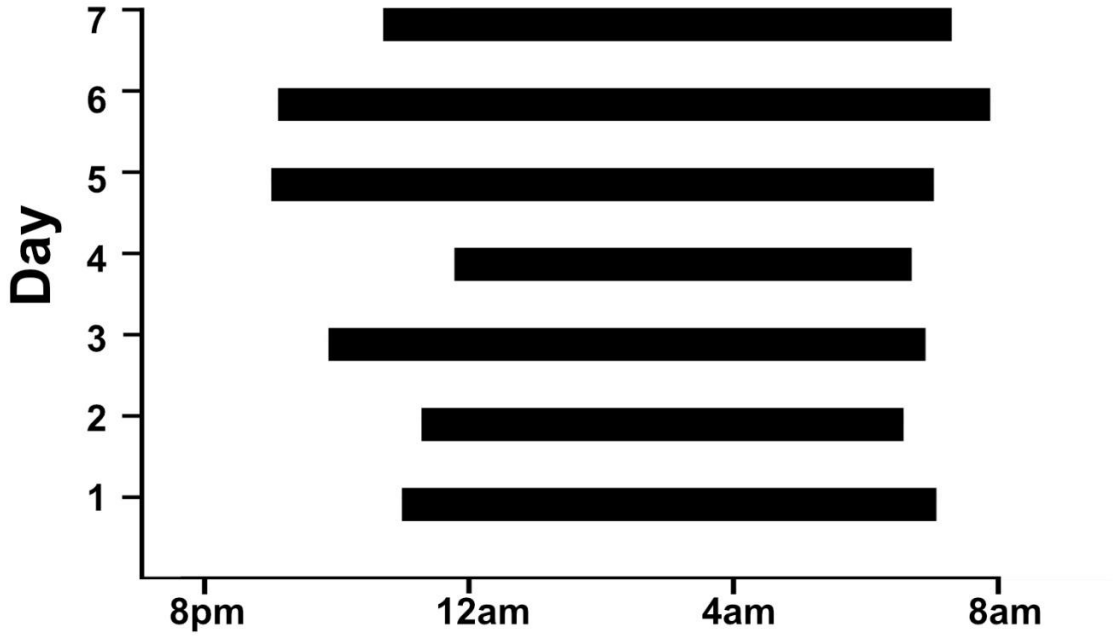

Figure 4.7: Sleep and Circadian Rhythms. Sleep-Wake graph showing that pigs tend to fall asleep between 10 pm and 12 am and tend to wake around 7 am. Pigs slept for an average of 8.7 hours per night.

4.2 Effects of Anesthesia on fMRI

The advancement of translational, large animal imaging procedures is essential for the study of various diseases in a non-invasive, quantitative, and replicable way, leading to improved quality of life for millions of patients worldwide. To date, the majority of preclinical imaging studies, including those in pigs, administer either inhaled isoflurane or sevoflurane for the maintenance of general anesthesia (GA). Although inhalational anesthetics are easy to administer, evidence from preclinical studies showed that they significantly diminish fMRI signals through altered neurovascular coupling. Human imaging studies have suggested that propofol may have a lesser effect on cerebral hemodynamics, therefore propofol has become a preferred substance for the maintenance of GA in pediatric fMRI studies. It remains critical to identify the optimal anesthetic regime to obtain accurate information about brain function. In this section, I compared visual- and tactile-evoked fMRI responses in the occipital and sensorimotor regions of Yucatan minipigs under sevoflurane versus propofol anesthesia.

Visual stimulation was delivered via fiber optic cables positioned with the light shining directly into the eye. The stimulation paradigm consisted of a 20-seconds-on/20-seconds-off block stimulus, with 4 sets over 160 seconds. The results, as shown in **Figure 4.8** and **Figure 4.9**, demonstrate that sevoflurane yielded a significantly more robust fMRI response to visual stimulation compared to propofol; The average number of activated voxels across the brain was 5275 ± 2265 for sevoflurane and 2886 ± 2213 for propofol (**Figure 4.8a**). The number of activated voxels in the cortex (**Figure 4.8b**) was significantly higher for sevoflurane (mean: 4284 ± 2043) compared to propofol (mean: 2162 ± 1780) (non-parametric, unpaired t-test, $p=0.026$). Isolation of the occipital lobe (**Figure 4.8c**) reveals that the number of activated voxels was significantly higher for sevoflurane (mean: 2540 ± 1680) than propofol (mean: 639 ± 434); (T-test, $p=0.0173$).

Brain activation maps comparing the response to visual stimulation in the occipital lobe under the two anesthetic agents can be seen in **Figure 4.9**. The response to visual stimulation was more widespread in pigs anesthetized with sevoflurane compared to pigs anesthetized with propofol. The scale bar for the signal overlay represents the F-statistic, which is the variance between the ideal fMRI signal curve and our true response signal.

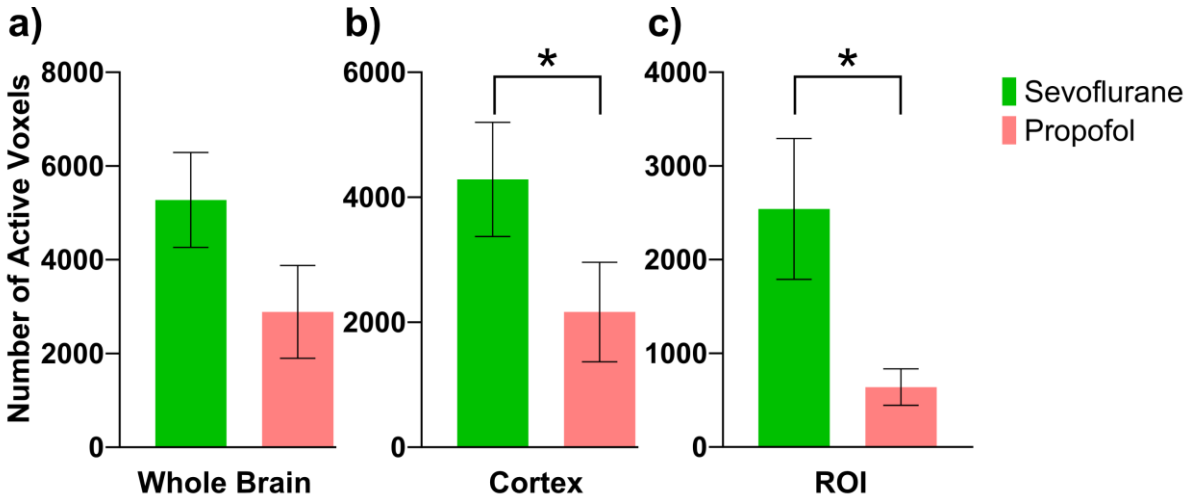

Figure 4.8: Group effects of sevoflurane (n=6) and propofol (n=6) on visual stimulation showing mean and SEM. **a)** Count of the total number of activated voxels throughout the whole brain during visual stimulation. Statistical t-test analysis suggests there are no significant differences in activation between pigs anesthetized with sevoflurane or propofol (p=0.0649). **b)** Count of the total number of voxels activated within the cortex during visual stimulation. Results from a t-test indicate there is a significant difference in the number of activated voxels in the cortex of pigs anesthetized with sevoflurane versus propofol (p=0.0260). **c)** Count of the number of activated voxels within the occipital lobe of the brain during visual stimulation. T-test analysis indicates that the neural response to sevoflurane is significantly greater than the response to propofol throughout visual areas (p=0.0173).

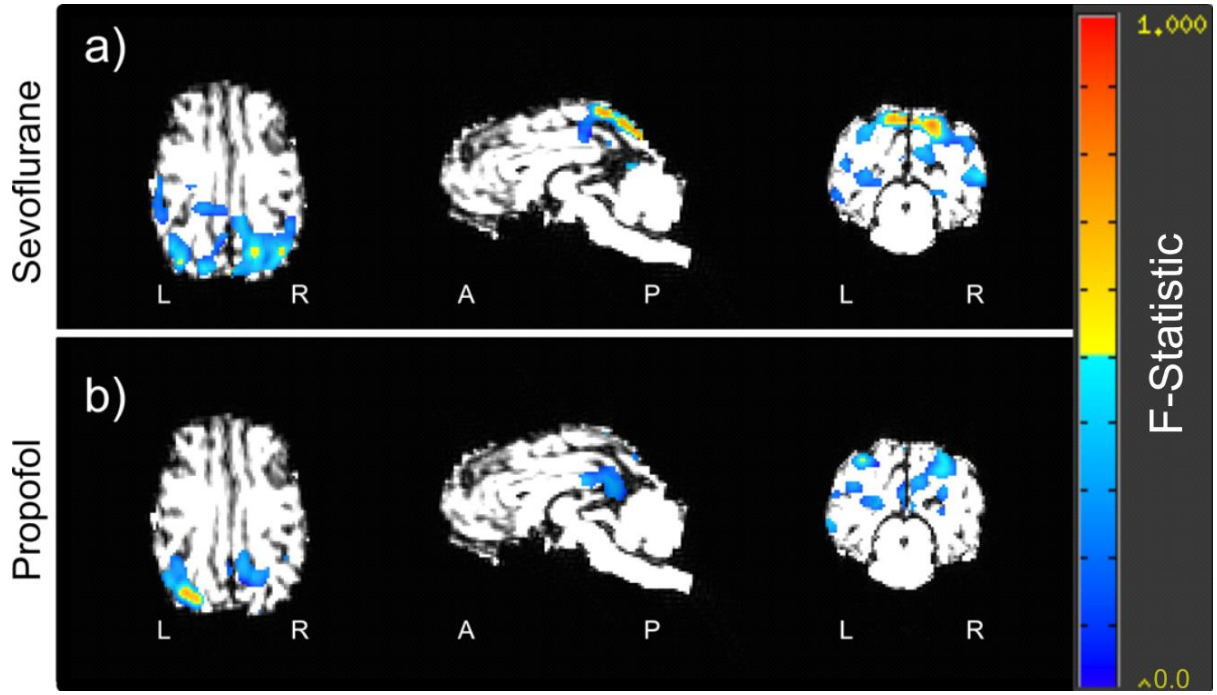

Figure 4.9: Brain maps showing extent of activation in the occipital lobe during visual stimulation. **a)** Average BOLD fMRI activation of pigs(n=6) under sevoflurane anesthesia. **b)** Average BOLD fMRI activation of pigs under propofol anesthesia (n=6). Scale bar represents the F-statistic, or the variance between our signal and the ideal fMRI response. Only positive values shown here.

Results from tactile stimulation reveal a different response profile. Tactile stimulation consisted of a 20-seconds-on/20-seconds-off block stimulus paradigm where the hind limb was scratched and pinched from the pad of the hoof to the calcaneus. fMRI data reveal a trend towards a heightened response to propofol compared to sevoflurane. **Figure 4.10a** illustrates the number of voxels activated throughout the whole brain under sevoflurane (2685 ± 653) and propofol anesthesia (3946 ± 1684). The number of active voxels in the cortex (**Figure 4.10b**) reveals a mean for sevoflurane of 1753 ± 528 and propofol 2655 ± 1039. Isolation of the somatosensory area (**Figure 4.10c**) illustrates the number of active voxels in pigs under sevoflurane (226 ± 165) and propofol (639 ± 434) anesthesia. **Figure 4.11** shows brain activation maps demonstrating the extent of the response to tactile stimulation under sevoflurane **(a)** and propofol **(b)**.

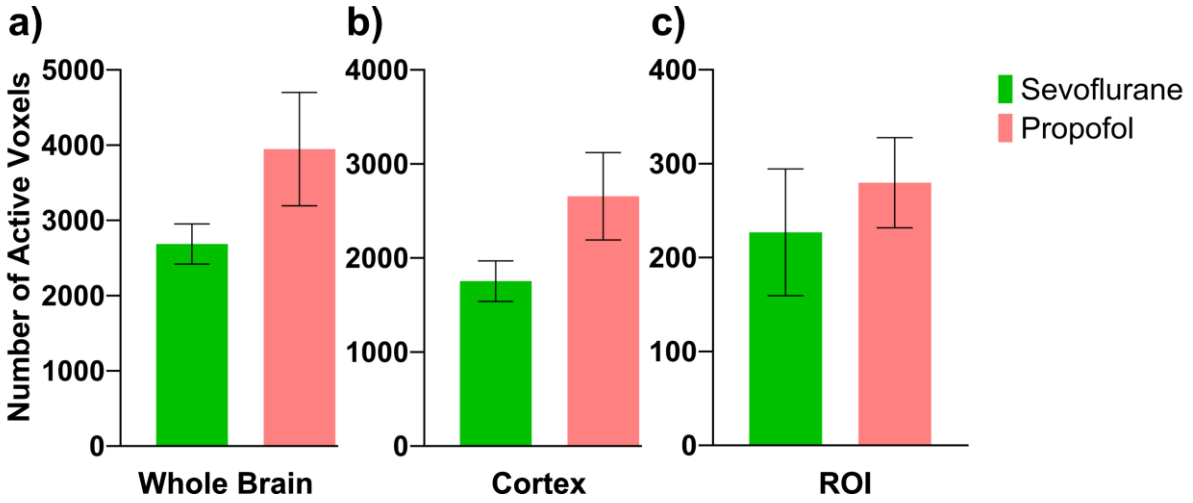

Figure 4.10: Group effects of sevoflurane (n=7) and propofol (n=6) on tactile stimulation showing mean and SEM. **a)** Count of the total number of activated voxels throughout the whole brain during tactile stimulation. Statistical t-test analysis indicates there are no significant differences between the responses to tactile stimulation under either sevoflurane or propofol anesthesia (p=0.2343). **b)** Count of the number of voxels active in the cortex during tactile stimulation. T-test analysis shows no significance (p=0.1807). **c)** Count of the number of voxels active in the sensorimotor area during tactile stimulation. Analysis using a t-test indicates there are no significant differences between groups (p=0.6544).

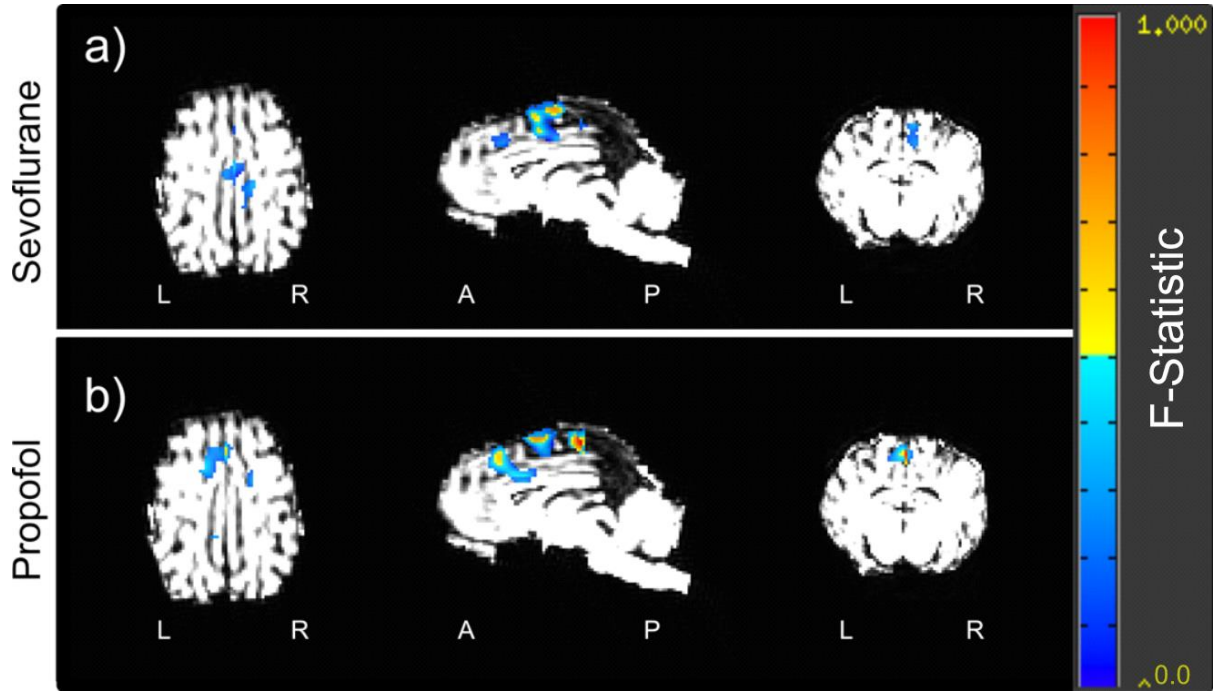

Figure 4.11: Brain maps showing extent of activation in the sensorimotor area during tactile stimulation. **a)** Average BOLD fMRI activation of pigs under sevoflurane anesthesia (n=7). **b)** Average BOLD fMRI activation of pigs under propofol anesthesia (n=6). Only positive values shown here.

Statistical analysis was also conducted to evaluate if there are sex differences in the fMRI response to the two anesthetic agents. During visual stimulation, male pigs exhibit a heightened average response (6074 ± 3219) compared to female pigs (4876 ± 1429) when anesthetized with sevoflurane throughout the whole brain (**Figure 4.12a**). This trend is conserved in the cortex (males:5143 ± 2809, females:3854 ± 13289) as shown in **Figure 4.12b**, and in the visual cortex (males:3241 ± 2340, females:2190 ± 1062) as shown in **Figure 4.12c**. Conversely, under propofol anesthesia, the average response of female pigs (3414 ± 2500) was greater than male pigs (2406 ± 1004) throughout the brain (**Figure 4.13a**). Once again, the trend is conserved in the cortex (males:1779 ± 690, females:2502 ± 2044), shown in **Figure 4.13b**, and the occipital lobe (males:646 ± 216, females:993 ± 984), shown in **4.13c**. These differences are not statistically significant.

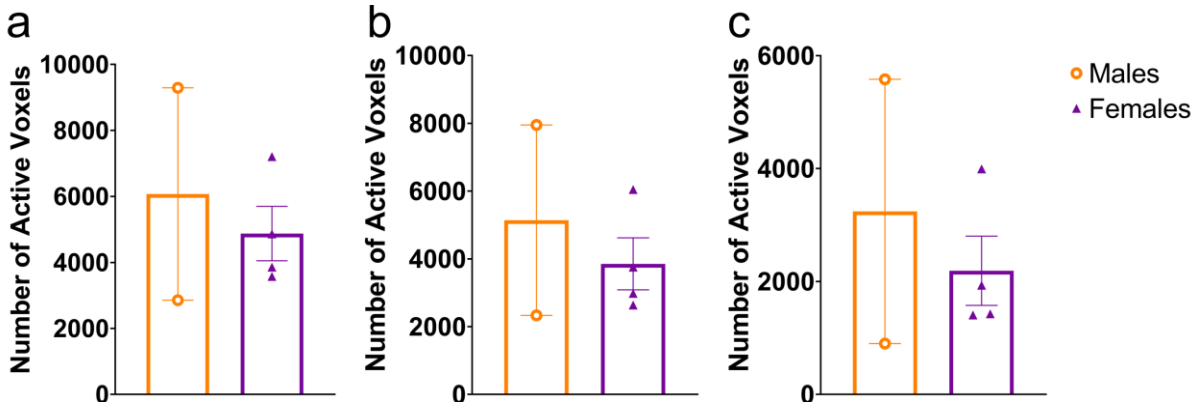

Figure 4.12: Effect of sex on the response to visual stimulation in pigs anesthetized with sevoflurane (Males: n=2; Females: n=4). **a)** Number of active voxels throughout the whole brain during visual stimulation under sevoflurane anesthesia. Statistical t-test analysis suggests there is no significant effect of sex on activation throughout the whole brain (p=>0.9999). **b)** Count of the total active voxels throughout the cortex during visual stimulation under sevoflurane anesthesia. Results from a t-test shows no significant differences between groups (p=>0.9999). **c)** Count of the total number of active voxels in the occipital lobe during visual stimulation. T-test suggests there are no statistical differences between groups (p=>0.9999).

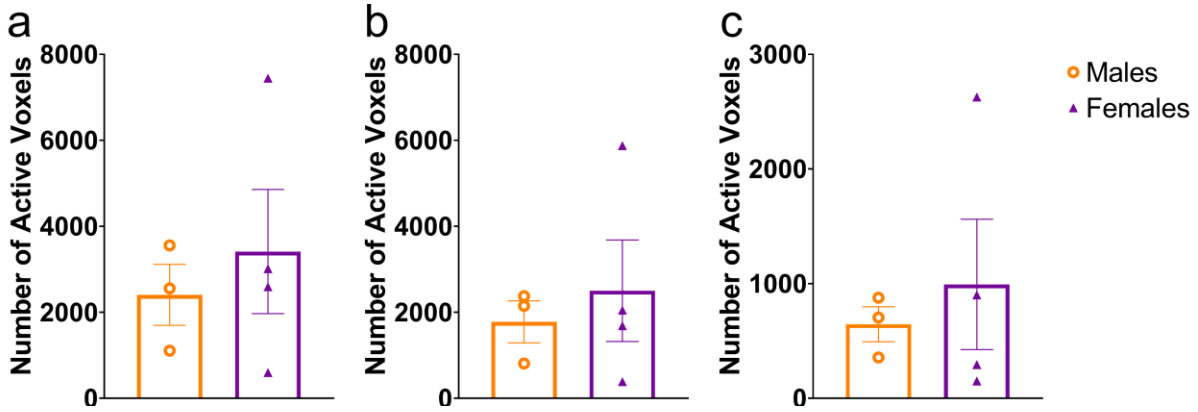

Figure 4.13: Effect of sex on the response to visual stimulation in pigs anesthetized with propofol (Males: open blue circles, n=3; Females: red triangles, n=4). **a)** Number of active voxels throughout the whole brain during visual stimulation under propofol anesthesia. T-test analysis suggests there is no significant effect of sex on activation throughout the whole brain (p=0.8571). **b)** Count of the total active voxels throughout the cortex during visual stimulation under propofol anesthesia. Results of a t-test show there is no significant differences between sexes (p=0.8571). **c)** Count of the total number of active voxels in the occipital lobe. Statistical analysis via t-test indicates there are no differences between sexes (p=>0.9999).

Tactile stimulation yielded similar results for pigs anesthetized with sevoflurane or with propofol. Under sevoflurane anesthesia, the response to tactile stimulation was similar between male pigs (2518 ± 426) and female pigs (2811 ± 757) throughout the whole brain (**Figure 4.14a**). Assessment of the cortex (**Figure 4.14b**) reveals the average response of male pigs was 1523 ± 397, while the average response of female pigs was 1926 ± 548. In the sensorimotor area (**Figure 4.14c**), females exhibited a higher average response (320.25 ± 161) compared to males (114 ± 20). Under propofol anesthesia, the average response of female pigs to tactile stimulation (4506 ± 1615) was higher than that of males (2749 ± 979) throughout the brain (**Figure 4.15a**). This trend is conserved in the cortex (males: 1799 ± 770, females: 2976 ± 966) as shown in **Figure 4.15b**, and in the sensorimotor area (males: 224 ± 70, females: 318 ± 164) as shown in **Figure 4.15c**). Overall, these differences are not statistically significant.

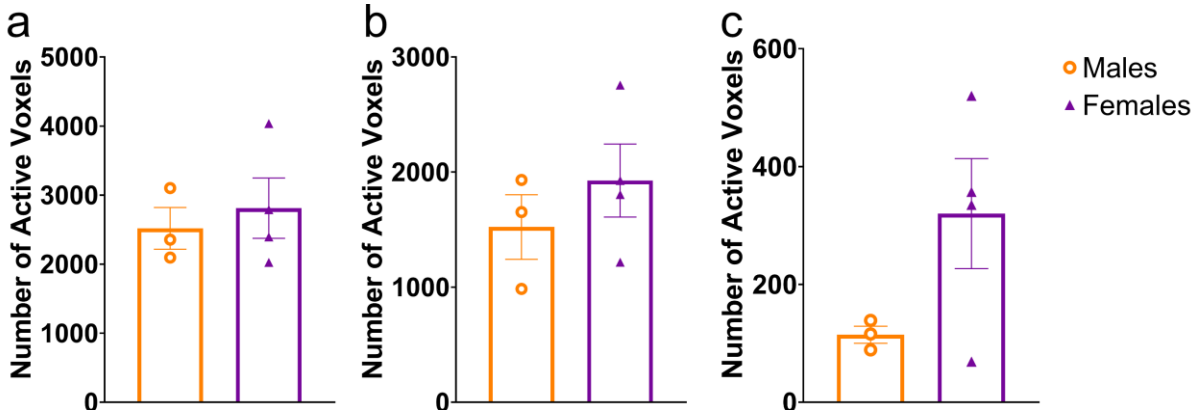

Figure 4.14: Effect of sex on the response to tactile stimulation in pigs anesthetized with sevoflurane (Males: open blue circles, n=3; Females: red triangles, n=4). **a)** Number of active voxels throughout the whole brain during visual stimulation under sevoflurane anesthesia. Analysis using a t-test suggests there are no effects of sex on the response to tactile stimulation (p=0.8571). **b)** Count of the total active voxels throughout the cortex. A t-test shows no significance between sexes (p=0.6286). **c)** Count of the total number of active voxels in the sensorimotor area. A t-test indicates no statistically significant differences between the male and female response to tactile stimulation under sevoflurane anesthesia (p=0.4000).

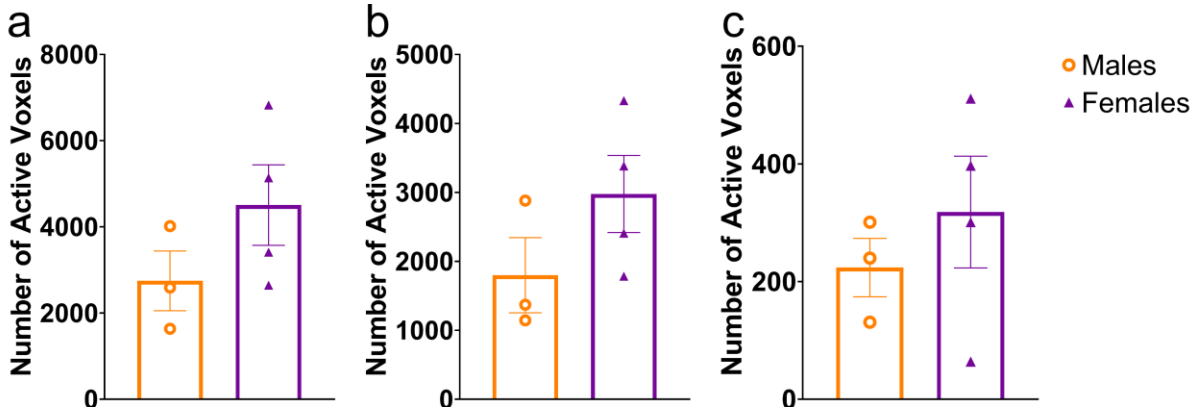

Figure 4.15: Effect of sex on the response to tactile stimulation in pigs anesthetized with propofol (Males: open blue circles, n=3; Females: red triangles, n=4). **a)** Number of active voxels throughout the whole brain during tactile stimulation under propofol anesthesia. A t-test shows no statistically significant differences between groups (p=0.2286). **b)** Count of the total active voxels throughout the cortex. Analysis using a t-test suggests there is no significant effect of sex on the response to tactile stimulation (p=0.2286). **c)** Count of the total number of active voxels in the sensorimotor area. A t-test indicates there are no statistically significant differences between males and females in the response to tactile stimulation under propofol anesthesia (p=0.4571).

4.3 Effects of Concussion in Yucatan Minipigs

Brain injury can have lasting effects on cognition and neural processing. While many individuals who sustain mild head injury often recover spontaneously without incident, a subset of patients continue to experience post-concussive changes in cognition, memory, mood, and executive functioning long after the acute clinical recovery period. For the purposes of this work, I was most interested in determining whether pigs who underwent the concussion procedure would exhibit post-concussive changes in behavior and fMRI response to stimulation.

Mild head injuries can be difficult to diagnose, but recent studies have suggested that fMRI can detect subtle changes in connectivity and function that cannot be identified using conventional imaging methods. Using task-based visual and tactile stimulation, I sought to determine whether pigs with concussion would exhibit differences in neural activation when compared to surgical sham animals.

Figure 4.16 illustrates the total number of activated voxels present within the occipital region of the brain in response to visual stimulation. At 24 hours post injury, sham pigs exhibited mean activation of 642 ± 286 voxels and mTBI pigs exhibited mean activation of 515 ± 389 voxels. Statistical analysis using a standard t-test comparing groups yields a p-value of 0.334 at this 24-hour time point. Mean activation of sham pigs at 5 weeks post injury was 1077 ± 383 voxels, whereas mTBI pigs mean activation was 600 ± 422 voxels. Standard t-test analysis shows that the difference between groups at 5 weeks is trending toward significance with a p-value of 0.099, however this difference is not great enough to objectively state that the difference is not simply due to chance. At 12 weeks post injury, statistical analysis reveals a p-value of 0.469 when comparing mean activation of sham pigs (1192 ± 921) and mTBI pigs (1243 ± 537). **Figure 4.17** shows representative BOLD fMRI brain maps comparing the extent of visual response to visual

stimulation in pigs with and without concussion. The extent of response to visual stimulation in sham pigs is demonstrated in **4.17a**, whereas **4.17b** illustrates the extent of visual response in pigs with concussion.

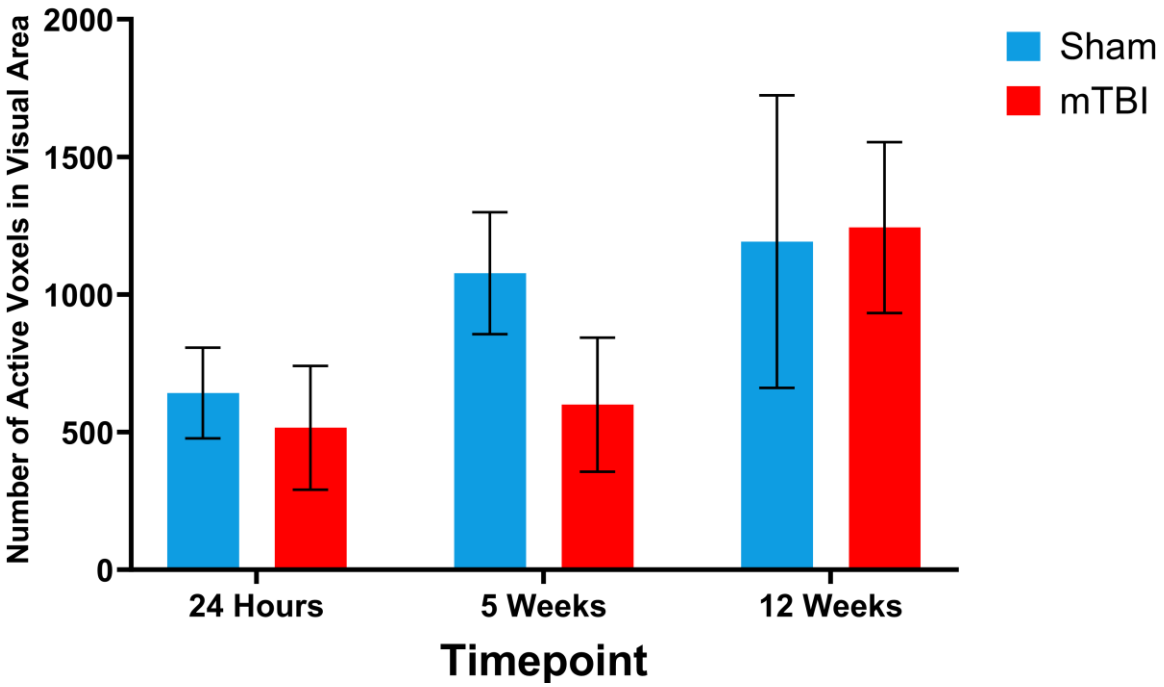

Figure 4.16: Neural Activation in Response to Visual Stimulation in the Visual Cortex. Bar graph comparing the number of voxels in the visual region of the brain activated in response to visual stimulation in sham pigs (n=4) and mTBI pigs (n=4) at three timepoints. T-test statistical analysis comparing the activation at the three timepoints yields p-vales of 0.334 at 24 hours, 0.099 at 5 weeks, and 0.469 12 weeks post-injury.

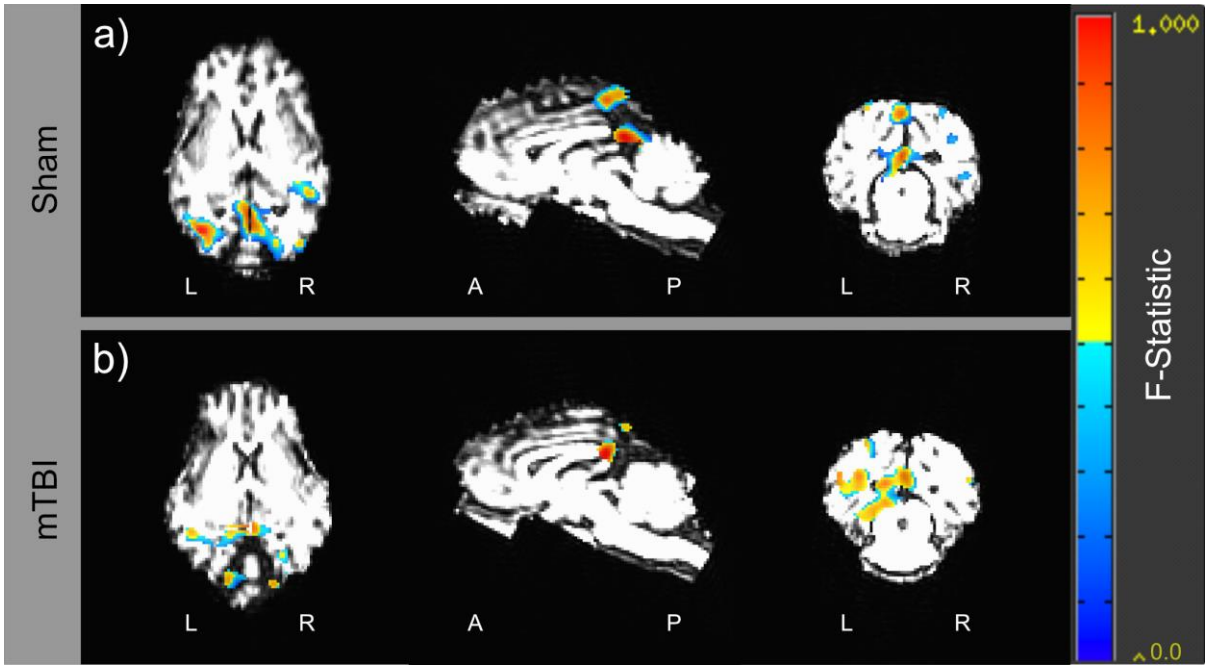

Figure 4.17: Representative brain maps showing the extent of activation in the visual cortex in response to visual stimulation in healthy and concussed pigs 5 weeks post-injury. **a)** BOLD fMRI response to visual stimulation in the visual region of interest (ROI) in surgical sham pigs (n=4). **b)** BOLD fMRI response to visual stimulation in the visual ROI in pigs with concussion (n=4). Only positive values shown.

Figure 4.18 shows the total number of voxels activated in the sensorimotor area in response to tactile stimulation. At 24 hours post-injury the mean activation of sham pigs was 185 ± 140, whereas mTBI mean activation was 273 ± 64. A t-test comparing these differences yields a p-value of 0.189. Activation of sham pigs at 5 weeks post-injury was 215 ± 70, and the activation of mTBI pigs was 328 ± 101. Statistical analysis comparing groups at 5 weeks yielded a p-value of 0.084, indicating there is a trend towards significance at this timepoint, however the differences are not great enough to indicate this difference is not due to chance. At 12 weeks, the mean activation of surgical sham pigs was 191 ± 78. The mean activation of mTBI pigs in response to tactile stimulation at 12 weeks post-injury was 355 ± 185. A t-test analysis of these differences yielded a p-value of 0.115. The BOLD fMRI response to tactile stimulation is illustrated in **Figure 4.19**.

Representative brain maps of sensory response to tactile stimulation in sham pigs is shown in **4.19a** and response to tactile stimulation in pigs with concussion is shown in **4.19b**.

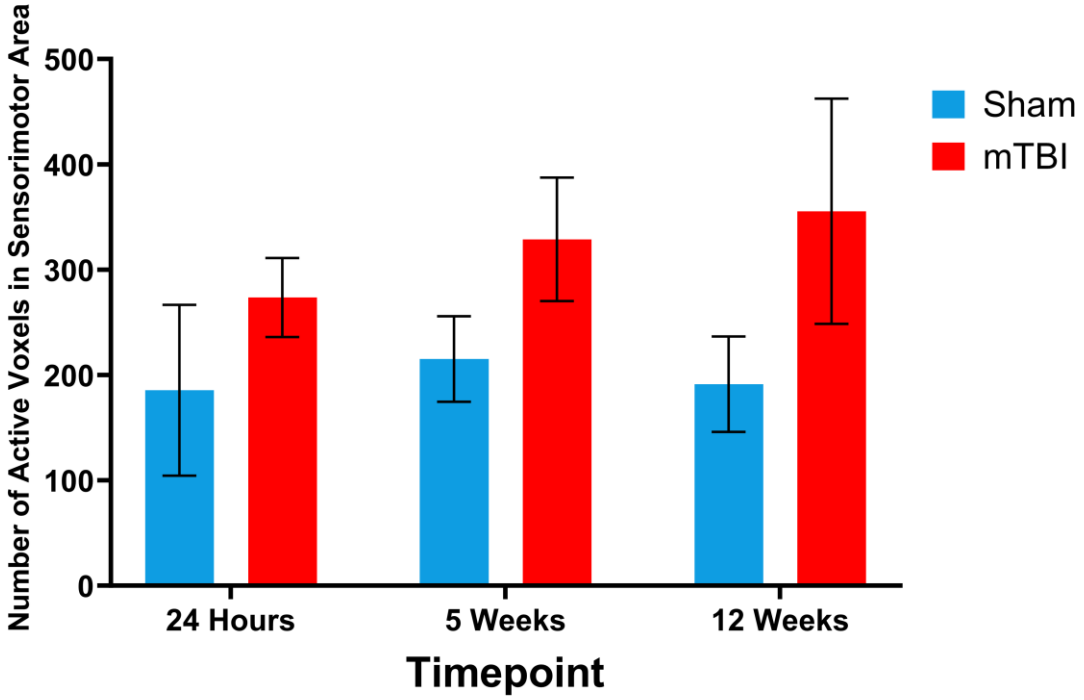

Figure 4.18: Neural Activation in Response to Tactile Stimulation in the Sensorimotor Cortex. Bar graph comparing the number of voxels in the sensorimotor region of the brain activated in response to tactile stimulation in sham pigs (n=4) and mTBI pigs (n=4) at three timepoints. T-test statistical analysis comparing the activation at the three timepoints yields p-vales of 0.189 at 24 hours, 0.084 at 5 weeks, and 0.115 12 weeks post-injury.

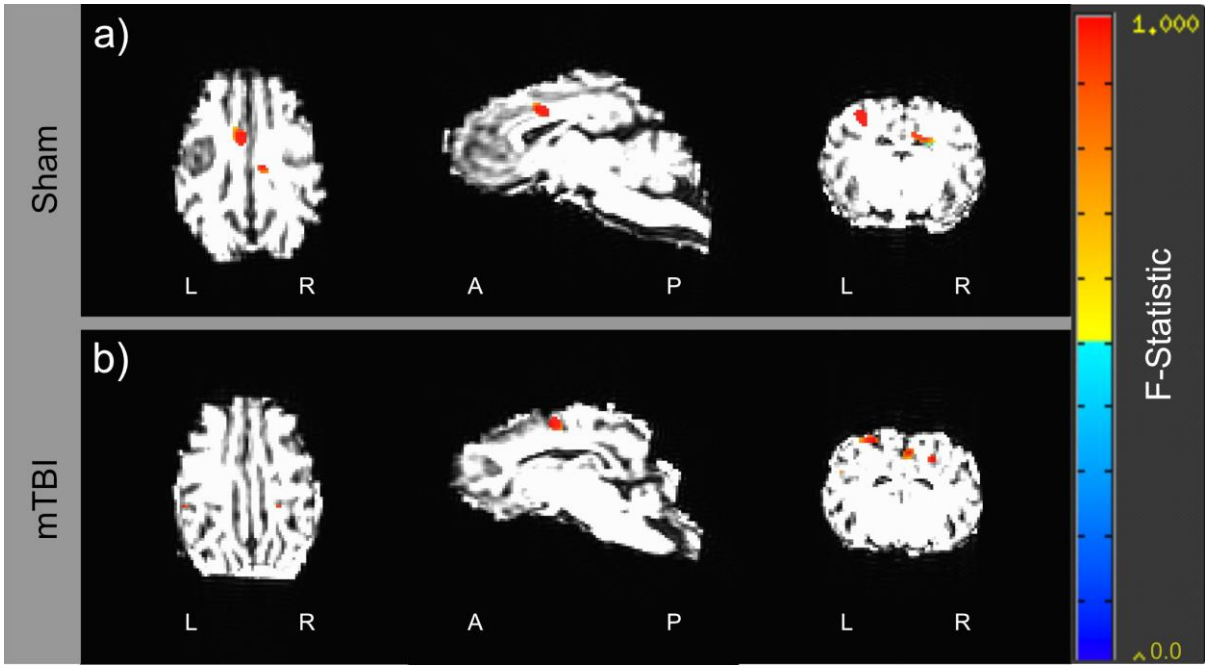

Figure 4.19: Representative brain maps showing the extent of activation in the sensorimotor area in response to tactile stimulation in healthy and concussed pigs 5 weeks post-injury. **a)** BOLD fMRI response to tactile stimulation in the sensorimotor region of interest (ROI) in surgical sham pigs (n=4). **b)** BOLD fMRI response to tactile stimulation in the sensorimotor ROI in pigs with concussion (n=4). Only positive values shown.

Psychological assessment for changes in locomotor activity, anxiety, and novelty seeking behaviors were performed using the open field test. **Figure 4.20** shows the percent change in total distance traveled by pigs in the open field. Data was normalized to baseline measurements taken one week prior to injury. **Table 4.1a** shows the p-value for t-tests calculated between groups in the acute phase and the chronic phase. **Table 4.1b** shows p-values for t-test of group means in comparison to baseline measurements.

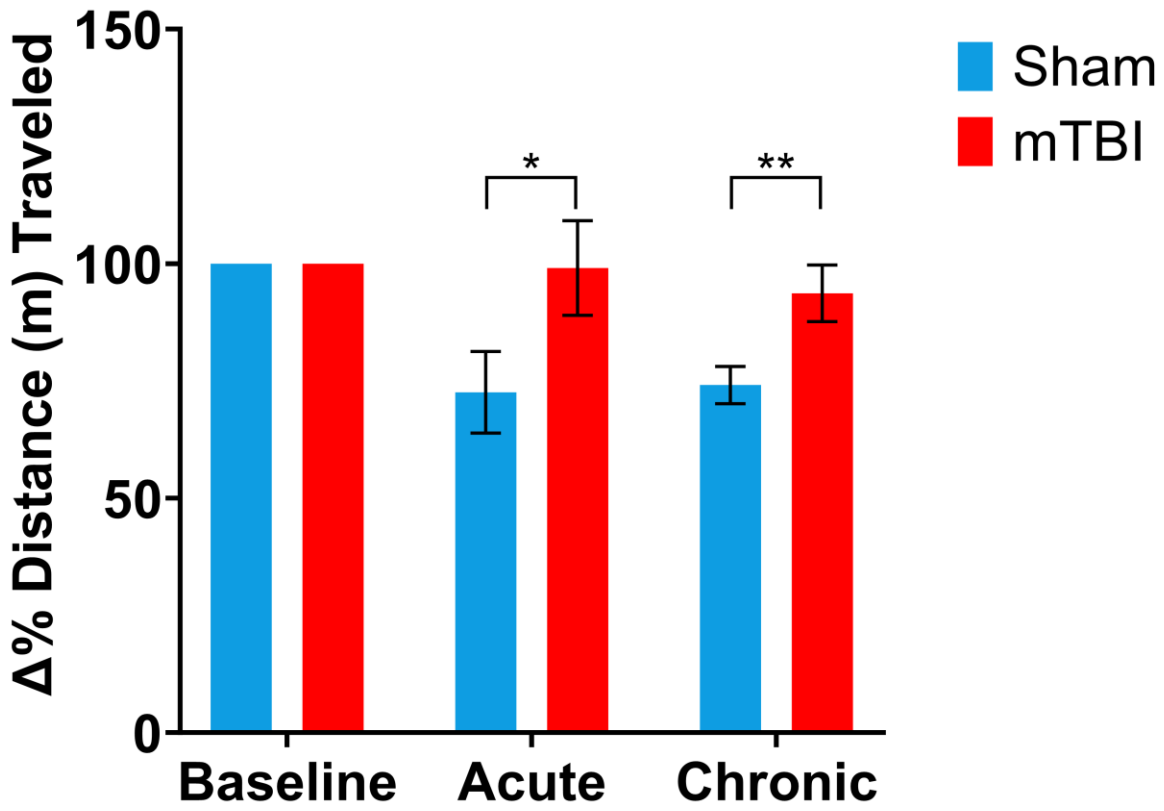

Figure 4.20: Percent change in distance (m) traveled in the open field in the acute phase and the chronic phase. Mean and SEM for distance measures of sham pigs (n=6) and mTBI pigs (n=6) in the open field over the course of 10 minutes. Data is normalized to baseline values measured 1 week prior to injury. Asterisks shown for between group comparisons only.

a)

Timepoint	Acute	Chronic
Sham vs mTBI	0.035	0.005

b)

Timepoint	Acute	Chronic
Sham vs Baseline	0.006	0.0000001
mTBI vs Baseline	0.465	0.153

Table 4.1: Statistical analysis for distance traveled in the open field. **a)** Between group statistics showing p-value results of t-test calculated in the acute phase and the chronic phase. **b)** Within group statistics showing t-test p-value comparison of means in the acute phase and chronic phase with pre-injury baseline values.

Anxiety of mTBI and sham pigs was assessed by tracking the number of individual escape attempts (**Figure 4.21**) and the total cumulative duration of escape attempts (**Figure 4.22**) made by pigs in the open field. Data was normalized to baseline measurements taken one week prior to injury. Statistical analysis of escape attempts is listed in **Table 4.2** (Number of attempts) and **Table 4.3** (time spent attempting escape).

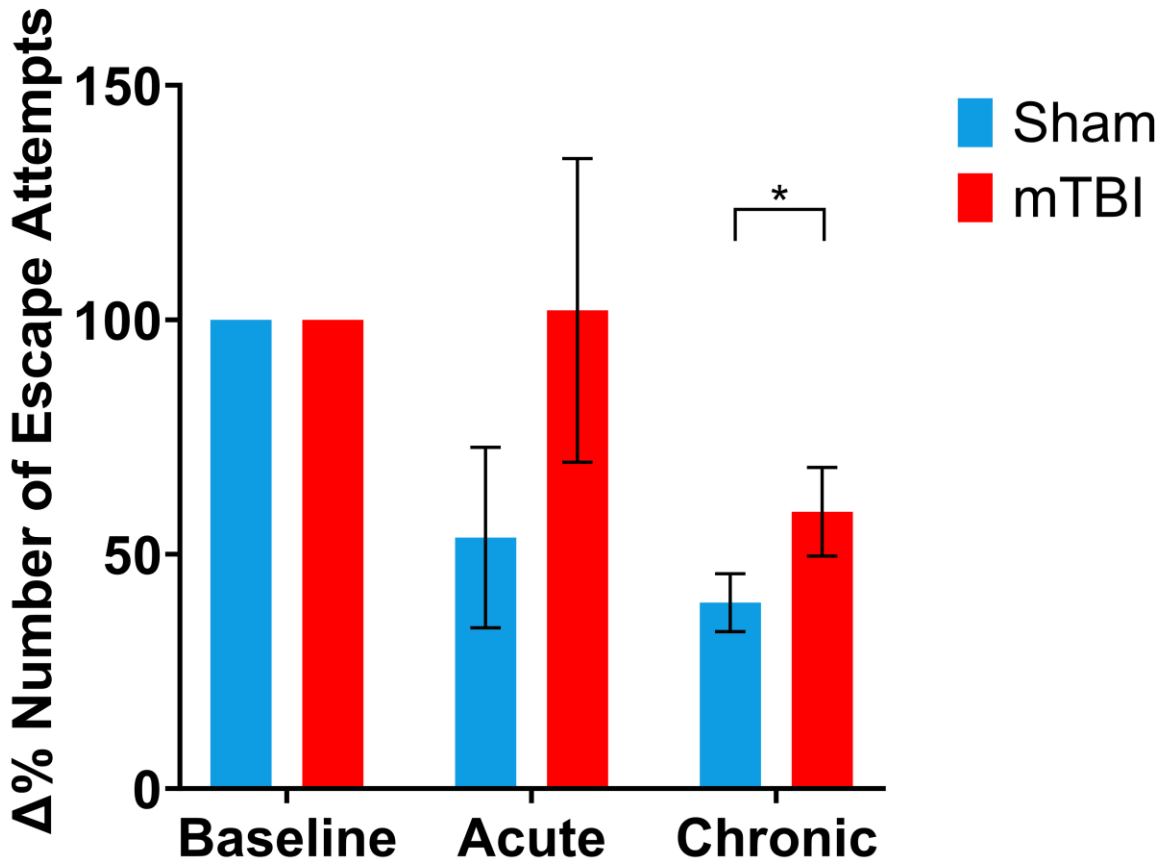

Figure 4.21: Percent change in the number of escape attempts made by pigs in the open field. Bar graph shows mean and SEM of data for sham pigs (n=6) and mTBI pigs (n=6) in the acute phase and the chronic phase. Data normalized to baseline values from pre-injury measurements 1 week before concussion procedure. Asterisks shown for between group comparisons only.

a)

Timepoint	Acute	Chronic
Sham vs mTBI	0.117	0.047

b)

Timepoint	Acute	Chronic
Sham vs Baseline	0.021	3.42E-12
mTBI vs Baseline	0.477	0.0001

Table 4.2: Statistical analysis of the change in number of escape attempts made by pigs during the open field test. **a)** Between group statistics showing p-value results of t-test calculated in the acute phase and the chronic phase. **b)** Within group statistics showing t-test p-value comparison of means in the acute phase and the chronic phase with pre-injury baseline values.

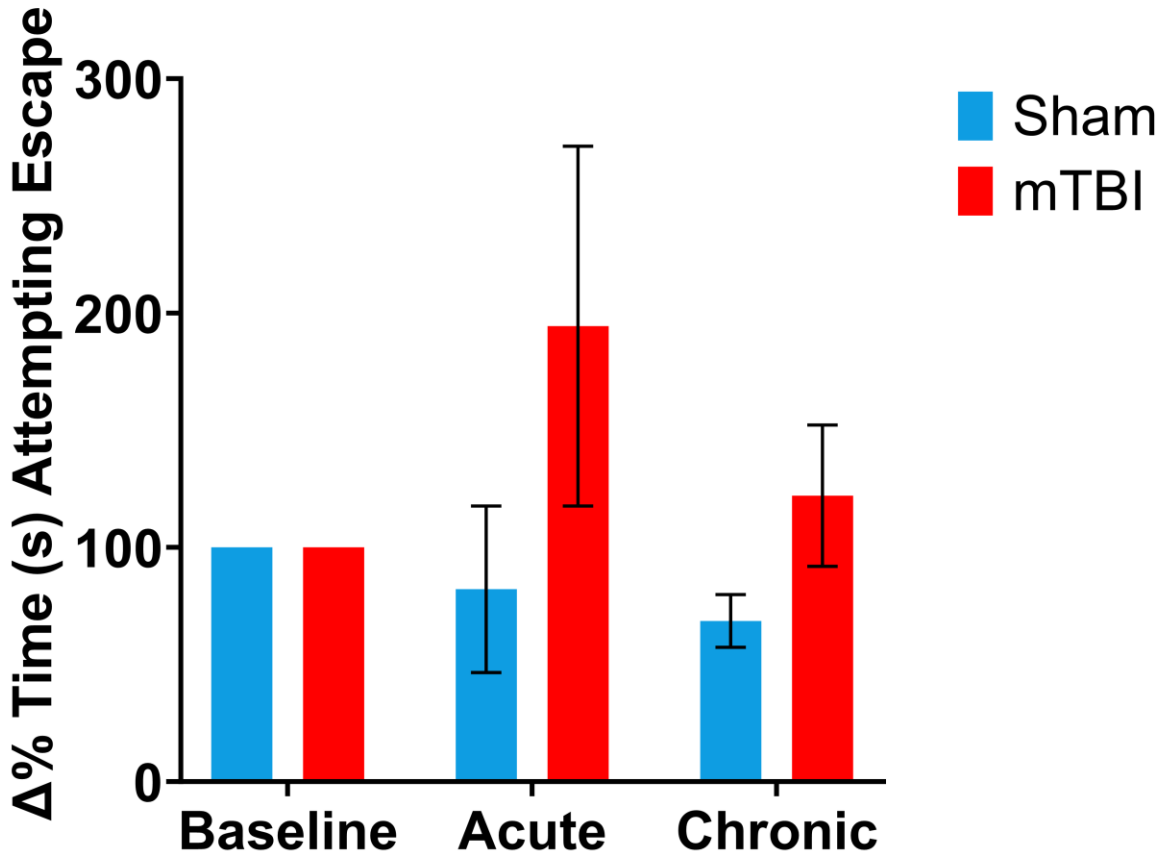

Figure 4.22: Percent change in the amount of time pigs spend attempting to escape the open field in the acute phase and the chronic phase. Mean and SEM for escape attempts of sham pigs (n=6) and mTBI pigs (n=6) in the open field over the course of 10 minutes. Data normalized to baseline measurements taken 1 week prior to injury. Asterisks not shown for within group significance.

a)

Timepoint	Acute	Chronic
Sham vs mTBI	0.111	0.054

b)

Timepoint	Acute	Chronic
Sham vs Baseline	0.320	0.004
mTBI vs Baseline	0.132	0.237

Table 4.3: Statistical analysis showing the change in the total cumulative duration pigs spend trying to escape the behavioral chamber during the open field test. **a)** Between group statistics showing p-value results of t-test calculated in the acute phase and the chronic phase. **b)** Within group statistics showing t-test p-value comparison of means in the acute phase and the chronic phase with pre-injury baseline values.

The novel object recognition test was used to assess depression and novelty-seeking behaviors in pigs. **Figure 4.23** illustrates the change in the number of contacts pigs made with the novel object during the test phase of the novel object recognition task. Statistical comparison between groups is shown in **Table 4.4a.** Comparison of sham group and mTBI group to baseline measurements is shown in **Table 4.4b.** Additionally, the total cumulative duration pigs spent interacting with the novel object during the test phase of the novel object recognition task is illustrated in **Figure 4.24**. Statistical analysis for duration of contact both between groups and within groups can be found in **Table 4.5a** and **Table 4.5b**.

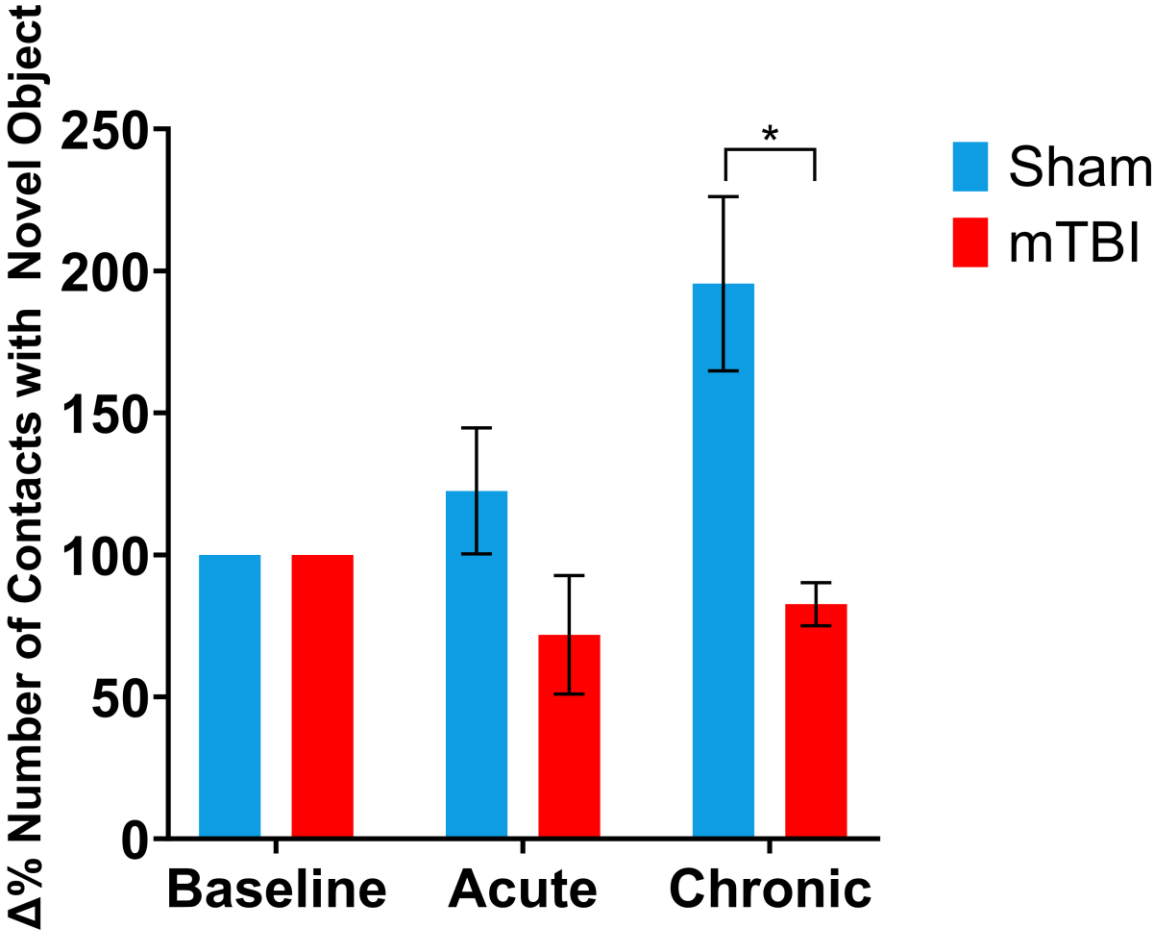

Figure 4.23: Percent change in the number of contacts with the novel object made by pigs during the novel object recognition test. Graphs show mean and SEM for sham pigs (n=6) and mTBI pigs (n=6) in the acute phase and the chronic phase. Data is normalized to baseline measurements taken 1 week prior to injury. Asterisks shown for between group comparisons only.

a)

Timepoint	Acute	Chronic
Sham vs mTBI	0.063	0.001

b)

Timepoint	Acute	Chronic
Sham vs Baseline	0.176	0.002
mTBI vs Baseline	0.112	0.016

Table 4.4: Statistical analysis of the number of contacts with the novel objects made by pigs during the test phase of the novel object recognition test. **a)** Between group statistics showing p-value results of t-test calculated in the acute phase and the chronic phase. **b)** Within group statistics showing t-test p-value comparison of means in the acute phase and the chronic phase with pre-injury baseline values.

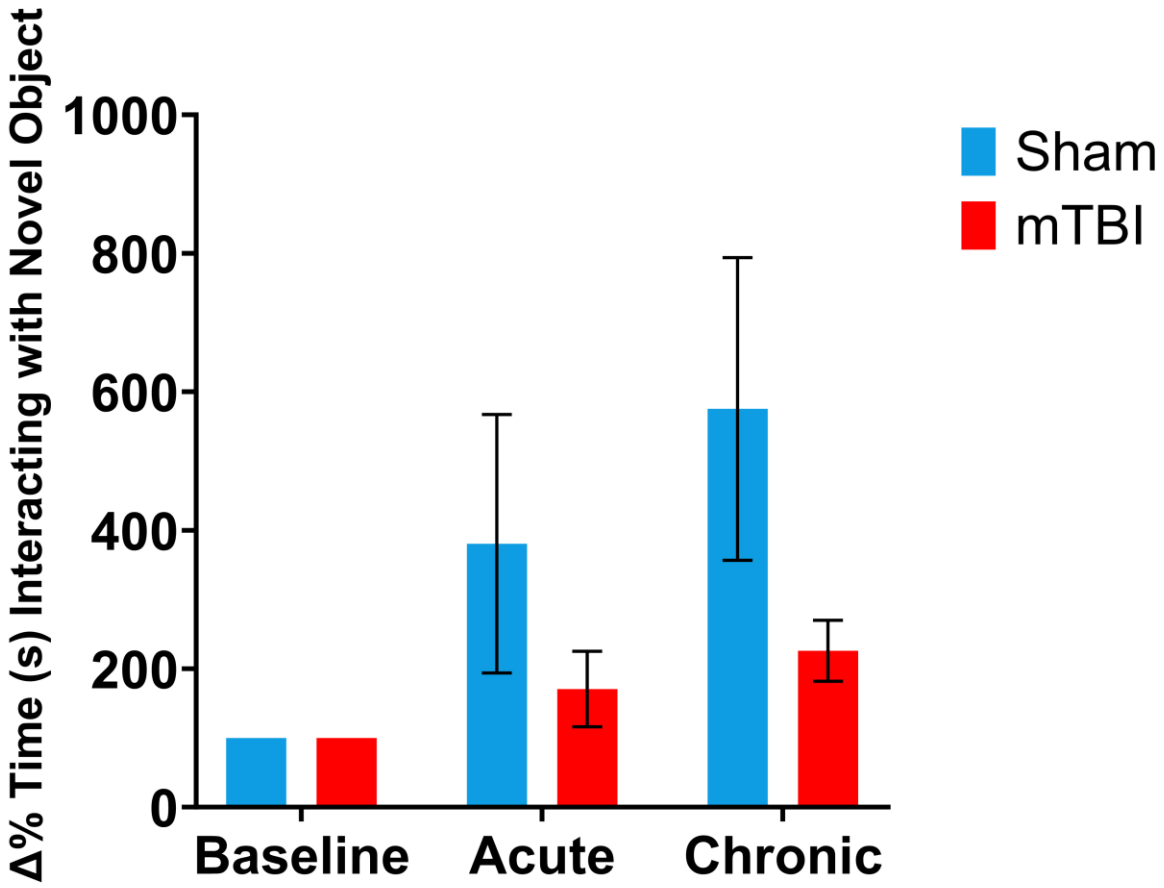

Figure 4.24: Percent change in the amount of time (s) pigs spend interacting with the novel object during the novel object recognition test. Graphs show mean and SEM for sham pigs (n=6) and mTBI pigs (n=6) in the acute phase and the chronic phase. Data is normalized to baseline measurements taken 1 week prior to injury. Asterisks not shown for within group significance.

a)

Timepoint	Acute	Chronic
Sham vs mTBI	0.162	0.067

b)

Timepoint	Acute	Chronic
Sham vs Baseline	0.091	0.021
mTBI vs Baseline	0.120	0.005

Table 4.5: T-test based statistical analysis of changes in the time pigs spend interacting with the novel object during the novel object recognition test. **a)** Between group statistics showing p-value results of t-test calculated in the acute phase and the chronic phase. **b)** Within group statistics showing t-test p-value comparison of means in the acute phase and the chronic phase with pre-injury baseline values.

The baited ball pit was used to assess differences in spatial learning and memory. **Figure 4.25** highlights the differences in the latency between sham and mTBI pigs to retrieve hidden food rewards in the ball pit. **Table 4.6** reveals between group (**4.6a**) and within group (**4.6b**) statistical analysis for the ball pit test.

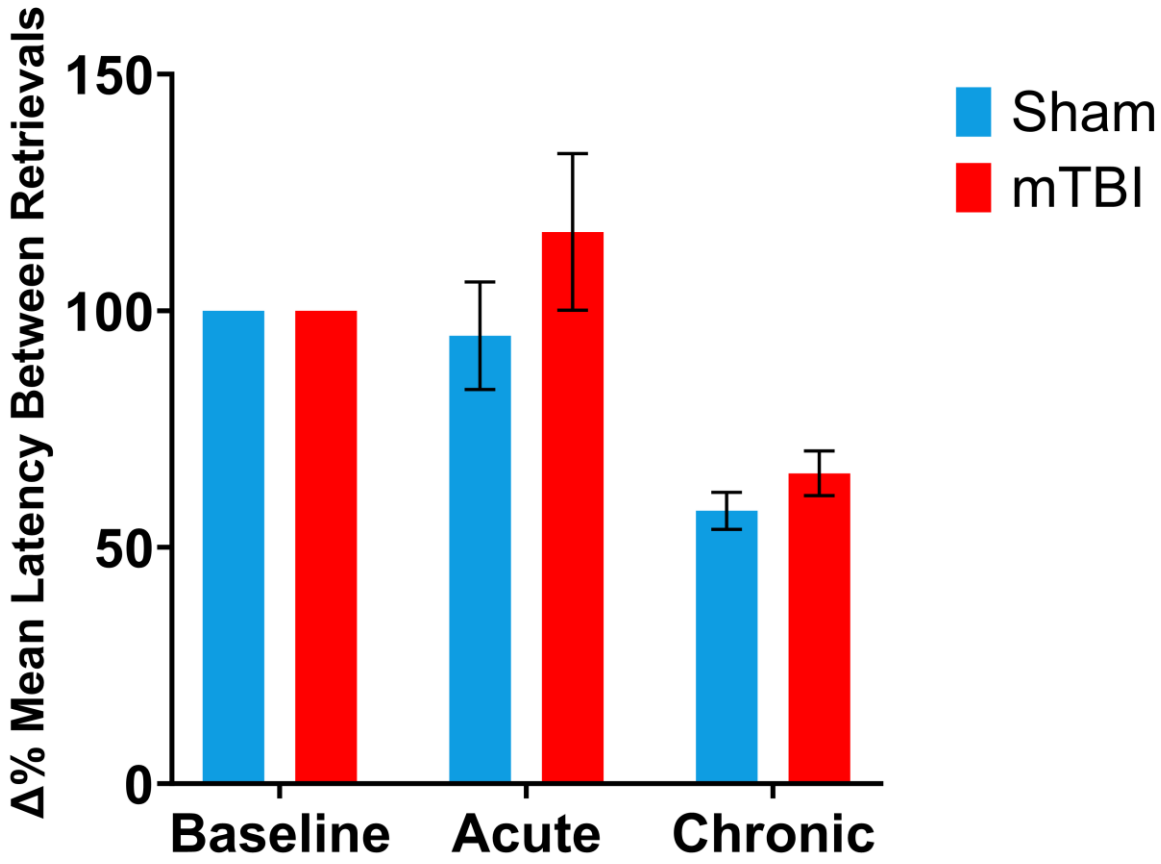

Figure 4.25: Percent change in average latency between successful retreival of food reward by pigs in the baited ball pit test. Bar graphs show mean and SEM for sham pigs (n=6) and mTBI pigs (n=6) in the acute phase and the chronic phase. Data normalized to baseline measurements taken 1 week prior to injury. Asterisks not shown for within group significance.

a)

Timepoint	Acute	Chronic
Sham vs mTBI	0.154	0.104

b)

Timepoint	Acute	Chronic
Sham vs Baseline	0.332	2.16E-13
mTBI vs Baseline	0.178	5.81E-09

Table 4.6: Statistical analysis revealing dynamics in the latency of pigs to retrieve apple slices in the baited ball pit. **a)** Between group statistics showing p-value results of t-test calculated in the acute phase and the chronic phase. **b)** Within group statistics showing t-test p-value comparison of means in the acute phase and the chronic phase with pre-injury baseline values.

A two-choice shape discrimination test was used as an assessment for short term memory in pigs with and without concussion. **Figure 4.26** shows the change in the amount of time pigs required before making a correct nose touch with the target shape in the presence of a distractor shape. Between group statistics are listed in **Table 4.7a**, and within group statistics in **Table 4.7b**. Additional analyses were conducted to determine the total amount of time pigs required to complete a 3-touch trial, shown in **Figure 4.27**. This analysis allows for the representation of a lack of focus by subjects. Complementary statistical analysis is shown in **Table 4.8**.

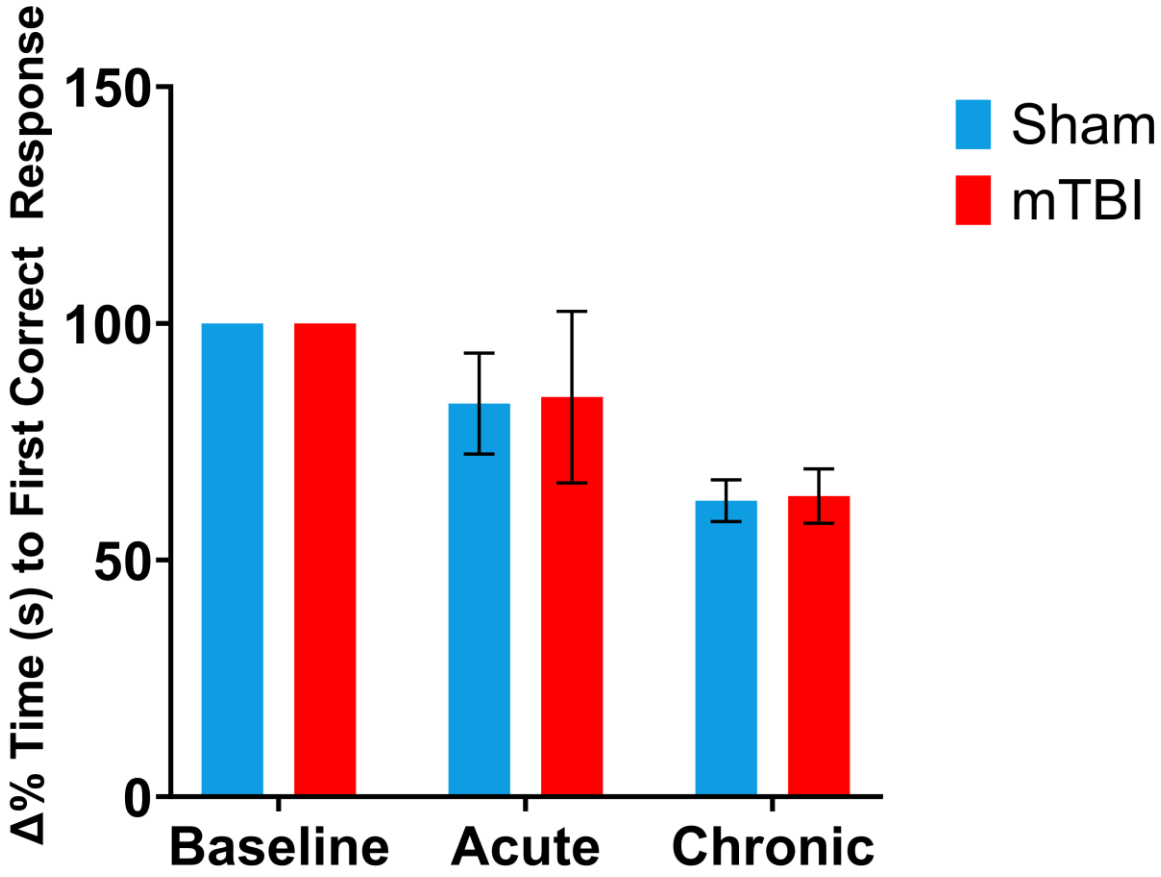

Figure 4.26: Percent change in the time (s) pigs spend before making a first correct response during the shape discrimination task. Graphs illustrate mean and SEM for sham pigs (n=2) and mTBI pigs (n=2) in the acute phase and the chronic phase. Data is normalized to baseline measurements taken 1 week prior to injury. Asterisks not shown for within group significance.

a)

Timepoint	Acute	Chronic
Sham vs mTBI	0.479	0.451

b)

Timepoint	Acute	Chronic
Sham vs Baseline	0.132	0.000003
mTBI vs Baseline	0.255	0.0001

Table 4.7: Statistical analysis of the percent change in the latency of pigs to make a first correct nose-contact **a)** Between group statistics showing p-value results of t-test calculated in the acute phase and the chronic phase. **b)** Within group statistics showing t-test p-value comparison of means in the acute phase and the chronic phase with pre-injury baseline values.

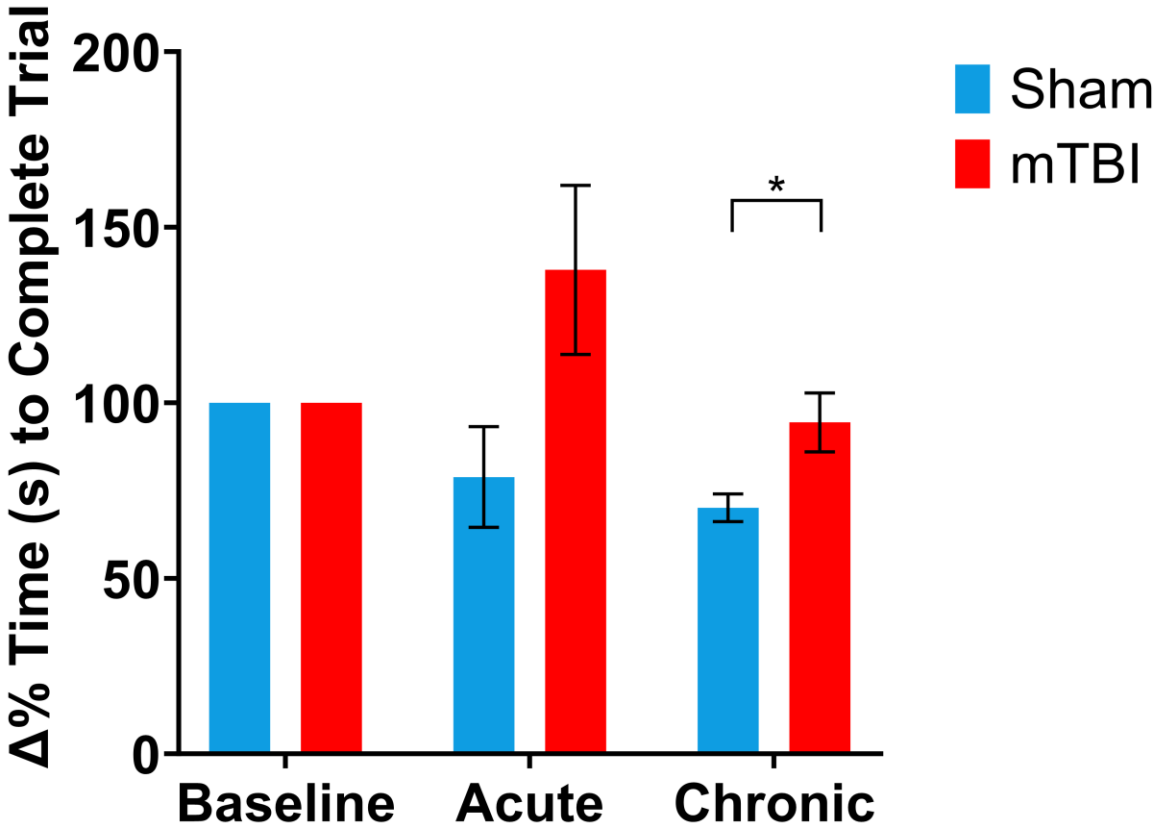

Figure 4.27: Percent change in time (s) pigs take to complete the three-nose-touch trial in the shape discrimination task. Graphs show mean and SEM for sham pigs (n=2) and mTBI pigs (n=2) in the acute phase and the chronic phase. Data is normalized to baseline measurements taken 1 week prior to injury. Asterisks shown for between group comparisons only.

a)

Timepoint	Acute	Chronic
Sham vs mTBI	0.066	0.046

b)

Timepoint	Acute	Chronic
Sham vs Baseline	0.146	0.000001
mTBI vs Baseline	0.133	0.265

Table 4.8: Statistical results from the percent time pigs require to complete a 3-contact trial. **a)** Between group statistics showing p-value results of t-test calculated in the acute phase and the chronic phase. **b)** Within group statistics showing t-test p-value comparison of means in the acute phase and the chronic phase with pre-injury baseline values.

Spatial awareness and recognition memory was additionally assessed using a color discrimination task. **Figure 4.28** shows graphical representation of the percent change in the latency in time pigs required before identifying and selecting the correct box. Statistics for this test are shown in **Table 4.9.**

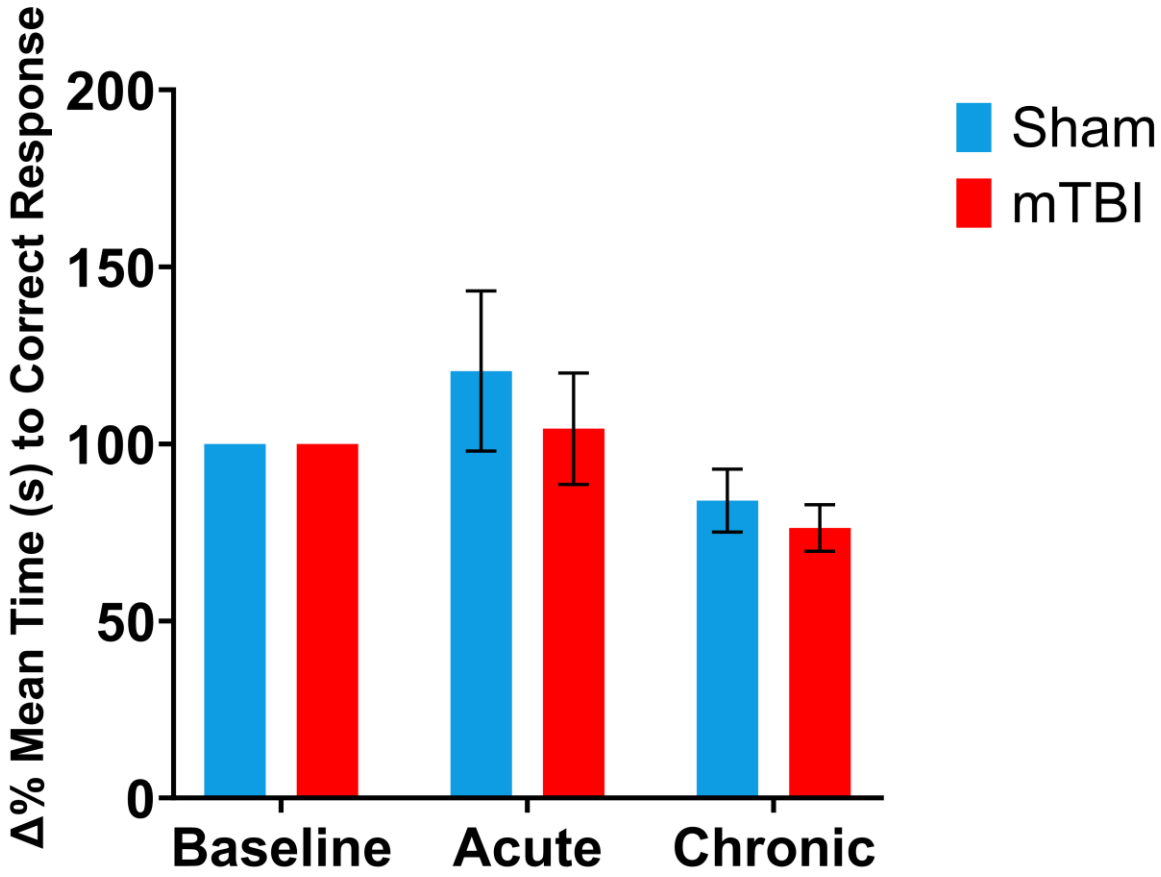

Figure 4.28: Percent change in time (s) to correct response in the color discrimination task. Graphs show mean and SEM for sham pigs (n=4) and mTBI pigs (n=4) in the acute phase and the chronic phase. Data is normalized to baseline measurements taken 1 week prior to injury. Asterisks not shown for within group significance.

a)

Timepoint	Acute	Chronic
Sham vs mTBI	0.295	0.250

b)

Timepoint	Acute	Chronic
Sham vs Baseline	0.211	0.046
mTBI vs Baseline	0.403	0.001

Table 4.9: Statistical analysis of latency to correct box selection. **a)** Between group statistics showing p-value results of t-test calculated in the acute phase and the chronic phase. **b)** Within group statistics showing t-test p-value comparison of means in the acute phase and the chronic phase with pre-injury baseline values.

Pigs are social animals that live within herds and maintain a strict hierarchical structure. A food aggression test was used to measure the incidence of aggressive behavior in pigs with and without concussion. **Figure 4.29** illustrates the changes in the total amount of time pigs spend with their head inside the food bowl. Between group statistical analysis is shown in **Table 4.10a**, whereas within group changes are illustrated by statistics in **Table 4.10b**.

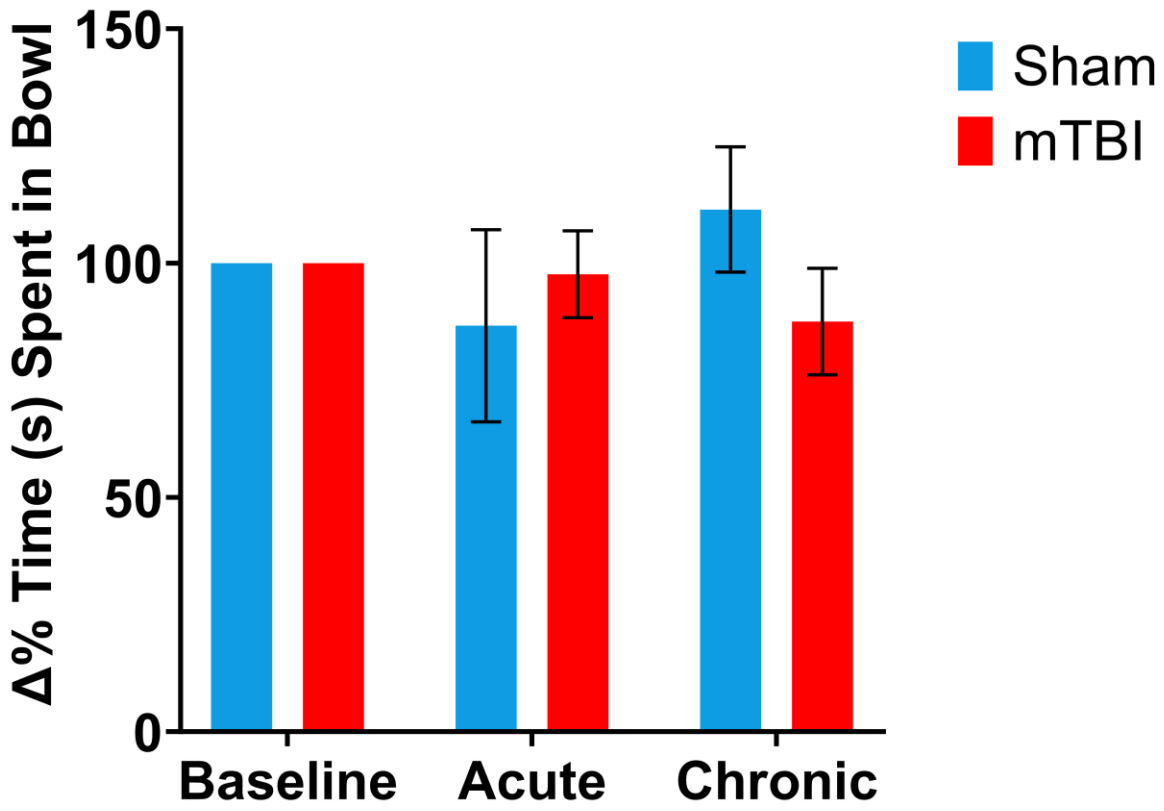

Figure 4.29: Percent change in time (s) pigs spend with their head in the bowl during the food aggression test. Graphs show mean and SEM for sham pigs (n=4) and mTBI pigs (n=4) in the acute phase and the chronic phase. Data is normalized to baseline measurements taken 1 week prior to injury. Asterisks not shown for within group significance.

a)

Timepoint	Acute	Chronic
Sham vs mTBI	0.329	0.099

b)

Timepoint	Acute	Chronic
Sham vs Baseline	0.281	0.210
mTBI vs Baseline	0.408	0.152

Table 4.10: Statistical results for the percentage of time pigs spend engaged with the food bowl during the food aggression test. **a)** Between group statistics showing p-value results of t-test calculated in the acute phase and the chronic phase. **b)** Within group statistics showing t-test p-value comparison of means in the acute phase and the chronic phase with pre-injury baseline values.

The final behavioral assessment measures changes in locomotion and overall levels of activity. **Figure 4.30** graphically denotes the number of steps taken by mTBI and sham pigs over the course of 8 weeks as recorded by commercially available activity trackers. Statistical results for this assessment can be found in **Table 4.11**.

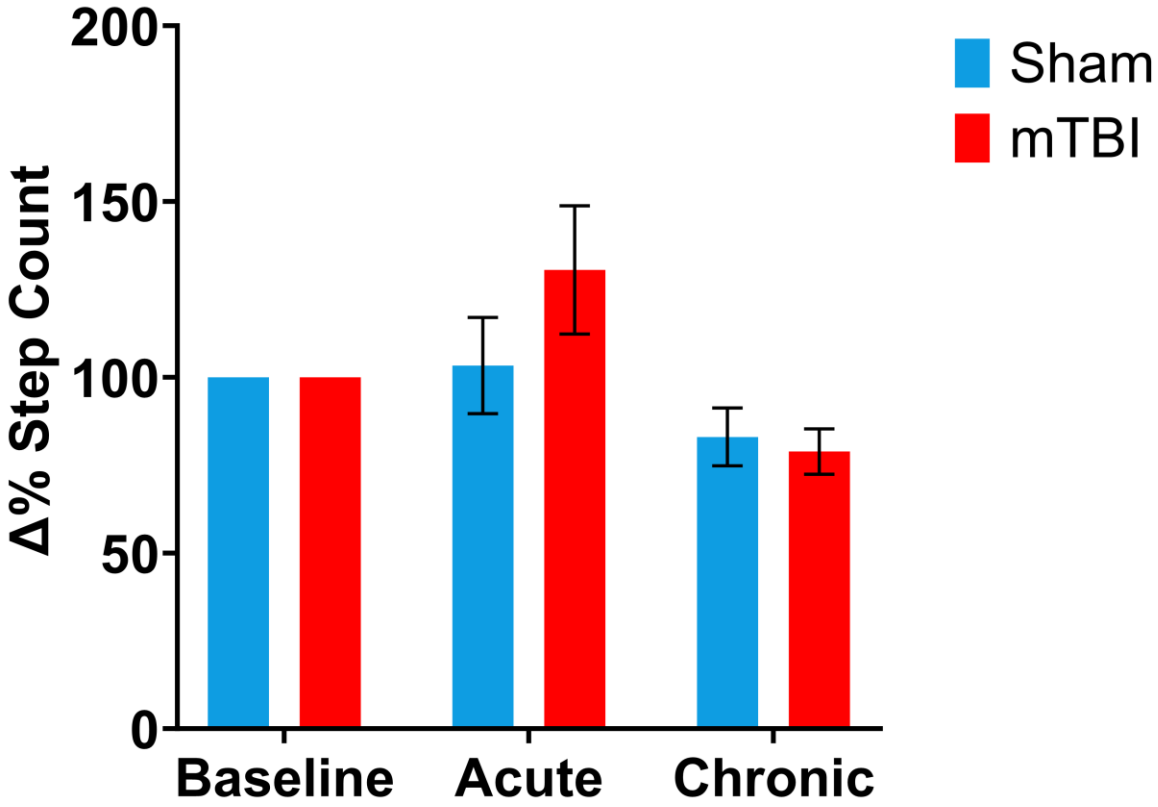

Figure 4.30: Percent change in the number of steps recorded using activity tracking devices. Bar graphs illustrate mean and SEM for sham pigs (n=4) and mTBI pigs (n=4). Data is normalized to baseline measurements taken 1 week prior to injury. Asterisks not shown for within group significance.

a)

Timepoint	Acute	Chronic
Sham vs mTBI	0.299	0.274

b)

Timepoint	Acute	Chronic
Sham vs Baseline	0.411	0.032
mTBI vs Baseline	0.071	0.003

Table 4.11: Statistical representation of the changes in number of steps taken by pigs throughout the day. **a)** Between group statistics showing p-value results of t-test calculated in the acute phase and the chronic phase. **b)** Within group statistics showing t-test p-value comparison of means in the acute phase and the chronic phase with pre-injury baseline values.

Overall assessment of behavioral results are summarized in **Table 4.12**. The table quickly summarizes whether each behavioral test yielded statistically significant differences between sham pigs and pigs with concussion. Tests which resulted in significant differences between groups in the acute and chronic phases are indicated with a score of 1. Those without significance are scored as 0.

Summary of Behavioral Results		
Clinical Symptoms	**Difference Between Groups**	
	Acute	Chronic
Depression		
Novel Object - Number of Contacts	0	1
Novel Object - Time in Contact	0	0
Anxiety		
Open Field - Escape Number	0	1
Open Field - Escape Time	0	1
Hyperactivity		
Open Field - Distance	1	1
Activity Tracking	0	0
Learning Deficits		
Shape Discrimination - First Touch	0	0
Shape Discrimination - Full Trial	0	1
Baited Ball Pit	0	0
Color Discrimination	0	0
Aggression		
Food Aggression	0	0
Total	**1**	**5**

Table 4.12: Table summarizing the presence of differences between sham pigs and pigs with concussion across various behavioral tests. A score of 1 indicates statistically significant differences were detected between groups. A score of 0 indicates no statistical differences between groups were detected. These results show there are more behavioral tests which show differences between groups in the chronic phase than in the acute phase.

4.4 Improving Outcomes using Non-Invasive Repetitive Transcranial Magnetic Stimulation

Evidence in rodent models of TBI show that delivery of repetitive transcranial magnetic stimulation improves long-term potentiation, multi-unit activity, and performance in cognitive and behavioral assessment. Based on this evidence, we sought to develop procedures to deliver rTMS therapy to pigs with brain injury. The objective of my efforts toward this goal was to develop preliminary procedures and determine whether pigs would be a viable candidate for neuromodulatory therapy in the future.

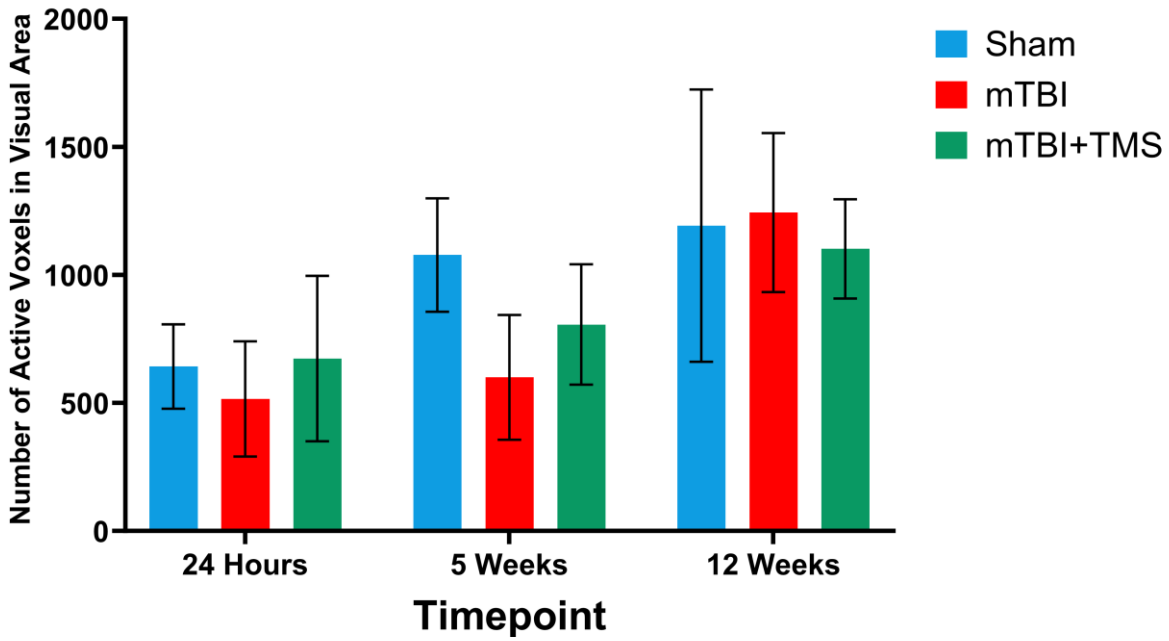

Figure 4.31: Neural Activation in Response to Visual Stimulation in the Visual Cortex. Bar graph comparing the number of voxels in the visual region of the brain activated in response to visual stimulation in sham pigs (n=4) mTBI pigs (n=4) and treatment pigs (n=4) at three timepoints.

Timepoint	24 Hours	5 Weeks	12 Weeks
TMS vs Sham	0.467	0.217	0.441
TMS vs mTBI	0.352	0.282	0.357

Table 4.13: T-test statistical analysis comparing activation of visual cortex in response to visual stimulation between treatment pigs and sham pigs as well as treatment pigs and injury only pigs.

Figure 4.31 illustrates the total number of activated voxels present within the occipital region of the brain in response to visual stimulation among all pigs. Specifically, the treatment pigs exhibited a mean activation of 673 ± 559 voxels at 24 hours post-injury. At 5 weeks post injury, the mean activation of treatment pigs was 806 ± 407 voxels. And the mean activation of treatment pigs 12 weeks post-injury was 1101 ± 336. T-test statistical analysis comparing the activation in treatment pigs with both sham pigs and mTBI only pigs is shown in **Table 4.13**. BOLD fMRI response to visual stimulation in mTBI pigs after 4 weeks of rTMS therapy are shown in **Figure 4.32**.

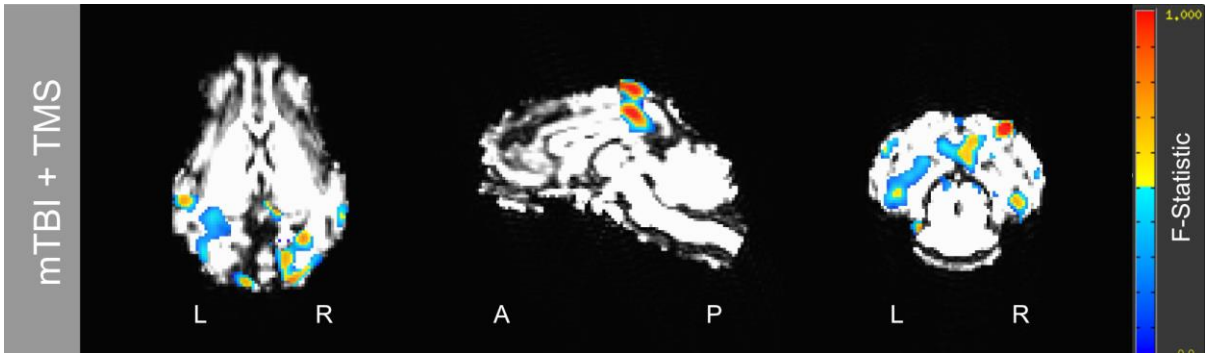

Figure 4.32: Representative brain maps showing the extent of BOLD fMRI signal in the visual region of the brain in response to visual stimulation in pigs with concussion who have received 4 weeks of rTMS therapy. Images taken at 5 weeks post injury (n=4). Only positive values shown.

Additionally, the number of activated voxels in the sensorimotor area in response to tactile stimulation in all pigs is shown in **Figure 4.33**. In this graph, the mean activation of treatment pigs at 24 hours post-injury was 358 ± 256 voxels. At 5 weeks post injury, the mean number of voxels active in the sensorimotor area of treatment pigs was 208 ± 88. At 12 weeks post-injury, the mean number of active voxels in the sensorimotor area of treatment pigs in response to tactile stimulation was 543 ± 230 voxels. **Table 4.14** shows t-test statistical analysis comparing the activation of treatment pigs across the three timepoints with the activation of

sham pigs and mTBI only pigs. Representative brain maps showing BOLD fMRI response to tactile stimulation in mTBI pigs following TMS therapy are illustrated in **Figure 4.34**.

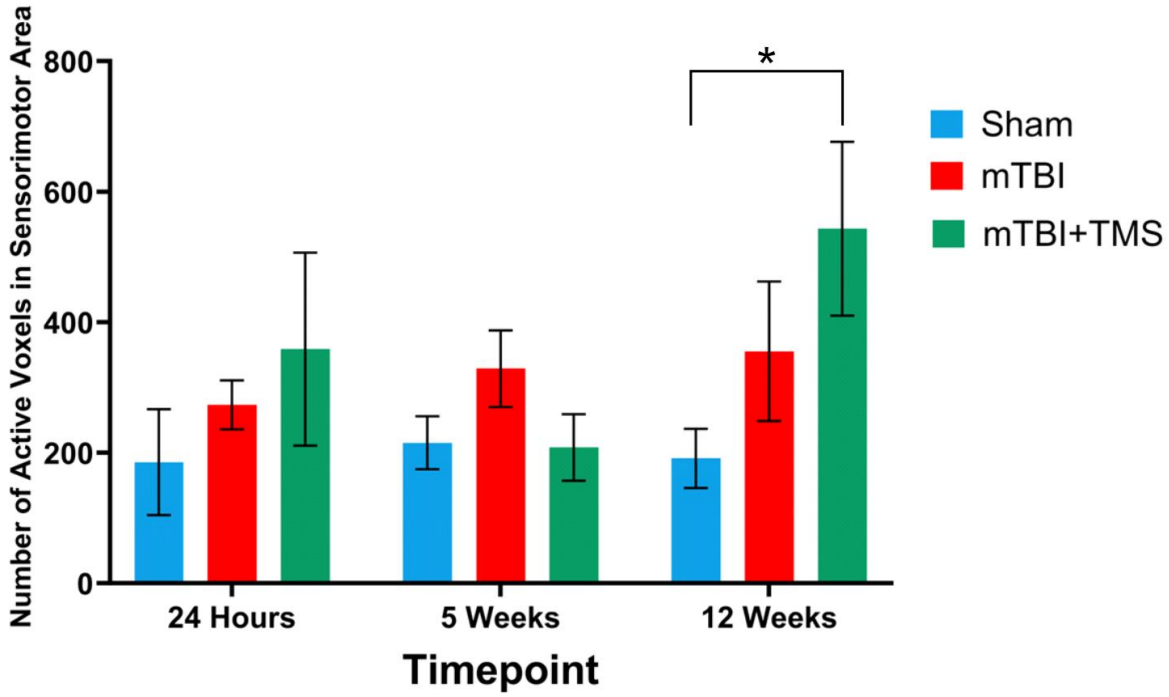

Figure 4.33: Neural Activation in Response to Tactile Stimulation in the Visual Cortex. Bar graph comparing the number of voxels in the visual region of the brain activated in response to visual stimulation in sham pigs (n=4) mTBI pigs (n=4) and treatment pigs (n=4) at three timepoints.

Timepoint	24 Hours	5 Weeks	12 Weeks
TMS vs Sham	0.177	0.458	0.036
TMS vs mTBI	0.306	0.086	0.158

Table 4.14: T-test statistical analysis comparing activation of sensorimotor cortex in response to tactile stimulation between treatment pigs and sham pigs as well as treatment pigs and injury only pigs.

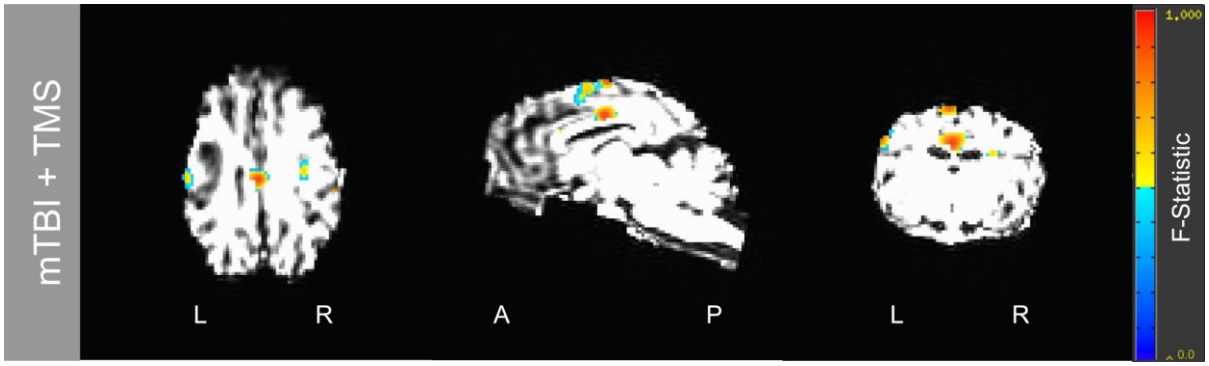

Figure 4.34: Representative brain maps showing the extent of activation in the sensorimotor area of the brain in response to tactile stimulation in pigs with concussion after receiving 4 weeks of rTMS therapy. Images taken at 5 weeks post-injury (n=4). Only positive values shown.

While not every behavioral test shows differences between healthy pigs and pigs with concussion, there are some interesting differences between groups in the open field and novel object recognition tests. In order to examine if rTMS therapy has an effect on post-concussive changes in these two assessments, I looked into differences between the treatment pigs and the aforementioned groups. **Figure 4.35** illustrates the mean and SEM for distance measures of pigs in the open field. In **Table 4.15a**, I list between group differences in the locomotor activity of treatment pigs with both sham and mTBI animals. Within group differences for treatment pigs comparing each week to baseline measurements are shown in **Table 4.15b**.

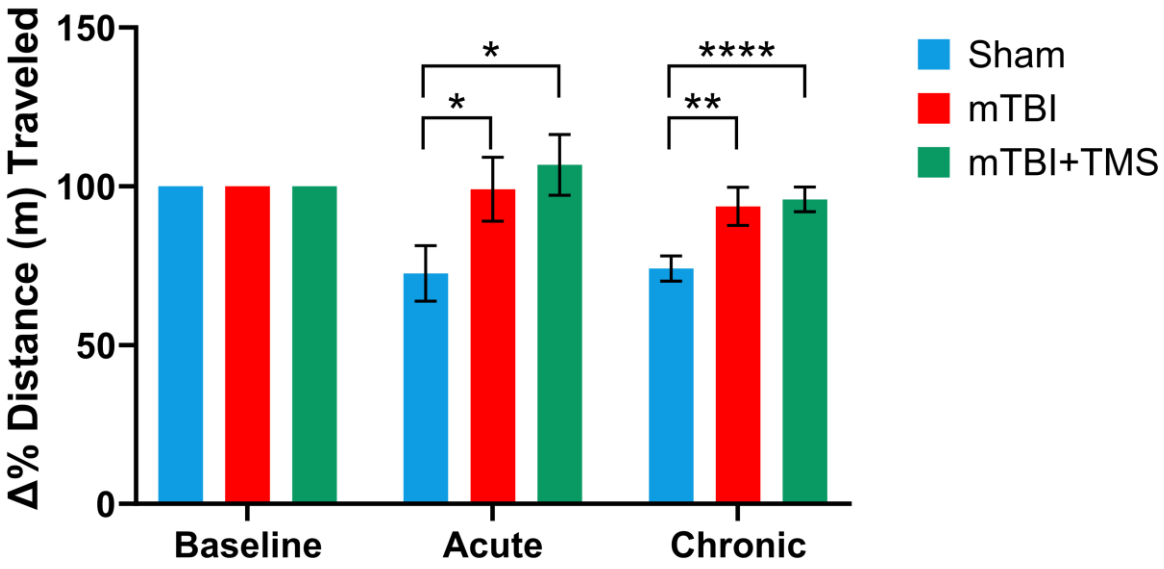

Figure 4.35: Percent change in distance (m) traveled in the open field. Mean and SEM for distance measures of sham pigs (n=6) mTBI pigs (n=6) and treatment pigs (n=4). Data is normalized to baseline values measured 1 week prior to injury. No data available for treatment pigs at 5 weeks.

a)

Timepoint	Acute	Chronic
TMS vs Sham	0.012	0.0001
TMS vs mTBI	0.304	0.381

b)

Timepoint	Acute	Chronic
TMS vs Baseline	0.266	0.154

Table 4.15: Statistical analysis for distance traveled in the open field. **a)** Between group statistics showing p-value results of t-test calculated in the acute phase and the chronic phase. **b)** Within group statistics showing t-test p-value comparison of means in the acute phase and the chronic phase with pre-injury baseline values.

Results for novel object recognition test are shown in **Figure 4.36**. The number of contacts with the novel object during the test portion of the novel object recognition tests shows that there is a pretty large change in the number of interactions by treatment pigs in comparison to other pigs as well as pre-injury scores. Between group statistics are shown in **Table 4.16a**, and within group statistics are represented in **Table 4.16b**.

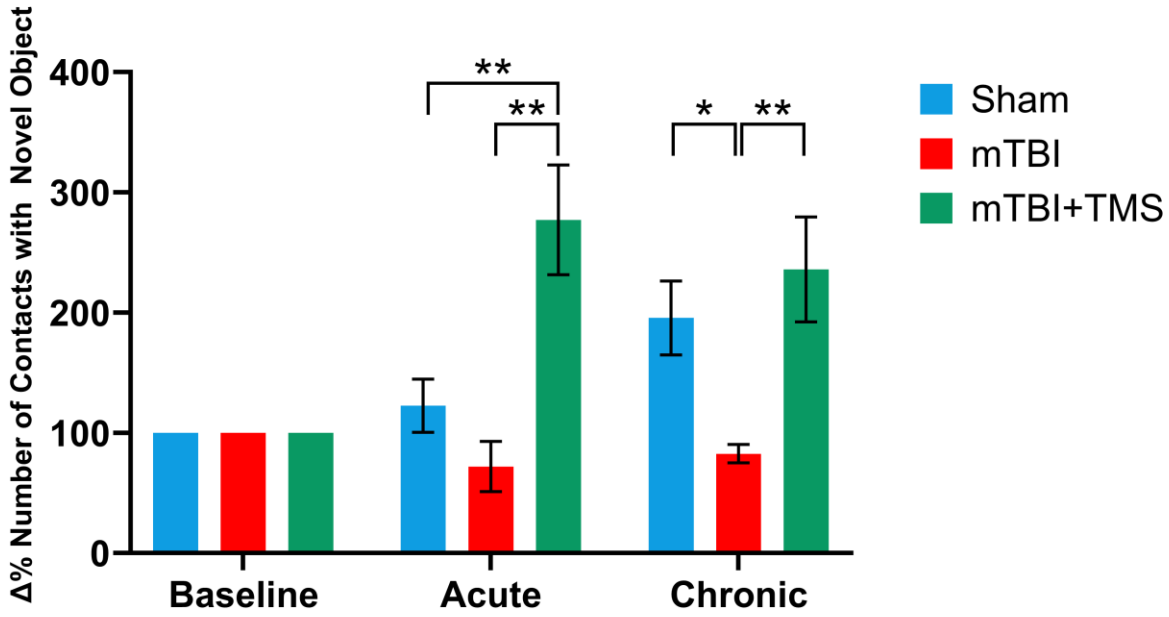

Figure 4.36: Percent change in the number of contacts with the novel object made by pigs during the novel object recognition test. Graphs show mean and SEM for sham pigs (n=6), mTBI pigs (n=6) and treatment pigs (n=4). Data is normalized to baseline measurements taken 1 week prior to injury.

a)

Timepoint	Acute	Chronic
TMS vs Sham	0.008	0.232
TMS vs mTBI	0.002	0.002

b)

Timepoint	Acute	Chronic
TMS vs Baseline	0.004	0.003

Table 4.16: Statistical analysis of the number of contacts with the novel objects made by pigs during the test phase of the novel object recognition test. **a)** Between group statistics showing p-value results of t-test calculated in the acute phase and the chronic phase. **b)** Within group statistics showing t-test p-value comparison of means in the acute phase and the chronic phase with pre-injury baseline values.

Results from the shape discrimination task are shown next. **Figure 4.37** demonstrates the mean and SEM results from sham pigs, pigs with concussion, and pigs with concussion who receive rTMS therapy for the first touch in the shape discrimination task. Statistical analysis of these results, as shown in **Table 4.17** indicate that there are no significant differences between

groups in terms of how quickly pigs make first contact with the target shape. The mean and SEM of pigs to complete a 3-touch trial of the shape discrimination task are illustrated in **Figure 4.38**. Statistical analysis of these results are shown in **Table 4.18**.

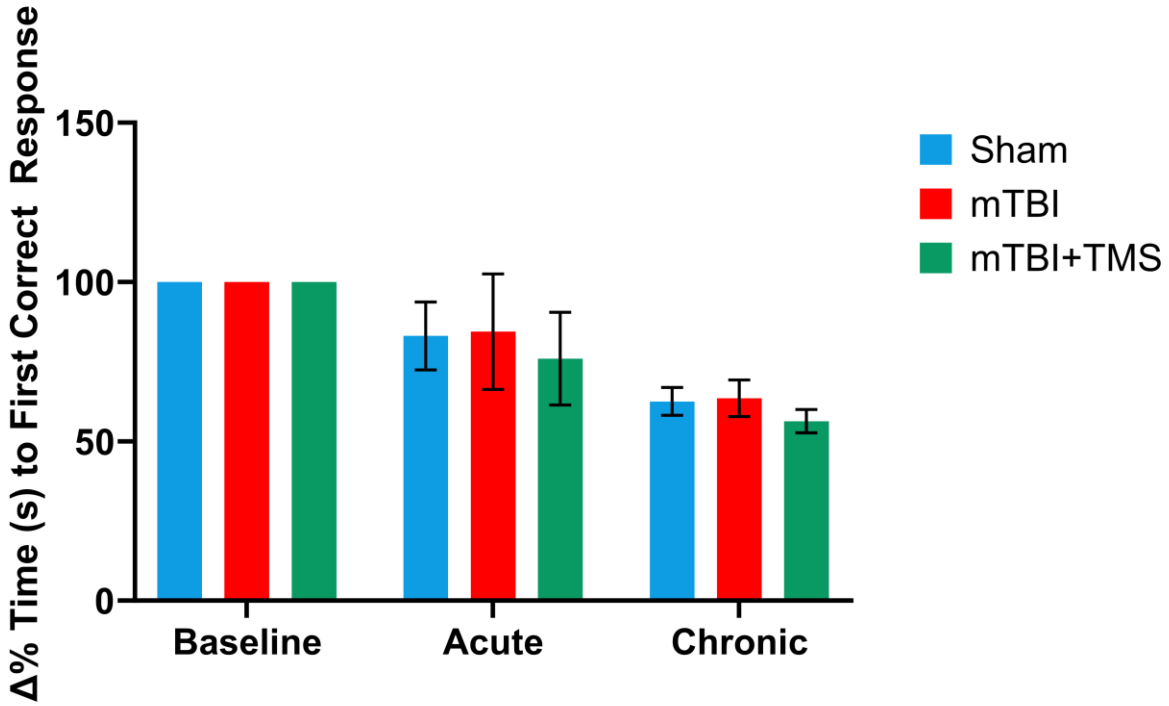

Figure 4.37: Percent change in the time (s) pigs spend before making a first correct response during the shape discrimination task. Graphs illustrate mean and SEM for sham pigs (n=2), mTBI pigs (n=2) and treatment pigs (n=4) in the acute phase and the chronic phase. Data is normalized to baseline measurements taken 1 week prior to injury. Asterisks not shown for within group significance.

a)

Timepoint	Acute	Chronic
TMS vs Sham	0.364	0.070
TMS vs mTBI	0.378	0.092

b)

Timepoint	Acute	Chronic
TMS vs Baseline	0.088	2.17E-10

Table 4.17: Statistical analysis of the percent change in the latency of treatment pigs to make a first correct nose-contact **a)** Between group statistics showing p-value results of t-test calculated in the acute phase and the chronic phase. **b)** Within group statistics showing t-test p-value comparison of means in the acute phase and the chronic phase with pre-injury baseline values.

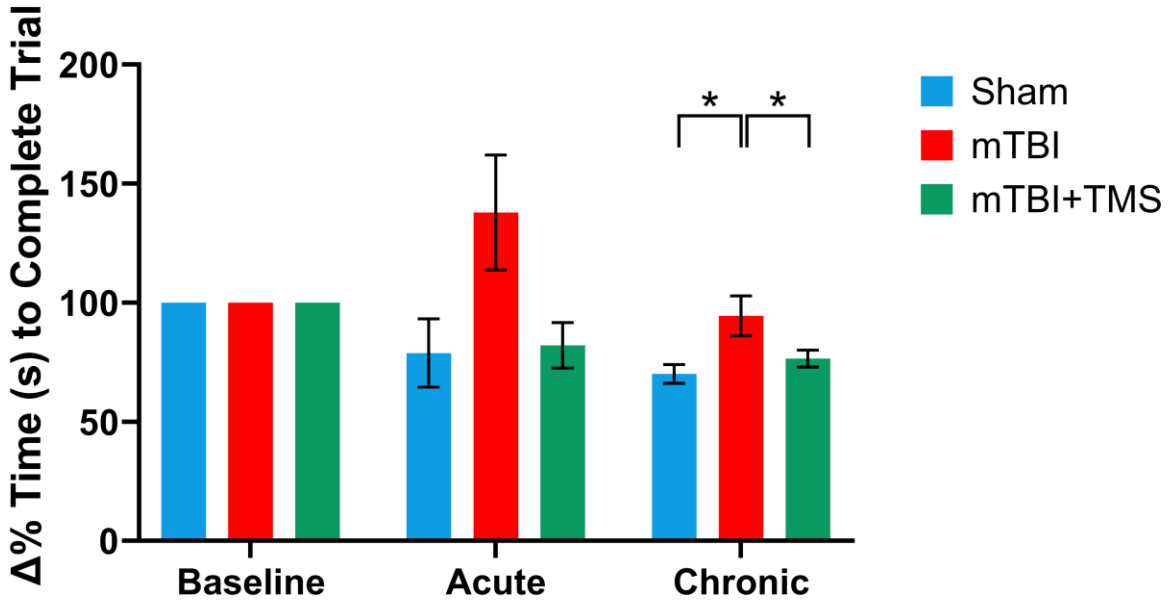

Figure 4.38: Percent change in time (s) pigs take to complete the three-nose-touch trial in the shape discrimination task. Graphs show mean and SEM for sham pigs (n=2), mTBI pigs (n=2), and treatment pigs (n=4) in the acute phase and the chronic phase. Data is normalized to baseline measurements taken 1 week prior to injury. Asterisks shown for between group comparisons only.

a)

Timepoint	Acute	Chronic
TMS vs Sham	0.437	0.125
TMS vs mTBI	0.068	0.036

b)

Timepoint	Acute	Chronic
TMS vs Baseline	0.066	0.000002

Table 4.18: Statistical results from the percent time pigs require to complete a 3-contact trial. **a)** Between group statistics showing p-value results of t-test calculated in the acute phase and the chronic phase. **b)** Within group statistics showing t-test p-value comparison of means in the acute phase and the chronic phase with pre-injury baseline values.

Figure 4.39 shows the mean and SEM of the number of steps pigs take as recorded by activity tracking devices over the course of an 8-hour day. Statistical representation of the changes in number of steps taken by pigs throughout the day is shown in **Table 4.19**.

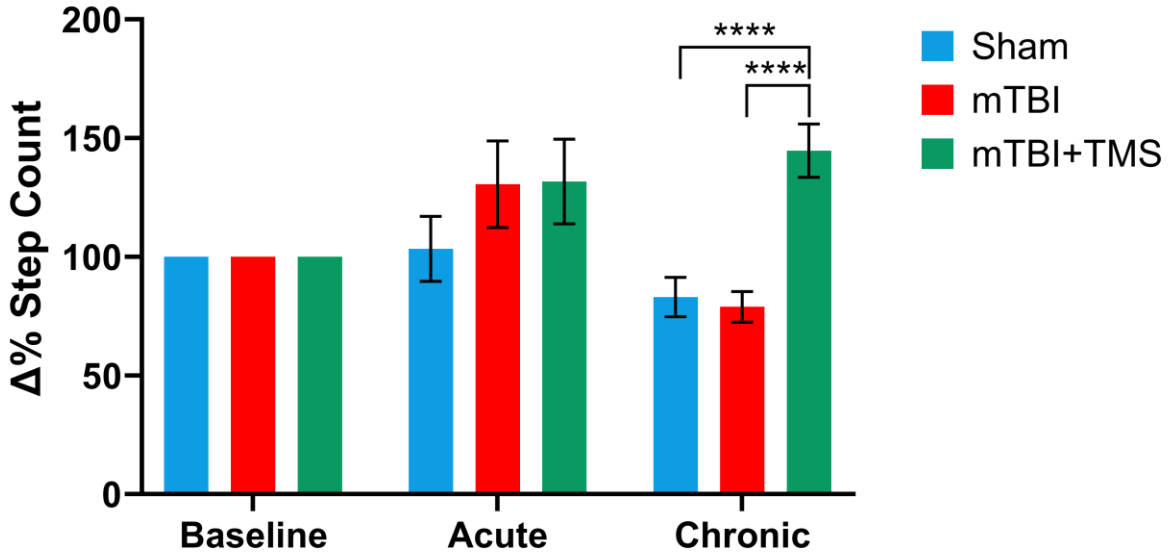

Figure 4.39: Percent change in the number of steps recorded using activity tracking devices. Bar graphs illustrate mean and SEM for sham pigs (n=4), mTBI pigs (n=4), and treatment pigs (n=4). Data shown for the acute and chronic phases is normalized to baseline measurements taken 1 week prior to injury. Asterisks not shown for within group significance.

a)

Timepoint	Acute	Chronic
TMS vs Sham	0.127	0.00001
TMS vs mTBI	0.483	0.000001

b)

Timepoint	Acute	Chronic
TMS vs Baseline	0.070	0.0001

Table 4.19: Statistical analysis of the number of steps recorded using an activity tracker in sham pigs, mTBI pigs, and treatment pigs. **a)** Between group statistics showing p-value results of t-test calculated in the acute phase and the chronic phase. **b)** Within group statistics showing t-test p-value comparison of means in the acute phase and the chronic phase with pre-injury baseline values.

A summary of behavioral results for treatment pigs compared to sham and mTBI only pigs are presented in **Table 4.20**. Statistical comparisons for behavioral tests which are significant are denoted by a score of 1. Those without statistical significance are given a score of zero. The table indicates that the greatest number of statistical differences are between treatment pigs and mTBI only pigs in the chronic phase.

Summary of Behavioral Results		
Clinical Symptoms	**Difference Between mTBI and TMS pigs**	
	Acute	Chronic
Depression		
Novel Object	1	1
Hyperactivity		
Open Field	0	0
Activity Tracking	0	1
Learning Deficits		
Shape Discrimination - First Touch	0	0
Shape Discrimination - Full Trial	0	1
Total	**1**	**3**

Table 4.20: Table showing a summary of behavioral differences between concussed pigs who received rTMS treatment, and both sham and mTBI only animals. A score of 1 was given for any behavioral test which exhibited statistical significance between groups, and a score of 0 indicates no statistically significant differences found. This table illustrates that rTMS therapy elicits significant changes in pigs with concussion in the novel object test, both in the acute phase and the chronic phase, and changes in the full trial of shape discrimination and in the number of steps taken throughout the day in the chronic phases.

CHAPTER 5: DISCUSSION

Throughout the history of biomedical research, identification and usage of the most clinically relevant animal models has been a key factor driving the successful translation of therapeutic strategies. The objective of my graduate work was to develop a platform through which to test the efficacy of novel treatments for brain injury in the form of a minipig model of pediatric concussion. Do pigs show signs of post-concussive syndrome? My hypothesis was that pigs with closed head injury would show post-injury changes in behavior indicative of post-concussive syndrome, such as deficits in memory and changes in mood. There are a number of commonly altered behavioral phenomena associated with brain injury in humans. Patients often experience hyperactivity, increased novelty seeking and impulsive decision making,

5.1 Development of Behavioral Assessments

In order to determine if pigs with concussion would exhibit changes in behavior, I first needed to adapt traditional behavioral assessments used in rodents for use in pigs. Pigs are clever and easily motivated, however, there are distinct challenges that come with conducting behavioral research in pigs. The most glaring limitation is the lack of pig research studies which utilize behavioral assessment, thus the number of published procedures and methods for pig behavior research is limited. There are very few, if any, commercially available devices for pig behavior research, therefore the majority of behavioral apparatuses must be custom engineered. Pigs are also limited in the types of tasks they can perform, while rodents are often tasked with completing a challenge ladder or balance beam, hooved mammals are not suitable for these types of assessments. I selected two of the most common behavioral assessments used in neuroscience research and scaled them up for use in pigs: the open field test and novel object recognition test.

In rodents, the open field test is used to measure novelty-seeking, locomotor activity and anxiety (Hall 1934; Lu et al. 2015; Cywiak et al. 2020). As prey animals, rats and mice do not like being exposed and tend to spend the majority of their time near the perimeter of the open field chamber, a phenomenon known as thigmotaxis. Rodents who spend more time in the center of the chamber are considered to be less anxious than those who remain near the walls. Domestic pigs do not have predators other than humans. Wild hogs on the other hand may occasionally fall prey to big cats but they act as predators themselves as they will kill and eat any small animals within reach. In this regard, I did not consider pigs to be prey, thus hypothesized that pigs would not exhibit thigmotaxis in the open field.

Interpretation of the heatmap generated by tracking the pig in the open field reveals that pigs seem unafraid to traverse the center of the chamber. This exploration of the center region supports my hypothesis that pigs are not thigmotaxic animals. These data also reveal that pigs spend more time investigating the area closest to the door of the chamber. While lack of thigmotaxis means that comparison of time spent in the center versus perimeter of the chamber is not a valid measure of anxiety in pigs, active time spent attempting to escape may be able to provide insight into pigs' emotional state and potential anxiety.

The open field test is conducted in a barren, inescapable environment, therefore increased locomotor activity can be an indication of novelty seeking. I was able to track the pig in the area and determine overall distance traveled during the 10-minute testing period. My results indicate that young pigs are less mobile in the open field compared to older, more confident animals.

The Novel Object Recognition task is another assessment traditionally performed in behavioral neuroscience research. This task is used to test recognition memory and novelty seeking

in rodents. Like what is observed in most healthy mammals, pigs show more interest in the novel object than the familiar object when given the choice to explore the two freely.

In addition to adapting traditional behavioral tests, I wanted to attempt to develop new procedures for testing various aspects of brain injury in pigs. Studies have shown that spatial relational memory is impaired following TBI. My objective was to conceptualize a test that pigs could perform which would engage their spatial learning and memory. The baited ball pit was the result of this conceptualization. My hypothesis for testing in healthy pigs throughout development was simple; pigs would become faster and more accurate at retrieving the apple slices over time, demonstrating active spatial learning. My results for average latency to retrieve apple slices support this hypothesis. As pigs age, and through repetition of testing, they are able to retrieve apple slices more quickly.

Attention Deficit Hyperactivity Disorder (ADHD) is highly correlated with concussion in children. Notably, children with ADHD are more likely to sustain a concussion, and children with concussion are more likely to develop ADHD. Behavioral assessments often include increased locomotor activity as a measurement for ADHD. While the open field can provide a snapshot of locomotor activity of a pig in an unnatural environment within the 10-minute period, I wanted to capture the locomotor activity of pigs in the home environment throughout the day. To do this, I attached Fitbit devices to miniature swine harnesses and observed the pigs over the course of 12 hours while the Fitbits recorded steps. I was successfully able to obtain activity tracking data from the pigs and determined that pigs are most active in the middle of the day.

Finally, it is known that patients with PCS often experience disturbances in sleep. I sought to determine the natural circadian rhythm of the pig by checking the time of night pigs fell asleep and the time in the morning when they awoke. We were able to do this my manually watching

videos of pigs in the home room and recording the times at which the pigs would lay down for sleep at night.

5.2 Behavior in Concussed Pigs

After characterizing a small battery of behavioral assessments for use in pigs and learning to properly handle the animals, work began to induce concussion in juvenile minipigs. I conducted extensive behavioral testing and collected an exorbitant amount of data. Here, I will go over the implications of my results.

Anxiety

Heightened anxiety is a common consequence of post-concussive syndrome. Behavioral results indicate that pigs with concussion exhibit more behaviors which suggest heightened anxiety post-injury. In the open field test, over time, sham animals exhibit a decrease in both the number and duration of escape attempts. This suggests that sham animals are less anxious in the open field as they grow and become used to the space. This could also be an indication of a lack of sensation-seeking or novelty seeking in sham animals. The continuation of escape attempts made by concussed pigs could suggest heightened anxiety or a desire to find a sense of stimulation. Studies have shown that patients with concussion are more likely to engage in sensation seeking behaviors (Liebel et al. 2020). This increase in a desire to escape a boring environment could be a valid predictor of post-concussive complications in pigs.

Depression

Following concussion, patients often experience depressive symptoms. Results from the novel object recognition test show that pigs with concussion interact significantly less with the novel object than sham pigs. Overall, pigs with concussion interact less with the novel object both in the number of individual contacts as well as the overall duration of contact. This decreased

interest in something that ordinarily would be very interesting could be indicative of depression in pigs with concussion. The novel object recognition test was primarily used as a strong indicator of novelty-seeking (Lueptow 2017). I hypothesized that pigs with concussion would show heightened interest in the novel object. Results show that in fact, the opposite is true. Mood disorders such as depression are a fairly common consequence of post concussive syndrome (Vargas et al. 2015). Depression is also highly correlated with measures of anxiety, and results from the open field test indicate that pigs with concussion are more anxious than sham pigs (Al-Kader et al. 2022).

Learning Deficit

Patients will often have trouble with memory and the ability to learn new things following concussion. The baited ball pit was designed as a measurement of spatial learning and memory. My hypothesis was that pigs with concussion would not perform as well in the ball pit as sham animals, with brain injured pigs being distracted and slow. My results from baited ball pit show that pigs with concussion may learn a bit more slowly than sham animals as sham animals become significantly faster in the ball pit 3 weeks post injury whereas mTBI pigs do not show significant improvement until week 5.

Trouble Concentrating

One of the most common symptoms of post-concussive syndrome is difficulty focusing and maintaining concentration. The shape discrimination test was developed as a measure of short-term memory. Studies have shown that short-term memory is often impaired in cases of brain injury, however, the majority of behavioral assessments utilize long-term memory (Willis, Bartlett, and Vukovic 2017). Results indicate that there are no differences in the amount of time sham pigs and pigs with concussion require to make an initial selection between shapes. Interestingly, results show that pigs with concussion take longer to complete a 3-touch trial of the shape discrimination

test when compared to sham pigs. This difference indicates that pigs with concussion have trouble staying focused when completing the full trial. It should be noted that this assessment was conducted on 2 sham pigs and 2 mTBI pigs, and these results are considered preliminary. These results are, however, very promising and it would be well worth it to attempt this assessment on additional pigs.

Hyperactivity

ADHD-like symptoms such as restlessness and hyperactivity are common consequences of concussion. My primary hypothesis for the open field test was that pigs with concussion will exhibit hyperactivity and heightened locomotor activity. Comparison of distance measures between mTBI and sham pigs reveals that pigs with concussion do move considerably more in the open field when compared to sham animals. While this provides evidence that mTBI pigs move more than sham animals, it does not necessary confirm my hypothesis that pigs with concussion will be hyperactive. Analysis comparing weekly distance measures of concussed pigs with their baseline measurements shows little to no chance in the amount of locomotion pigs are engaging in. However, sham pigs show a significant decrease in locomotor activity as time progresses. The results therefore show that pigs with concussion exhibit a conservation of locomotor activity as they age, whereas pigs without concussion decrease activity over time.

Similar to my hypothesis for the open field, I hypothesized that pigs with mTBI would exhibit ADHD-like hyperactivity throughout the day. My findings indicate little to no differences between pigs with brain injury and those without. There could be a bit of hyperactivity exhibited by mTBI pigs 2 weeks after injury, but it is not a very big difference and certainly is not conserved over time.

Mood Changes

One of the common consequences of brain injury includes changes in mood. These affective changes can span from irritability to depression (Yang et al. 2012). I personally hypothesized that pigs with concussion would be more irritable, and therefore exhibit more aggressive and dominant behaviors. Specifically for the food aggression test, I hypothesized that pigs with brain injury would spend more time with their heads in the food bowl. The results show no indication that pigs with concussion are more aggressive. If anything, my results show that over time sham pigs spend more time dominating the herd, and mTBI pigs less. This could corroborate findings from other tests which indicate pigs with concussion experience depression-like symptoms. Depressed pigs may be less likely to fight for the opportunity to eat food, regardless of how enticing the treats may be. Lack of motivation is a classic sign of depression in humans (Yang et al. 2018).

Other

The color discrimination task was adapted from procedures published by Halaweish et al (2015) (Halaweish et al. 2015). The results indicate little to no difference between groups in the completion of this task. This could suggest that the color discrimination task, as it is, is not a valid assessment for post-traumatic changes in neural processing. Potentially, the difference between yellow and blue color was too great, making the test too simple thus not necessitating much cognitive prowess on the pigs behalf.

5.3 fMRI in Pigs

In order to develop a platform which could assess the presence and extent of neurological damage non-invasively, I sought to use functional magnetic resonance imaging to identify regions and pathways in the brain which were damaged by concussion procedures. Considering the long-

term nature of this work, live imaging procedures were essential for the potential identification of altered neural connectivity in the pig brain. This assessment of post-concussive damage through non-invasive imaging procedures has high clinical relevance, as tissue samples are not typically collected from living human patients following a single concussion.

In preparation for imaging procedures, work was conducted to determine which anesthetic protocol would be optimal for neuroimaging procedures in pigs. The results of this study reveal two important aspects: primarily, that these anesthetic agents do have a differential affect the extent of the BOLD fMRI response, significantly in the case of visual stimulation, and secondly that female pigs exhibit a heightened response to stimulation on average, however, due to a small sample size, this difference does not yield statistical significance. These two findings can serve as critical pieces of information when designing biomedical research studies and interpreting the results.

These results suggest that sevoflurane may be the more optimal anesthetic agent for investigating visual stimulation. However, the results could also indicate that propofol is best for tactile stimulation. For our purposes, we also noticed that pigs anesthetized with sevoflurane would experience emergence complications. Specifically, the pigs would vomit when waking up from procedures. This was distressing for not only the pigs, but for me as I try to take the welfare of my animals into account when planning experiments. When pigs were anesthetized with propofol, they no longer exhibited any signs of emergence complications, and thus we chose to continue to use propofol for the maintenance of general anesthesia for the remainder of the study.

Investigative comparison of the extent of the BOLD signal of surgical sham pigs and pigs with mTBI provides inconclusive evidence to determine whether fMRI could be used as a valid diagnostic tool for concussion. There is an interesting trend towards a reduction in response to

visual stimulation 5 weeks post injury in the pigs with concussion, but the effect is not powerful enough to yield statistical significance. Statistically speaking, there is a 10% chance this effect is random and not due to the injury. It would be worth exploring this interaction with a higher number of animal subjects, thus, the results here are greatly affected by the limited number of animals used. By 12 weeks post injury, there is no noticeable difference between groups in response to visual stimulation. This suggests that pigs with concussion, if they did experience any changes in visual response, are fully recovered by 12 weeks. Based on our results assessing the effect of anesthetic agents on the BOLD response, perhaps the differences between mTBI and sham pigs would be more pronounced had these animals been anesthetized under sevoflurane anesthesia instead of propofol. By this line of thinking, results from tactile stimulation should be more obvious as anesthetic protocols were optimized for tactile response. Unfortunately, there are no statistically significant differences between groups at any timepoint for tactile stimulation. This could mean that our concussion procedure has little to no effect on the neural response to tactile stimulation. Moving forward, I would likely be more apt to conduct visual stimulation in brain injured pigs as opposed to tactile stimulation, regardless of the anesthetic agent used.

Developing a new animal model for the study of brain injury is a complex process that must take into consideration various benefits and limitations of experimental subjects as well as available expertise and resources.

5.4 Preliminary Results for rTMS Therapy

The objective of my efforts involving the delivery of rTMS therapy to pigs with concussion was primarily for the purpose of developing procedures which can be used to deliver treatments to pigs with more severe injuries in the future. I was successfully able to complete experiments and did not notice any unfortunate side effects such as the onset of seizures in pigs treated with rTMS.

Behavioral results show that rTMS therapy greatly improves performance in several behavioral tests. Results from the open field test show that therapy has little to no effect on the distance pigs travel during the 10-minute testing period. The distance traveled by pigs who have received therapy is comparable to the distance traveled by mTBI pigs who did not receive therapy. Previous studies conducted in rodents showed that rTMS therapy reduced hyperactivity in brain injured animals, but the results presented in this work suggest that this may not be the case in pigs. In fact, step tracking data shows that throughout the day pigs with brain injury who undergo rTMS therapy take more steps than either sham or mTBI only pigs. These results could suggest that pigs who receive treatment are more playful or less depressed than pigs who do not receive therapy. While sham pigs did not exhibit obvious symptoms of depression, for the purposes of this work, sham and mTBI-only pigs were housed together, thus sham pigs could have adapted to the low-energy state of the other members of the herd, resulting in less movement throughout the day. Results from the novel object recognition test indicate that pigs who received therapy did not exhibit symptoms of depression, interacting much more with the novel object compared to either sham or mTBI-only pigs. As rTMS is an FDA approved treatment for depression, these results support the use of rTMS therapy for depressive symptoms. In the two-choice shape-discrimination task, there are no differences between groups when tasked with making a first response to a target shape. We do see, however, that while pigs in the mTBI only group perform more slowly when asked to make 3 consecutive nose touches of the target shape, pigs with concussion who receive rTMS therapy perform much more similarly to healthy sham pigs. These results indicate that rTMS may have the potential to enhance focus and restore short-term-memory function.

Analysis of preliminary imaging results showed that there were few detectable effects of therapy on response to visual stimulation. There are no notable differences between groups at any

timepoints. This suggests that rTMS therapy may not have an effect on visual differences post injury. The results from tactile stimulation are more interesting. While there are no obvious differences between groups at 24 hours and 5 weeks post injury, the response of rTMS pigs at 12 weeks post injury is much greater than that of healthy pigs. The results show that pigs who receive therapy, while exhibiting no differences to sham pigs immediately after the termination of therapy, show an exaggerated response to tactile stimulation 7 weeks later. These results could indicate that rTMS therapy has the potential to increase sensitivity to touch in pigs with concussion.

5.5 Limitations of the Study

While this body of work successfully filled a gap in the field of large animal neurotrauma research, there were some limitations within the study which must be discussed. The primary limitation is the low number of animal subjects. This is a common limitation in large animal research as working with large animals is extremely difficult. Compared to rodent studies, large animals require much larger, specialized spaces dedicated to housing. Pigs and other large animals also require specialized veterinary care and research equipment as well as well trained and dedicated handlers. These considerations often make it difficult to conduct studies with more than a few pigs at a time. We are fortunate at Michigan State to have a team of large animal veterinarians, and animal care staff as well as a veterinary hospital which regularly sees large animal patients. As an agricultural college, Michigan State is equipped with several specialized facilities dedicated to large animal husbandry.

Though the small number of animal subjects had a limiting effect on the statistical power of the results of this work, we can use the data generated in this study to calculate a more accurate power analysis which can help to determine the number of animal subjects needed to complete behavioral studies like this in the future. Statistical power analysis based on the behavioral results

presented in this work calculated using an online power analysis calculator (http://hedwig.mgh.harvard.edu/sample_size/js/js_parallel_quant.html) show that the minimum number of pigs needed to generate statistically significant results is 10 pigs per experimental group.

5.6 Potential Future Directions

Repetitive injury model

The platform which I have developed has served as excellent practice for a young investigator who is just breaking into the field of neurotrauma research. However, as a model of sports-related concussion, the work presented here is limited. The biggest challenge which sports-related concussion is that incidences tend not to be a singular event, but the result of multiple concussions occurring over time. A more relevant model of sports-related concussion would involve multiple impact procedures. Differences could be investigated based on the timing of multiple injuries, whether occurring in rapid succession or the overall number of incidences.

More severe injury model

While PCS can be a frustrating and challenging condition to live with, only a small subset of the population will ever experience PCS, and even then, it may resolve itself spontaneously after a few months. Patients who experience moderate to severe TBI tend to have more complex and debilitating complications post injury. In order to gather more concrete information about post-injury cognitive differences in pigs, a more severe injury paradigm should be investigated.

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